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Stephen C. Wood
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Cardio-Respiratory Control in Vertebrates

Comparative and Evolutionary Aspects

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Preface

Hopefully, this book will be taken off of the shelf frequently to be studied carefully over many years. More than 40 researchers were involved in this project, which examines respiration, circulation, and metabolism from fish to the land vertebrates, including human beings. A breathable and stable atmosphere first appeared about 500 million years ago. Oxygen levels are not stable in aquatic environments and exclusively water-breathing fish must still cope with the ever-changing levels of O_2 and with large temperature changes. This is reflected in their sophisticated counter-current systems, with high O_2 extraction and internal and external O_2 receptors. The conquest for the terrestrial environment took place in the late Devonian period (355–359 million years ago), and recent discoveries portray the gradual transitional evolution of land vertebrates. The oxygen-rich and relatively stable atmospheric conditions implied that oxygen-sensing mechanisms were relatively simple and low-gain compared with acid–base regulation. Recently, physiology has expanded into related fields such as biochemistry, molecular biology, morphology and anatomy. In the light of the work in these fields, the introduction of DNA-based cladograms, which can be used to evaluate the likelihood of land vertebrates and lungfish as a sister group, could explain why their cardio-respiratory control systems are similar. The diffusing capacity of a duck lung is 40 times higher than that of a toad or lungfish. Certainly, some animals have evolved to rich high-performance levels.

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Overview of the Respiratory System

S.C. Wood

This monograph comprises a diverse collection of chapters dealing with gas exchange, circulation, and metabolism in species ranging from fish to man. As you read the chapters, the unifying theme that emerges is one that was proposed 18 years ago by Weibel et al. (1991), i.e., the hypothesis of symorphosis.

> *The concept that animals, and humans as well, should be designed economically (i.e., that structural design should be matched to functional demand) follows from common sense, but it is also supported by many observations.*

The respiratory system is often depicted as four processes; ventilation, diffusion to blood, circulation, and diffusion to cells that are arranged in series. This series arrangement means that the total resistance to gas transport is the sum of the four resistances and that any of the steps can be rate-limiting. Many authors liken this system to an "oxygen cascade", referring to the progressive drop in PO_2 that occurs at each step of transport. Kuper and Soni (2003) have likened oxygen transport to a whirlpool instead of a cascade. They pointed out that mitochondria "suck" oxygen out of cells, generating an oxygen flux to meet the demand. The drop in PO_2 between arterial blood and venous blood leaving tissues depends on the O_2 content removed from the blood. The venous PO_2 is then a dependent variable of venous O_2 content and, due to the shape of the $PO_2 = f(\text{venous } [O_2])$ curve, is held at fairly constant value over a wide range of venous O_2 contents.

For a given species, each of the four steps in the oxygen cascade (or whirlpool) is adaptable to changes in demand for oxygen uptake and carbon dioxide output. The passive steps of diffusion to blood and diffusion to cells can increase acutely with increased surface areas due to recruitment and distension of capillaries, and can increase chronically with increased capillary density, increased mitochondrial density and increased oxidative enzyme activity (Andersen 1975; Holloszy and Booth 1976). Likewise, the active step of circulation can increase acutely by increasing heart rate and stroke volume, and chronically by increasing maximum stroke volume. This step also includes O_2 transport properties of hemoglobin, which show adaptive changes both acutely and chronically. The other active step, ventilation, shows the same capacity to increase frequency and tidal volume acutely, but does not normally show responses to chronic increases in O_2 demand (Ekblom 1969). When different species or animal groups are compared, the same pattern of adaptation

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emerges as structure is matched quite closely with differences in oxygen demand. An additional variable is now important, i.e., genetic differences.

An alternate approach to examining plasticity of the oxygen transport system is to focus on diminished oxygen supply, i.e., hypoxia. For healthy individuals, this normally becomes a problem only at high altitude. The adaptations of animals to acute and chronic exposure to hypoxia differ somewhat from the adaptations to exercise. For example, a key aspect of adaptation to hypoxia is increased ventilation. Unlike exercise, where the ventilation increases to match increased CO_2 production, the hypoxia-induced increase in ventilation is not related to increased CO_2 production and is, in fact, hyperventilation. Without this hyperventilation, the tolerance to hypoxia would be greatly diminished. Perhaps the clearest example of this is man on the summit of Mt. Everest. Alveolar PCO_2, normally kept at about 40 mmHg at sea level, is reduced by hyperventilation to about 7 mmHg (West et al. 1983). With this hyperventilation, alveolar PO_2 on the summit was about 35 mmHg. Without this hyperventilation, alveolar PO_2 would have been only about 2 mmHg. The downside of this acute response to hypoxia is a pronounced respiratory alkalosis, a condition with medical risks of cerebral and coronary vasoconstriction and cardiac arrhythmias. A chronic response to hypoxia is stimulation of red blood cell production, leading to increased O_2-carrying capacity. The downside of this chronic response is increased blood viscosity and in some natives to altitude, chronic mountain sickness or Monge's disease (Monge-Medrano et al. 1928).

For many species, coping with hypoxia elicits the interesting and effective strategy of hypothermia, which reduces oxygen demand by roughly 11% per degree centigrade (Wood 1991). The mechanism in mammals and birds is disruption of the normal thermogenesis responses to lower body temperature. In ectothermic species, the mechanism is behavioral, i.e., seeking out cooler ambient temperatures. The downside of this response is loss of normal fight or flight speed or, more dramatically, becoming a popsicle by seeking a freezing temperature.

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Part I
Control of Respiration in Aquatic Vertebrates

Gas Transport and Gill Function in Water-Breathing Fish

S.F. Perry, A. Esbaugh, M. Braun, and K.M. Gilmour

Abstract This review focuses on four areas of fish gill function: oxygen transport and transfer, carbon dioxide transport and transfer, oxygen and carbon dioxide sensing, and ammonia excretion. Each section presents a synthesis of previous work while also highlighting recent and ongoing studies that are shaping the growth of these research fields. Where possible, we will comment on the utility of using emerging technologies, including gene knockdown in zebrafish, to evaluate the function of the fish gill.

1 Introduction

Is another review chapter on gas transport across fish gills really necessary? We asked ourselves the same question before taking on this task, and decided to try and determine what impact previous scholarly reviews of fish respiration were having in educating the public at large. A quick Google search using the key words 'fish AND gill' produced 319,000 hits (about half the number of hits obtained by Googling 'rat AND lung'). The very first hit (arguably the most popular) directed us to a site about respiration in fish where we learned that 'fish breathe by drinking'... Clearly, there is still work to be done! Here, we try to address this need while avoiding competition with other recent reviews, notably the ambitious and comprehensive tome on fish gills by Evans et al. (2005), which has soared to Google hit number 12 of 319,000 in only 3 years. For a wealth of detail on the structure and function of the fish gill, we urge the reader to consult Evans et al. (2005). In this review, we have focused on four areas of gill function: oxygen transport and transfer, carbon dioxide transport and transfer, oxygen and carbon dioxide sensing, and ammonia excretion.

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In each section, we have tried to synthesize previous work while highlighting recent studies that we feel are shaping the growth of these research fields.

2 Blood Oxygen Transport and Transfer Across the Gill

The processes of blood O_2 transport and transfer across the fish gill have been investigated intensely over the past 40 years, resulting in a comprehensive understanding of the underlying mechanisms, at least in the few so-called 'model' species that have been examined (e.g. rainbow trout; *Oncorhynchus mykiss*). Numerous detailed reviews have been written on various aspects of this broad topic (e.g. Randall et al. 1982; Jensen 1991, 2004; Weber and Jensen 1988; Perry and Wood 1989; Nikinmaa and Tufts 1989; Piiper 1989; 1998; Randall 1990; Thomas and Motais 1990; Swenson 1990; Thomas and Perry 1992; Nikinmaa 1992; 2001; 2002; 2006; Piiper and Scheid 1992; Fritsche and Nilsson 1993; Brauner 1995; Nikinmaa and Boutilier 1995; Val 1995; 2000; Brauner and Randall 1996; Ultsch 1996; Gilmour 1997; Malte and Lomholt 1998; Perry and Reid 2002; Graham 2006). Given this wealth of pre-existing review material, we aim to focus on the processes involved in optimizing blood O_2 transfer and transport during stress, as well as the more recent discoveries that are catalyzing further research.

2.1 Carriage of O_2 in the Blood

Except for the haemoglobin-lacking Antarctic ice fish (*Chaenocephalus aceratus*; Holeton 1970), typically about 95% of blood O_2 is carried within red blood cells (RBC) chemically bound to haemoglobin, with only a small fraction carried as physically dissolved O_2 in blood plasma. The concentration of haemoglobin in RBCs is relatively constant among those species that have been examined (Perry and McDonald 1993), such that arterial blood O_2 content (CaO_2) is essentially determined by haematocrit, the O_2-binding affinity of haemoglobin, and arterial blood O_2 partial pressure (PaO_2). At any given ambient PO_2, the PaO_2 is set by the combined properties of diffusive conductance, ventilation and perfusion (see Sect. 2.2. in this chapter, O_2 *transfer across the gill*).

2.1.1 Haematocrit

Large inter-specific variation in haematocrit exists among fish species. Active fish of high metabolic scope typically exhibit high haematocrit (e.g. Pacific blue marlin *Makaira nigicans*; Dobson et al. 1986), whereas more sluggish fish tend to have lower haematocrit (e.g. starry flounder *Platichthys stellatus*; Wood et al. 1979). An elevated haematocrit, while affording an increase in blood O_2 carrying capacity,

can be disadvantageous for two reasons. First, increasing blood viscosity (especially in fish inhabiting colder water) will increase the energetic costs associated with cardiac pumping and second, the increase in capacitance of the blood for O_2 may tend (depending on gill transit times and any existing diffusion limitations) to lower branchial O_2 transfer efficiency, leading to a lowering of PaO_2. On the other hand, moderate or high resting metabolic rates in fish of low haematocrit can be achieved only through elevation of cardiac output (Wood et al. 1979; Perry and McDonald 1993), which will constrain scope for activity and limit exercise performance.

Intuitively, it seems reasonable to assume that an optimal haematocrit (or range of haematocrits) exists that allows adequate O_2 carrying capacity without evoking diffusion limitations or impairing cardiac function because of elevated viscosity (Wells and Weber 1991). Surprisingly however, the intriguing question of whether an optimal haematocrit exists for any given species has rarely been addressed. Gallaugher et al. (1995) experimentally manipulated haematocrit to values between 8 and 55% in rainbow trout, and then challenged these anaemic, normocythaemic or polycythaemic fish with exercise trials to determine critical swimming speeds. In accordance with theory, O_2 uptake and critical swimming velocities were reduced in fish with lowered haematocrit ($<22\%$). Surprisingly, however, critical swimming velocity increased with increasing haematocrit (up to 55%) and O_2 uptake peaked at an abnormally elevated haematocrit of 42%. Clearly, the data of Gallaugher et al. (1995) do not support the notion of an optimal haematocrit in rainbow trout. Noteworthy, however, was the observation that PaO_2 during exercise was reduced to a greater extent in fish with elevated haematocrit, implying that a detrimental consequence of excessively increased O_2 carrying capacity is the imposition of diffusion limitations on O_2 transfer when cardiac output is elevated and transit times for gas exchange are reduced.

O_2 carrying capacity can be increased either acutely or chronically via elevation of haematocrit. Acute changes in haematocrit primarily reflect the release of sequestered RBCs from the spleen in response to activation of splenic α-adrenergic receptors by circulating catecholamines (Perry and Vermette 1987; Vermette and Perry 1988b; Perry and Kinkead 1989) or sympathetic nerves (Nilsson and Grove 1974). Conditions during which contraction of the spleen lead to an increase in blood O_2 carrying capacity include hypoxia (Yamamoto et al. 1985; Wells and Weber 1990), hypercapnia (Perry and Kinkead 1989) and exhaustive exercise (Yamamoto et al. 1980; Yamamoto 1988; Yamamoto and Itazawa 1989; Pearson and Stevens 1991b; Gallaugher et al. 1992). While it is uniformly accepted that the elevated blood O_2 carrying capacity associated with increasing haematocrit serves to increase CaO_2 during hypoxia, hypercapnia (Vermette and Perry 1988a) and exercise (Pearson and Stevens 1991b), the physiological benefit of the polycythaemia, at least during exercise, is unclear. For example, exercise-induced increases in haematocrit are reliably prevented by splenectomy, but conflicting consequences on exercise performance have been documented, with Pearson and Stevens (1991a) reporting a diminishment of aerobic swim performance in splenectomized rainbow trout, whereas Gallaugher et al. (1992) demonstrated that splenectomy was without

effect on aerobic swimming. Considering that the release of RBCs from the spleen is a common response to exercise, it is somewhat surprising that the physiological significance of this response is not more apparent.

Blood O_2 carrying capacity is chronically regulated during hypoxia (Wood and Johansen 1973; Lai et al. 2006; Rutjes et al. 2007) and sustained exercise (Thorarensen et al. 1993; Gallaugher et al. 2001) by mechanisms independent of splenic contraction. During sustained hypoxia, erythropoiesis is probably stimulated within the kidney by erythropoietin (EPO) (Lai et al. 2006), probably under the control of hypoxia inducible factor (HIF) (Soitamo et al. 2001; Semenza 2004).

2.1.2 Haemoglobin O_2 Binding Affinity

The relationship between CaO_2 and PaO_2 is dictated by the shape of the O_2 equilibrium curve (OEC). Except for the monomeric haemoglobins of agnathans, fish haemoglobins are tetramers that exhibit cooperativity of O_2 binding and hence yield sigmoidal OECs. The O_2-binding affinity of haemoglobin, estimated by the P_{50} (the PO_2 at which haemoglobin is 50% saturated with O_2), exhibits tremendous variation among the species that have been examined. At the extremes are those fish with unusually low or high affinities (i.e. high and low P_{50}s respectively). There are obvious advantages to high-affinity haemoglobins. Most importantly, PaO_2 can be maintained at lower levels than might otherwise be possible, resulting in reduced ventilatory convection requirements and accompanying energetic savings. The ability to saturate haemoglobin at a low PO_2 allows greater flexibility with respect to habitat selection, and may permit residence in environments that experience fluctuating O_2 levels. Additionally, a low PO_2 within the blood perfusing the gills will increase the overall water-to-blood PO_2 gradient, enhancing diffusive conductance. Low-affinity haemoglobins require a higher PaO_2, necessitating increased ventilation convection requirements and constraining fish to habitats with relatively high PO_2 levels.

As in other vertebrates, haemoglobin–O_2 (Hb–O_2) binding affinity is regulated acutely via a suite of intracellular allosteric modulators, including H^+, CO_2, organic phosphates and several anions including lactate and chloride. Increased RBC pH and reduced organic phosphate levels are the principal mechanisms underlying increased Hb–O_2 binding affinities during hypoxia or systemic acidosis. In rainbow trout and other teleosts (the number as yet undetermined; Berenbrink et al. 2005), increasing RBC pH or defending RBC pH during extracellular acidosis (e.g. hypercapnia) stems from the activation, via mobilization of circulating catecholamines, of a β-adrenergic Na^+/H^+ exchange protein (βNHE) (Borgese et al. 1992) on the RBC membrane. Upon binding to β_{3b} receptors (at least in trout; Nickerson et al. 2003, 2004), catecholamines cause cAMP-mediated activation of protein kinase A and phosphorylation-induced stimulation of βNHE. The pioneering studies more than 20 years ago of several researchers including Mikko Nikinmaa, René Motais and Andrew Cossins revealed that adrenergic activation of βNHE results in the (relative) alkalization of the RBC owing to the extrusion of H^+ coupled to the

inward movement of Na^+ (Nikinmaa 1982; Nikinmaa and Huestis 1984; Baroin et al. 1984; Cossins and Richardson 1985). This process either raises RBC pH (e.g. during severe hypoxia) (Boutilier et al. 1988) or effectively uncouples RBC pH from plasma pH, allowing RBC pH to be maintained during extracellular acidosis (Boutilier et al. 1986; Primmett et al. 1986; Vermette and Perry 1988a). The net consequence of RBC alkalization is increased Hb–O_2 affinity (Nikinmaa 1983) via the Bohr effect. Maintenance of RBC pH during systemic acidosis also can prevent reductions in CaO_2 that otherwise might occur because of Root effects (Vermette and Perry 1988a). Moreover, stimulation of RBC Na^+/H^+ exchange results in an inward flux of Na^+ and a compensatory activation of Na^+/K^+-ATPase. The resultant decline in cellular ATP levels also serves to increase Hb–O_2 binding affinity (see review by Nikinmaa and Boutilier 1995). Finally, the increase in RBC osmolarity associated with Na^+ entry causes osmotic water influx and cell swelling, leading to dilution of cellular organic phosphates and a further increase in Hb–O_2 affinity. Increases in Hb–O_2 binding affinity also occur independently of adrenergic phenomena. For example, hyperventilation induced by hypoxia may cause respiratory alkalosis and thereby raise RBC pH to evoke a Bohr effect. Deoxygenation of haemoglobin may promote RBC alkalization via the Haldane effect and so contribute to a decrease in P_{50}. Long-term increases in Hb–O_2 binding affinity associated with exposure of fish to hypoxia appear to be mediated predominantly by reductions in RBC organic phosphate levels (Wood and Johansen 1973; Greaney and Powers 1978; Soivio et al. 1980).

2.2 O_2 Transfer Across the Gill

The rate of O_2 transfer across the gill is governed by diffusive conductance, convection (ventilation and perfusion), and the blood-to-water PO_2 gradient (ΔPO_2). The importance of each of these factors in controlling gas transfer has been extensively detailed in previous reviews (Randall and Daxboeck 1984; Perry and Wood 1989; Randall 1990; Perry and McDonald 1993; Gilmour 1997; Piiper 1998; Malte and Lomholt 1998; Perry and Gilmour 2002; Evans et al. 2005; Graham 2006). Briefly, diffusive conductance is determined by functional surface area, diffusion distance and Krogh's permeation coefficient (diffusion constant · capacitance). Functional surface area and diffusion distance are labile, and can be dynamically adjusted according to metabolic requirements or environmental conditions. Under resting and normoxic conditions, diffusive conductance typically is kept as low as possible to reduce obligatory salt and water movement across the gill. Thus, the strategy of matching diffusive conductance to gas transfer requirements (the so-called osmorespiratory compromise) offers considerable energetic savings, particularly considering the relatively high costs of actively absorbing salts in freshwater and actively excreting salts in seawater. While it has long been known that fish are able to alter functional surface area by recruiting previously unperfused lamellae (lamellar recruitment) or by more uniformly perfusing individual

lamellae (Booth 1979; Farrell et al. 1980), only recently was it discovered that some species can dramatically alter gill functional surface (in some cases reversibly) by physical covering/uncovering of lamellae (Sollid et al. 2003, 2005; Brauner et al. 2004; Ong et al. 2007). Species exhibiting this strategy of gill remodelling include Crucian carp (*Carassius carassius*), goldfish (*Carassius auratus*), mangrove killifish (*Kryptolebias marmoratus*) and *Arapaima gigas*. In all cases, the gill remodelling consists of the invasion or retraction of an inter-lamellar cell mass (ILCM). The signalling mechanisms underlying proliferation of the ILCM or its removal by apoptosis are unknown (Sollid and Nilsson 2006; Nilsson 2007). In Crucian carp and goldfish, the ILCM is present in fish exposed to cold water but is retracted in fish exposed to increasing temperature (Sollid et al. 2005) or hypoxia (Sollid et al. 2003). In this manner, diffusive conductance is enhanced during periods of increased metabolism or hypoxia, conditions that require optimization of gill O_2 extraction. In the amphibious mangrove killifish, the ILCM appears when fish are exposed to aerial conditions where the gill is not functional (Ong et al. 2007). Appearance of the ILCM in *Arapaima* is associated with a developmental transition from water- to air-breathing (Brauner et al. 2004). Intuitively, the benefit of ILCM appearance and the associated loss of functional surface area should be a reduction in obligatory movements of ions and water. Surprisingly, however, only scarce, indirect data (plasma Cl^- levels in Crucian carp with or without ILCM; Sollid et al. 2003) exist to support this notion. Clearly, this area warrants future research.

A different type of gill remodelling occurs when freshwater fish are placed into ion-poor environments. In an attempt to increase branchial ion uptake capacity, fish placed into ion-poor water experience proliferation of mitochondria-rich cells on the lamellae (Laurent et al. 1985; Leino et al. 1987; Avella et al. 1987; Perry and Laurent 1989; Greco et al. 1996). The proliferation of mitochondria-rich cells causes a marked increase in the lamellar blood-to-water diffusion distance (Bindon et al. 1994b; Greco et al. 1996), thereby negatively affecting gas transfer (Bindon et al. 1994a; Greco et al. 1995), albeit in a relatively subtle manner (Perry et al. 1996; Perry 1998). CO_2 transfer is impeded because CO_2 movement across the gill behaves as a diffusion-limited system (reviewed by Perry and Gilmour 2002), but O_2 transfer is impaired only under conditions of hypoxia.

One of the more intriguing theories related to modulation of gas transfer is that hypoxic bradycardia, the reduction in heart rate observed in many fish upon exposure to hypoxic conditions, serves to increase gill gas-transfer efficiency (i.e. to raise PaO_2 or lower $PaCO_2$). Theoretically, the mechanisms underlying improved gas-transfer efficiency with bradycardia are a reduction in gill transit time (if cardiac output is lowered) and/or increased arterial pulse pressures (which may cause lamellar recruitment or increased gas permeability) (Davie and Daxboeck 1982). Empirical studies, however, have yielded conflicting results, with evidence both for (Taylor and Barrett 1985) and against (Short et al. 1979; Perry and Desforges 2006) a beneficial role of hypoxic bradycardia (reviewed by Farrell 2007). As suggested by Farrell (2007), the main benefit of the hypoxic bradycardia may be to enhance cardiac performance, because increased diastolic residence time may serve to increase O_2 delivery to the myocardium and improve cardiac contractility.

Equally puzzling is the physiological benefit (if any) on gas transfer of the elevation of blood pressure that may accompany hypoxia (Holeton and Randall 1967; Wood and Shelton 1980) or hypercapnia (Perry et al. 1999). While it has been demonstrated that increased blood pressure can promote lamellar recruitment (Farrell et al. 1979) and thus theoretically can enhance gas transfer, empirical data do not support this idea (Kinkead et al. 1991; Perry and Desforges 2006).

3 Blood Carbon Dioxide Transport and Transfer Across the Gill

As with oxygen, our basic understanding of the processes of blood CO_2 transport and transfer across the fish gill has been developed through concentrated research attention spanning many years (e.g. see reviews by Cameron and Polhemus 1974; Randall et al. 1982; Randall and Daxboeck 1984; Perry 1986; Perry and Wood 1989; Piiper 1989; Randall 1990; Brauner 1995; Randall and Val 1995; Brauner and Randall 1996; Tufts and Perry 1998; Henry and Swenson 2000; Perry and Gilmour 2002). However, incorporation of molecular approaches into these studies is opening up exciting new research directions, including recognition and characterization of the diversity of carbonic anhydrase isoforms and, following on from this discovery, awareness of species-to-species differences in patterns of CO_2 excretion. These new directions will form the main focus of our discussion of CO_2 excretion.

3.1 Carriage of CO_2 in the Blood

Carbon dioxide is transported within the blood of fish in three distinct chemical forms, as physically dissolved CO_2, carbamino CO_2, and bicarbonate ions (HCO_3^-). Physically dissolved CO_2 usually makes up less than 5% of the total, largely due to the low solubility of gaseous CO_2 in plasma (Boutilier et al. 1984). The contribution of carbamino CO_2 also appears to be quite low in both teleost and agnathan species owing to few binding sites for CO_2 on haemoglobin (Heming et al. 1986; Fago and Weber 1998), although this may not be true for elasmobranchs (Jensen 2004). The vast majority of CO_2 therefore is transported as HCO_3^-, with estimates exceeding 90% of the total circulating CO_2 pool. Consequently, blood CO_2 transport is dependent upon the conversion of CO_2 to HCO_3^-, a reaction catalyzed by the enzyme carbonic anhydrase (CA) (Brinkman et al. 1932; Meldrum and Roughton 1933). Because the conversion of CO_2 to HCO_3^- produces a proton, the CO_2 capacitance of whole blood is related to buffering capacity which is largely determined by the concentration of haemoglobin, the primary non-HCO_3^- blood buffer.

In teleosts, CO_2 transport begins with molecular CO_2 flooding into the blood from the tissues. In the RBC, the CA-catalyzed hydration of CO_2 yields HCO_3^-,

which is exchanged for plasma Cl^- via the band 3 anion exchange protein (AE1 or SLC26A1; Obaid et al. 1979; Heming et al. 1986; Perry 1986; Tufts et al. 1998), and H^+ that is buffered by haemoglobin. Dual end-product removal sustains the conversion of CO_2 to HCO_3^- within the RBC. In teleost fish as well as lamprey, some of the protons that bind to haemoglobin act as Bohr protons and the resultant decrease in Hb–O_2 binding affinity (Bohr effect) aids in O_2 delivery to the tissues (Fig. 1a). These reactions are reversed at the branchial epithelium, where CA catalyzes the dehydration of HCO_3^- retrieved from the plasma via band 3 (Fig. 1a). The production of CO_2 is initiated both by the loss by diffusion of molecular CO_2 across the gill, and by the large Haldane effect of teleost haemoglobins (Brauner and Randall 1998), in which haemoglobin oxygenation results in the release of Bohr protons into the RBC cytoplasm. The rate-limiting step of this process is the relatively slow rate of anion exchange between the plasma and RBC (Wieth et al. 1982; Perry 1986; Perry and Gilmour 1993; Tufts et al. 1998; Desforges et al. 2001). Indeed, CO_2 excretion in teleosts behaves as a diffusion-limited system, largely due to the chemical equilibrium constraints within the blood during the 0.5–2.5 s (Cameron and Polhemus 1974) gill transit time (Desforges et al. 2002); by contrast, O_2 uptake is perfusion-limited (Perry and Gilmour 2002). The process is nonetheless sufficient for successful matching of CO_2 excretion rates to the rate of CO_2 production by the tissues under steady-state conditions, with up to 35% of total blood CO_2 being removed in a single passage through the gills (Perry 1986).

3.2 Molecular Mechanisms Underlying CO_2 Transport and Transfer in Teleost Fish

The success of the CO_2 excretion pathway is largely predicated on the interplay between CA and the anion exchange protein, band 3. All teleost RBC CA enzymes examined to date are high-activity isozymes that are catalytically similar to mammalian CA II, one of the fastest known naturally occurring enzymes. Biochemical characterization of fish RBC CA isozymes suggested that teleost RBCs exhibited CA II, whereas agnathan RBCs contained the low activity CA I and a high-activity intermediate was present in elasmobranch RBCs (Henry and Heming 1998). This pattern led to the attractive hypothesis that high-activity RBC CA isozymes evolved only after the incorporation of band 3 into the RBC membrane (Henry et al. 1993). However, sequencing of several fish CA isozymes and ensuing phylogenetic analyses of the α-CA gene family indicate that fish cytoplasmic CA isozymes are evolutionarily distinct from their mammalian counterparts (Lund et al. 2002; Esbaugh et al. 2004; 2005; Esbaugh and Tufts 2006). Moreover, the RBCs of the agnathan lamprey were found to express a high-activity isozyme that was basal to both the derived fish and mammalian RBC CA groups (Esbaugh and Tufts 2006). Thus, high-activity RBC CA appears to have arisen early in the evolution of vertebrates, although hagfish and elasmobranch CA isozymes have yet to be investigated to complete this picture. Interestingly, the catalytic efficiency of the enzyme has

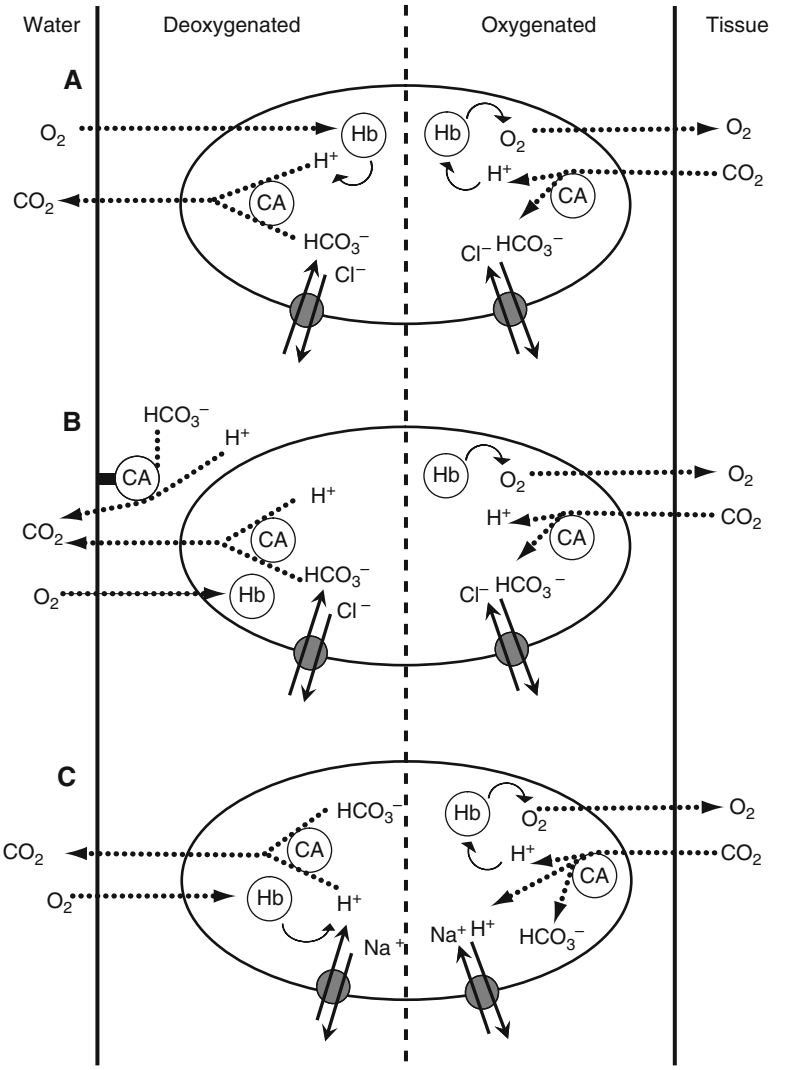

Fig. 1 A schematic model of CO_2 excretion in (**a**) teleost fish, (**b**) elasmobranch fish, and (**c**) the lamprey, an agnathan fish. Oxygen movement is from left to right, i.e. from water to blood at the gill, and then from blood to tissue, while CO_2 movement is in the opposite direction. Carbonic anhydrase (CA) is present in the cytosol of the red blood cells, and is also found associated with the branchial epithelium in elasmobranch fish. A Haldane effect, oxygenation-linked H^+ binding to haemoglobin (Hb), contributes to CO_2 excretion in teleost fish and lamprey but not in elasmobranch fish. In teleost and elasmobranch fish, HCO_3^- shuttles between red blood cell and plasma via the band 3 anion exchanger. This exchanger is absent from lamprey red blood cells, but a Na^+/H^+ exchanger contributes to end-product removal

changed very little through the evolution of vertebrates, whereas the RBC enzyme concentration has increased dramatically in more derived vertebrate groups (i.e. teleosts). This observation led to the idea that CA may be limiting at sub-cellular locations during specific physiological circumstances (Esbaugh et al. 2004, 2005;

Esbaugh and Tufts 2006). The available evidence from mammals, however, suggests that RBC CA exceeds the amount required for CO_2 excretion by 17-fold under steady state conditions, and 6-fold during intense exercise (Swenson and Maren 1978). Similarly, CA limitations on O_2 delivery (via the Bohr effect) appear only if CA inhibition is nearly complete (Maren and Swenson 1980). Indeed, in species examined to date, CA appears to be present in excess of 20-fold that needed for a functional Bohr effect during capillary transit, with an excess upwards of 300-fold in humans (Maren and Swenson 1980).

Although the physiological significance of the apparent excess of RBC CA in fish is unclear, in its presence the rate-limiting step in CO_2 excretion is the relatively slow rate of anion exchange (Wieth et al. 1982; Perry 1986; Tufts and Perry 1998; Desforges et al. 2001). Recently, it was suggested that RBC CA and band 3 form a physical association that could increase the efficiency of anion exchange. Initial support for this idea came from several studies on mammalian RBCs that posited an association of human CA II and AE1 (Vince and Reithmeier 1998; 2000; Vince et al. 2000; Reithmeier 2001; Sterling et al. 2001), but similar associations between other CA isozymes and ion transporters in other tissues have also been proposed; e.g. $Na^+ - HCO_3^-$ cotransporter isoform 1 (NBC1 or SLC4A4) (Gross et al. 2002), NBC3 (SLC4A7) (Loiselle et al. 2004), Na^+/H^+ exchanger isoform 1 (NHE1 or SLC9A1) (Li et al. 2002), monocarboxylate transporter 1 (MCT1 or SLC16A1) (Becker et al. 2005), and Cl^-/anion exchanger protein downregulated in adenoma (DRA or SLC26A3) (Sterling et al. 2002). In a recent and comprehensive study of the SLC4 HCO_3^- transporter family, however, Piermarini et al. (2007) did not find functional associations between human CA II and any member of the SLC4 family, and suggested that previously described associations may be attributed to CA II binding directly to the GST tag to which the recombinant anion exchange transporter C-terminal tails were bound. The results of other studies that used GST tags, such as those on NHE1 and DRA, therefore require re-examination. Nevertheless, whether functional associations occur between CA and various transporters is still debatable. For example, conflicting results were obtained in recent studies on whether the co-expression of CA II and NBCe1 resulted in increased membrane ion transport in oocytes (Lu et al. 2006; Becker and Deitmer 2007). Further research is needed to clarify whether CA may play a direct role in increasing the efficiency of anion exchange across RBC membranes in fish.

Aquaporins, specifically aquaporin 1, constitute another group of proteins integrally involved in the transport and excretion of CO_2. Several studies over the past decade have challenged the traditional view that CO_2 diffuses freely across lipid membranes (Cooper et al. 2002). Initial studies using oocytes demonstrated that CO_2 could enter cells via aquaporin 1 (Cooper and Boron 1998; Nakhoul et al. 1998; Prasad et al. 1998). A more recent series of studies indicated that aquaporin 1, and to a lesser extent rhesus A glycoprotein, are responsible for 50–80% of CO_2 permeability in human RBCs (Endeward et al. 2006a, b, 2007). Several studies on aquaporin 1 knock-out mice, on the other hand, have failed to reveal any effect of null mutations on CO_2 permeability of RBCs or red cell ghosts, lung or kidney tissues, or reconstituted liposomes (Yang et al. 2000; Fang et al. 2002; Ripoche

et al. 2006). Thus, the debate over the contribution of aquaporins to membrane CO_2 permeability continues. To date, neither the possible contribution of aquaporins to CO_2 permeability in non-mammalian RBCs nor their role in CO_2 movement across the branchial epithelium has been investigated.

3.3 Alternative Strategies of CO_2 Transport and Excretion

In several groups of fish, CO_2 excretion differs from the typical teleost pattern (Fig. 1a). For example, anion exchange activity is absent from agnathan RBCs, so that HCO_3^- formed from the hydration of CO_2 is transported within the RBC (see review of Tufts and Perry 1998) (Fig. 1c). Cellular accumulation of end-products should in theory reduce the capacity for CO_2 hydration, but the arterio-venous differences in blood total CO_2 of lamprey are comparable to those of rainbow trout (Tufts and Perry 1998), suggesting the existence of an efficient excretion pathway. The efficiency of CO_2 transport in lamprey is aided by two main characteristics, the large Bohr and Haldane effects of lamprey haemoglobins, and the involvement of RBC Na^+/H^+ exchange. Lamprey haemoglobins exhibit Bohr/Haldane effect coefficients comparable to those of many teleost species (Tufts and Perry 1998), and therefore provide substantial non-bicarbonate buffering upon deoxygenation. This trait not only effectively removes protons from the cytoplasm favouring CO_2 hydration, but it is also integral to CO_2 excretion at the respiratory epithelium, since O_2 uptake causes the release of Bohr protons that drive the dehydration of HCO_3^-. In addition, Na^+/H^+ exchange allows lamprey RBCs to maintain a high intracellular pH (Nikinmaa 1986, 1997; Nikinmaa et al. 1986; Tufts 1992), which also effectively lowers the proton concentration in the cytoplasm. These two mechanisms of single end product (H^+) removal are sufficient to maintain the hydration of CO_2, even at high intracellular HCO_3^- concentrations (Nikinmaa 1986, 1997; Nikinmaa et al. 1986).

Much less is known of CO_2 transport in hagfish. Although hagfish RBCs lack anion exchange and contain CA, the majority of HCO_3^- is found in the plasma. Unlike in lamprey, the RBC non-bicarbonate buffer capacity in Atlantic hagfish (*Myxine glutinosa*) is not greatly elevated (Tufts and Perry 1998). Hagfish RBC membranes are also devoid of appreciable Na^+/H^+ exchange (Nikinmaa et al. 1993), implying that there is little in the way of H^+ removal from the RBCs. Thus, although hagfish are similar to lamprey in lacking RBC anion exchange, their mechanisms of CO_2 carriage appear to differ. Interestingly (and unlike in other fish species), hagfish haemoglobin binds HCO_3^- in an oxygenation-dependent fashion (Fago et al. 1999), an effect that would not only increase the efficiency of CA-catalyzed CO_2 hydration, but would also favour HCO_3^- dehydration at the gill as haemoglobin is oxygenated. In vitro work on hagfish RBCs documented a significant increase in RBC but not whole blood CO_2 content with deoxygenation; the large pool of plasma HCO_3^- may have masked any effect of deoxygenation on whole blood CO_2 content (Tufts et al. 1998). It is unclear whether this unusual HCO_3^--based Haldane effect alone is

sufficient to drive CO_2 transport through the RBCs of hagfish in the short gill transit time, but the very low metabolic rates of hagfish (Malte and Lomholt 1998) may allow it.

Elasmobranch fish also constitute an exception to the typical model of CO_2 excretion, in that a substantial proportion of CO_2 excretion occurs directly from the plasma rather than via the RBC (Gilmour 2001; Gilmour and Perry 2004) (Fig. 1b). Two characteristics permit this excretion pathway. First, significant branchial membrane-bound CA activity with an extracellular orientation is present, allowing HCO_3^- dehydration to occur in the plasma as it passes through the gills (Henry et al. 1997; Gilmour et al. 2002; Gilmour and Perry 2004). The enzyme responsible for this activity is a CA IV isozyme bound to the membranes via a GPI-anchor (Gilmour et al. 2002, 2007). The relatively high non-bicarbonate buffering capacity of elasmobranch plasma is also integral to this model, by providing H^+ for HCO_3^- dehydration (Gilmour et al. 2002). Studies in rainbow trout in which both bovine CA and non-bicarbonate buffers were added to the plasma provided support for the elasmobranch model, by demonstrating that CO_2 excretion can be driven through the plasma given the availability of plasma CA (Desforges et al. 2001), to an extent that depends on plasma buffering (Gilmour et al. 2004). Why an alternative pathway is present in elasmobranchs is, however, unclear, particularly given the presence in elasmobranch RBCs of both CA and anion exchange (Obaid et al. 1979). One possible explanation stems from the absence of an appreciable Haldane effect in these animals (Lai et al. 1989; Wood et al. 1994), which may compromise the effective removal of CO_2 during capillary transit. For example, the Haldane effect in trout is directly responsible for approximately 30–40% of CO_2 excretion in vitro (Perry and Gilmour 1993).

Unlike most teleost fish examined to date, two Antarctic species (*Chaenocephalus aceratus* and *Notothenia coriiceps*) possess branchial membrane-bound CA (Tufts et al. 2002). Interestingly, *C. aceratus* lacks RBCs and exhibited approximately three times more membrane-bound CA than did *N. coriiceps*. Although complete characterization of CO_2-excretion pathways in these species remains to be carried out, Tufts et al. (2002) suggested that branchial membrane-bound CA was unlikely to contribute substantially to CO_2 excretion, owing to the very low metabolic rates of these species and the predominant role of the RBC route in most teleost fish.

Air-breathing fish also provide an interesting dilemma owing to the spatial uncoupling of O_2 uptake, which occurs via the air-breathing organ, and CO_2 excretion, the bulk of which typically occurs via the gills or skin (Brauner and Randall 1998). In keeping with the spatial uncoupling, functional uncoupling of O_2 uptake and CO_2 excretion is achieved by the presence of only small Haldane effects, with *Arapaima gigas* being a notable exception in this regard. This adaptation could, however, in theory reduce the efficiency of CO_2 excretion by eliminating the contribution of Bohr protons to HCO_3^- dehydration, which would then be driven solely by the fall in PCO_2 as molecular CO_2 diffuses across the gills. The generally small surface area and high blood-to-water diffusion distances found in the gills of many obligate air-breathers may compound this problem. An increased contribution

of dissolved CO_2 to overall CO_2 transport in the blood may provide a way around this difficulty (Brauner and Randall 1998), and in fact, the blood total CO_2 concentrations of obligate air-breathers are typically much higher than in water-breathing fish species. Interestingly, CO_2 excretion in the African lungfish *Protopterus dolloi* was shown to be unaffected by complete blood CA inhibition, suggesting that HCO_3^- dehydration is not limiting in this species (Perry et al. 2005). However, this species is unique among lungfish in excreting the majority of CO_2 through the lung, and CO_2 excretion in many other air-breathing species is reduced by blood CA inhibition (Burggren and Haswell 1979; Daxboeck and Heming 1982; Smatresk and Cameron 1982; Pelster et al. 1988). Although branchial membrane-bound CA that contributes significantly to CO_2 excretion could provide an alternative mechanism to supplement CO_2 excretion in obligate air-breathers, there is no evidence to support this possibility in the two species examined to date (Gervais and Tufts 1998; Perry et al. 2005), albeit neither exhibits spatially uncoupled gas exchange.

4 Sensing of Respiratory Gases at the Gill

The ability of fish to mount appropriate cardiorespiratory adjustments during fluctuations of the gaseous composition of the environment requires effective gas-sensing mechanisms or chemoreception. The gill is a critical site of gas sensing owing to the presence of both O_2 and CO_2 chemoreceptors that are able to detect changes in external and/or internal gas levels. Numerous detailed reviews have been written on chemoreception in fish (Shelton et al. 1986; Milsom 1989, 1995a, b, 2002; Smatresk 1990; Burleson et al. 1992; Fritsche and Nilsson 1993; Burleson 1995; Milsom et al. 1999; Gilmour et al. 2001; Perry and Gilmour 2002; Gilmour and Perry 2006). In this chapter, while briefly summarizing some of the classical concepts of chemoreceptor control of cardiorespiratory function, we will focus predominantly on relatively recent developments regarding the cellular mechanisms of O_2 and CO_2 sensing and chemoreceptor plasticity.

4.1 Downstream Responses Associated with Chemoreceptor Activation

Despite marked species variation in the thresholds required to elicit physiological responses and in the magnitude of those responses that do occur, there are several well-documented outcomes of chemoreceptor activation. Hyperventilation in response to hypoxia or hypercapnia is probably the most robust of responses, occurring in the vast majority of species that have been examined (Gilmour and Perry 2006). The physiological significance of hyperventilation during hypoxia is obvious, at least in those species attempting to maintain a constant metabolic rate. In

addition to lamellar recruitment and gill remodelling (see above), hyperventilation is an effective (yet costly) strategy for increasing branchial gas transfer while raising arterial PO_2. For the latter, the benefit stems from the fact that the increased water flow decreases the inspired–expired PO_2 difference, allowing the arterial blood to approach equilibrium with ventilatory water of higher mean PO_2. The benefit of hyperventilation during hypercapnia is to reduce the extent of the associated respiratory acidosis, since even a slight lowering of $PaCO_2$ can have a significant impact in raising blood pH.

Common cardiovascular responses to hypoxic and hypercapnic exposure are elevated blood pressure owing to increased systemic vascular resistance, and bradycardia (see Tables 3.1 and 3.2 in Gilmour and Perry 2006). Increased blood pressure during hypoxia (Holeton and Randall 1967; Wood and Shelton 1980) or hypercapnia (Perry et al. 1999) reflects peripheral vasoconstriction arising from stimulation of vascular smooth muscle α-adrenergic receptors by sympathetic nerves or circulating catecholamines (Fritsche and Nilsson 1990; Kinkead et al. 1991; Perry et al. 1999). Bradycardia arises from increased activity of cardiac parasympathetic nerves (Taylor et al. 1977; Wood and Shelton 1980).

In rainbow trout, the secretion of catecholamines (adrenaline and noradrenaline) into the circulation, a response intricately linked to cardiovascular control, is at least in part initiated by activation of branchial chemoreceptors during hypoxia (Reid and Perry 2003) and hypercapnia (Perry and Reid 2002).

4.2 Location and Orientation of Branchial Chemoreceptors

O_2 chemoreceptors sense changes in both water PO_2 and blood PO_2, suggesting that two populations of O_2 chemoreceptors are present, one that is oriented to sense the external environment and another positioned to sense the internal milieu (Milsom and Brill 1986; Burleson and Milsom 1993). Alternatively, a single population of O_2 chemoreceptors may be strategically located within the gill epithelium to sense changes in both water and blood PO_2. It has largely been accepted that activation of externally-oriented O_2 receptors stimulates cardiovascular and ventilatory adjustments, whereas stimulation of internally-oriented O_2 receptors elicits only ventilatory responses. However, a close inspection of the available data (see Table 3.3 in Gilmour and Perry 2006) reveals that this generalization probably oversimplifies a more complex situation in which a diversity of response patterns exist.

Fewer data are available for CO_2 chemoreceptors. More recent studies have provided evidence for the presence of branchial CO_2 chemoreceptors that are exclusively oriented towards the external environment and respond to PCO_2 rather than pH (McKendry and Perry 2001; Perry and McKendry 2001; Perry and Reid 2002; Gilmour et al. 2005), but data from earlier studies suggest the additional presence of internal receptors that may be stimulated by changes in body fluid CO_2 and/or pH (see review by Gilmour 2001).

4.3 Cellular Mechanisms of O_2 and CO_2 Sensing

Gill neuroepithelial cells (NECs) closely resemble the O_2- and CO_2-sensing glomus (Type I) cells of the mammalian carotid body (Dunel-Erb et al. 1982; Bailly et al. 1992; Goniakowska-Witalinska et al. 1995; Zaccone et al. 1997; Sundin et al. 1998; Jonz and Nurse 2003; Saltys et al. 2006). Typically, NECs are enriched with serotonin and possess dense-cored vesicles containing synaptic vesicle protein (Dunel-Erb et al. 1982; Bailly et al. 1992; Jonz and Nurse 2003), features that are characteristic of neurosecretory cells. NECs are occasionally found on lamellae, but are concentrated along the leading edge of distal regions of gill filaments. Based on the anatomical and chemical similarities between NECs and glomus cells and their favourable location to sense water and blood gases, Dunel-Erb et al. (1982) suggested that NECs may function as O_2 chemoreceptors. The first evidence to support their claim of an O_2-sensory function was the observation that NECs undergo degranulation (indicative of neurotransmitter release) in response to severe hypoxia (Bailly et al. 1992). Additional indirect evidence that the NEC acts as an O_2 sensor has accumulated in recent years. In adult zebrafish, the number of NECs is increased by hypoxic exposure (Jonz et al. 2004) and decreased during hyperoxia (Vulesevic et al. 2006). In larval zebrafish, the magnitude of the hypoxic ventilatory response correlates with the maturation of the NEC, becoming maximal as the NEC becomes fully innervated (Jonz and Nurse 2005). The most compelling evidence that gill NECs act as O_2 chemoreceptors stems from studies in which zebrafish (Jonz et al. 2004) or channel catfish (*Ictalurus punctatus*) (Burleson et al. 2006) NECs were cultured and subjected to patch clamp electrophysiology experiments. As in the glomus cells of the carotid body, NECs exposed to hypoxia exhibited membrane depolarization owing to inhibition of K^+ conductance. An important next step in this research area is to determine whether membrane depolarization is accompanied by neurotransmitter release. Although it is now clear that NECs are able to sense O_2, and that their response resembles the well-characterized response of carotid body cells, direct data linking NECs to the initiation of cardiorespiratory adjustments when ambient O_2 levels are altered remain to be collected.

Recently, it was demonstrated that NECs of zebrafish, like mammalian carotid body glomus cells, are bimodal sensors able to respond to both hypoxia and hypercapnia (Zhaohong Qin, J. Lewis and S.F. Perry, unpublished observations). The mechanisms of O_2 and CO_2 signal transduction appear to be similar, at least in part, as both involve inhibition of background K^+ conductance.

4.4 Chemoreceptor Plasticity

The zebrafish has emerged as an important resource for studying the ontogeny and plasticity of chemoreceptor-mediated cardiorespiratory responses (Pelster 2002). Although hyper-ventilatory responses to hypoxia in zebrafish are observed at 2 days post-fertilization (dpf), maximal ventilatory responses to hypoxia are elicited only

after 7 dpf, coinciding with the full innervation of gill NECs (Jonz and Nurse 2005). Interestingly, the zebrafish cardiac M_2 muscarinic receptor can initiate bradycardia in response to cholinergic stimulation at 3 dpf (Hsieh and Liao 2002), well before the full maturation of branchial NECs. Thus, if dependent on fully functional NECs, hypoxic bradycardia may only occur several days after maturation of the cardiac M_2 receptor. Peripheral vasoconstriction, often observed during hypoxia or hypercapnia (see above), can be elicited by α-adrenergic receptor agonists at 8 dpf (Bagatto 2005). Thus, maturation of the α-adrenergic receptor appears to coincide closely with the development of a functional NEC. The rate at which these cardiovascular control mechanisms develop can be influenced by environmental factors including water oxygen levels and temperature (Bagatto 2005). For example, the development of adrenergic tachycardia and peripheral vasoconstriction are accelerated by hypoxia. It is unclear, however, whether development of the branchial chemoreceptors controlling these functions is similarly affected.

The developmental plasticity of respiratory control in zebrafish recently was investigated by exposing fish to hypoxia, hyperoxia or hypercapnia during the first week of development (Vulesevic and Perry 2006). As adults, the responses of these same fish to acute ventilatory stimuli were assessed. The results indicated that chemoreceptor-mediated responses in adult fish could be markedly affected by the rearing environment. For example, the respiratory responses of fish reared under hyperoxic conditions to acute hypoxia, hypercapnia or external cyanide were blunted (hypoxia, cyanide) or eliminated (hypercapnia). Future studies should attempt to link the plasticity of these ventilatory responses to changes in chemoreceptor function.

Adult fish also are capable of exhibiting chemoreceptor plasticity that can influence cardiorespiratory responses. For example, adult zebrafish exposed for 28 days to hyperoxic water ($P_WO_2 = 350\,mmHg$) exhibited a blunting of the ventilatory responses to acute hypoxia or hypercapnia, which was associated with a significant reduction in the density of gill filament NECs (Vulesevic et al. 2006). Although long-term (60-day) exposure of zebrafish to hypoxia ($P_WO_2 = 35\,mmHg$) caused hypertrophy of gill filament NECs in zebrafish (Jonz et al. 2004), their response to acute hypoxia (at least after 28 days) was actually blunted (Vulesevic et al. 2006). This finding is in marked contrast to the results of Burleson et al. (2002), who demonstrated that prior exposure of channel catfish to moderate hypoxia for 7 days increased the ventilatory response to acute severe hypoxia.

5 Ammonia Excretion

The gills are structurally and functionally suited not only to exchange of the respiratory gases, O_2 and CO_2, but also for the excretion of gaseous ammonia. While ammonia excretion has received considerable attention in the context of nitrogenous waste excretion and/or acid–base balance (e.g. see reviews by Cameron and Heisler 1985; Randall and Wright 1989; Heisler 1990; Walsh and Henry 1991;

Mommsen and Walsh 1992; Wood 1993; Ip et al. 2001; Wilkie 2002), the recent emergence of rhesus proteins as an ammonia transporter mechanism has renewed interest in the excretion of gaseous ammonia at the fish gill.

The biological oxidation of amino acids and proteins produces nitrogenous waste, the most reduced and energy-efficient form of which is ammonia (Smith and Rumsey 1976; Wood 1993). The liver is the primary source of ammonia in fish, responsible for up to 70% of total production (Randall and Ip 2006). While the majority of the ammonia produced is a direct result of the deamination of amino acids to provide substrates that can be used in energy production (Brown and Cameron 1991; Wood 1993), an important secondary source of ammonia occurs within muscle fibres via the deamination of adenylates in exercising fish (Driedzic and Hochachka 1976). However, much of this ammonia is not excreted (Wood 1988), but rather acts to buffer the pH depression caused by the build-up of lactic acid (Dobson and Hochachka 1987), and may help maintain glycolytic flux by stimulating phosphofructokinase (Wood 1993).

The ability to act as a biological buffer is only one of several properties that ammonia shares with carbon dioxide, as it, too, occurs in both gaseous (NH_3) and ionic (NH_4^+) forms in aqueous solution, with the sum of both forms known as total ammonia (T_{amm}): $NH_3 + H^+ \leftrightarrow NH_4^+$. In fish plasma, this relationship has a pK of approximately 9.5 (Boutilier et al. 1984), meaning that at physiological pH approximately 95% of T_{amm} is carried as NH_4^+.

5.1 Toxicity

Ammonia is the most toxic of the respiratory gases and must be continually removed from the body through either conversion into less toxic compounds (urea, uric acid) or excretion. Most terrestrial animals make use of the former strategy, and only encounter elevated levels of ammonia when experiencing pathological conditions such as hepatic encephalopathy. With the exception of the ureotelic elasmobranchs and a few unusual teleosts such as the Gulf toadfish (*Opsanus beta*) (Mommsen and Walsh 1989) and the Lake Magadi tilapia (*Alcolapia grahami*) (Randall et al. 1989), most fish are ammonotelic, excreting up to 90% of their nitrogenous waste as ammonia (Wood 1993). Perhaps as a consequence, they tend to have a higher tolerance for ammonia than terrestrial vertebrates (Wilkie 2002), but fish will also succumb when exposed to high concentrations of ammonia.

High internal levels of T_{amm} cause severe central nervous system disruptions, including convulsions, coma and death (Iles and Jack 1980; Cooper and Plum 1987; Raabe 1987; Norenberg et al. 1992; Rama Rao et al. 2003). While much remains to be learned about mechanisms of ammonia toxicity, it appears, ironically, that it is the predominant ionic form, NH_4^+, that is most dangerous. NH_4^+ has long been known to substitute for K^+ in ion transporters and channels (Binstock and Lecar 1969) and can therefore affect ionic homeostasis. NH_4^+ interferes with the currents underlying excitatory and inhibitory signalling in synapses (Iles and Jack 1980; Raabe 1987)

and depolarizes membranes (Allert et al. 1998), both of which actions can lead to activation of *N*-methyl-D-aspartate (NMDA) glutamate receptors (Rose 2002). In fact, excessive glutamate release via activation of the NMDA receptor appears to underlie many of the damaging effects usually linked with ammonia toxicity (Felipo et al. 1998; Rose 2002; Klejman et al. 2005). Hyperammonaemia has been associated with increased release of free radicals (Albrecht and Wegrzynowicz 2005), high levels of intracellular calcium (Randall and Tsui 2002; Rama Rao et al. 2003), opening of the mitochondrial permeability transition pore (Rama Rao et al. 2003) and apoptosis (Rose 2002; Svoboda et al. 2007), all of which are downstream effects of glutamate excitoxicity (Bickler et al. 2002).

Injection of NMDA receptor blockers appears to protect mammalian neurons from ammonia intoxication (Marcaida et al. 1992), and injection of the NMDA receptor antagonist MK-801 also reduced ammonium-induced mortality in the weatherloach *Misgurnus anguillicaudatus* (Tsui et al. 2004). However, MK-801 did not give similar protection to the mudskippers *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti* (Ip et al. 2005), suggesting that NMDA receptors may not be involved in ammonia toxicity in all fish.

5.2 Ammonia Excretion Pathways

Despite the relatively high tolerance of fish for ammonia (Wilkie 2002), efficient ammonia excretion is vital for survival. While it is accepted that the majority of ammonia is lost at the gills, evidence exists for several different mechanisms of ammonia excretion, including passive diffusion of NH_3, passive diffusion of NH_4^+, apical Na^+/NH_4^+ exchange, and basolateral Na^+/NH_4^+ (K^+) ATPases (Fig. 2).

5.2.1 Passive Diffusion of NH_3

Due to the interplay of pH, T_{amm} and electrical potential, a complete understanding of the mechanisms of ammonia excretion has been difficult to attain. However, while questions still remain, the consensus opinion is that the majority of ammonia excretion takes place at the gills as simple diffusion of NH_3 from the blood to the water (Wood 1993; Wilkie 2002). The concentration of total ammonia found as NH_3 intracellularly is low, but sufficient to maintain excretion down the NH_3 partial pressure (PNH_3) gradient from the blood to the water. When the pH of the water is increased (increasing PNH_3 in the water), T_{amm} excretion falls in rainbow trout (McGeer and Eddy 1998). Indeed, a favourable PNH_3 blood-to-water gradient is maintained in large part via acidification of the gill boundary layer, either through CO_2 excretion or direct excretion of H^+. Water pH can drop significantly (up to 1.5 pH units) in passing over the gills (Wright et al. 1986), and Wright et al. (1989) proposed that H^+ released from the hydration of excreted CO_2 'trapped' NH_3 as NH_4^+, preventing backflux of NH_3 and maintaining the PNH_3 blood-to-water gradient.

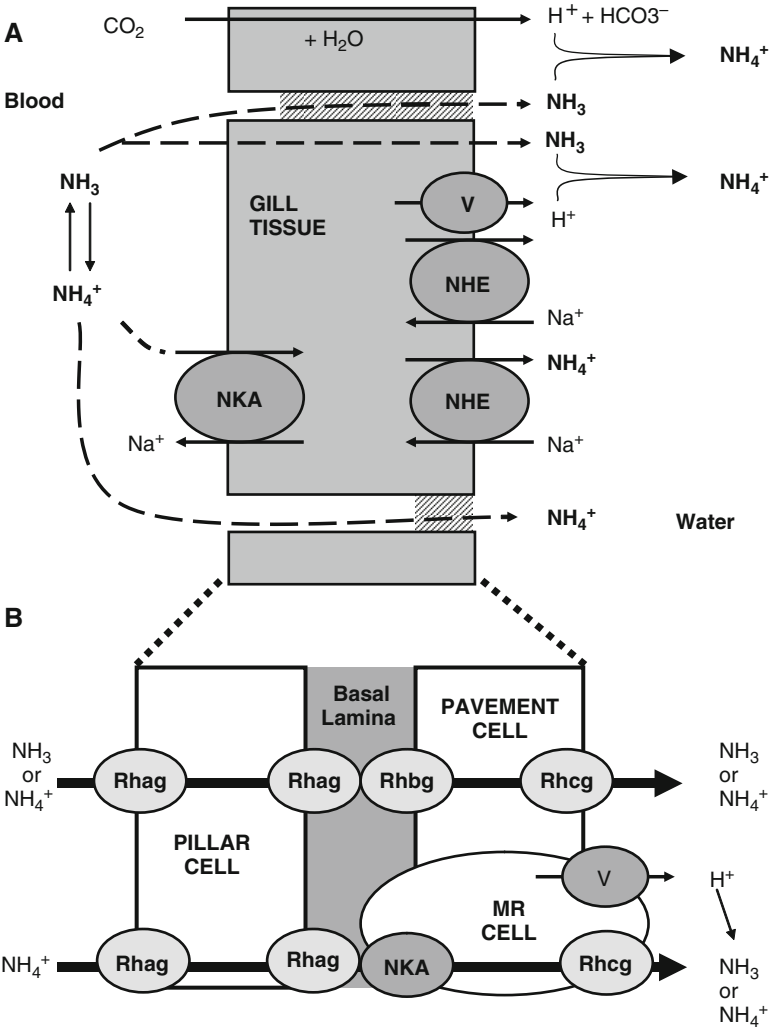

Fig. 2 **A** Schematic model of ammonia movement through fish gills. *Dashed lines* indicate diffusion, while movement through pumps and exchangers is represented with *solid lines*. NH_3 diffuses across the gills down the blood-to-water partial pressure gradient, where in many fish it is trapped as NH_4^+. The H^+ required for 'acid trapping' is produced by the hydration of CO_2, or is secreted directly into the water via the V-type H^+-ATPase (*V*), or in exchange for Na^+ through a Na^+/H^+ exchanger (*NHE*). NH_4^+ can diffuse out through the permeable paracellular spaces of marine fish gills (small tight junction) or it can be actively excreted by means of a basolateral Na^+/K^+-ATPase (*NKA*) and an apical *NHE*. **B** An expanded view of the gill tissue depicts the discrete tissue layers and the arrangement of ammonia transporting rhesus (Rh) proteins. *Rhag* is found on both surfaces of pillar cells, while *Rhbg* and *Rhcg* occur on the basolateral and apical surfaces respectively of pavement cells. Rh proteins appear to increase membrane permeability to both NH_3 and NH_4^+. Additional orthologues of Rhcg are expressed in mitochondria-rich (*MR*) cells, and NH_4^+ can enter these cells in exchange for Na^+ via NKA before leaving through the Rhcg transporter. Often closely associated with V-type H^+-ATPases, this coexpression provides the acidification required for the efficient acid trapping of NH_3. Modified from Wilkie (2002) and Nakada et al. (2007a)

Evidence supporting the linkage between boundary-layer acidification and T_{amm} excretion has come from studies demonstrating that changes in acidification of the boundary layer changed the rate of excretion of NH_3. NH_3 excretion fell drastically when CO_2 excretion was inhibited with the CA inhibitor acetazolamide (Wright et al. 1989), or when TRIS or HEPES buffer was added to the ventilatory water (Wright et al. 1989; Wilson et al. 1994). The increased buffer capacity of the water prevents boundary-layer acidification and limits the potential for acid trapping. However, as the internal ammonia levels rise, the blood-to-water gradient is re-established and excretion rates return to normal (Wilson et al. 1994). Similarly, maintenance of a high plasma ammonia level allows the creation of a favourable blood-to-water PNH_3 gradient in freshwater environments where boundary-layer acidification is impossible (e.g. in the heavily buffered water of Pyramid Lake, pH 9.4) (Wright et al. 1993).

Acidification of the boundary layer may not be limited to hydration of CO_2. The presence of V-type H^+-ATPases on the apical membrane of certain mitochondria-rich cells (Lin et al. 1994) or pavement cells (Sullivan et al. 1995) provides an alternative proton source for boundary layer acidification in freshwater fish. While the activity of these ATPases has been linked with Na^+ uptake in freshwater fish, their importance in ammonia excretion can be distinguished using amiloride. Blockade of Na^+ uptake alters the apical membrane potential, inhibiting electrogenic H^+-ATPase activity, and resulting in a drop in NH_3 excretion (Wilkie 2002).

Due to the heavily buffered nature of seawater, marine fish are unlikely to benefit from boundary-layer acidification. However, evidence of NH_3 diffusion remains. Injection of NH_4Cl into spiny dogfish (Wood et al. 1995), Atlantic hagfish (McDonald et al. 1991) or sculpin (*Myoxocephalus octodecimspinosus*) (Claiborne and Evans 1988) resulted in an increase in T_{amm} excretion and metabolic acidosis, suggesting that the NH_4^+ dissociated, crossing the gills as NH_3 and leaving behind the excess protons.

5.2.2 Passive Diffusion of NH_4^+

NH_4^+ diffusion is unlikely to be significant in freshwater teleosts owing to its ionic nature. The deep tight junctions (Sardet 1980) and low permeability to cations of freshwater fish gills probably preclude NH_4^+ diffusion (Evans et al. 2005). By contrast, the shallow junctions between epithelial pavement cells of marine fish can have high cation permeability, potentially allowing NH_4^+ diffusion (Evans et al. 1989). Evidence for this route comes from the failure of longhorn sculpin exposed to high water T_{amm} concentrations to exhibit metabolic alkalosis, which suggests that the ammonia species entering the fish was NH_4^+, rather than the basic NH_3 (Claiborne and Evans 1988).

5.2.3 Na^+/NH_4^+ Exchange

Although amiloride blockade decreases T_{amm} excretion in freshwater fish, as noted above this outcome probably reflects disruption of the apical membrane potential rather than a direct linkage between Na^+ and NH_4^+ (Avella and Bornacin 1989). In freshwater fish, altered membrane potential may impact activity of the proton pump, limiting T_{amm} excretion by decreasing boundary-layer acidification. In marine fish, however, the large inward Na^+ gradient and apical Na^+/H^+ exchangers (NHEs) provide the potential for Na^+/NH_4^+ exchange (Evans et al. 2005). Although marine fish possess the necessary exchangers, they may not be required, owing to the favourable blood-to-water gradients for passive diffusion of T_{amm}. There is very little evidence for a significant Na^+/NH_4^+ exchange under normal conditions; neither amiloride treatment nor Na^+ removal have an effect on T_{amm} excretion in a variety of marine species (Evans et al. 2005). Whether NHEs become more important during exposure to high external ammonia levels is unknown.

5.2.4 Active Excretion of T_{amm}

Certain fish possess the capacity to excrete ammonia against an unfavourable concentration gradient, a possibility that exists because NH_4^+ can substitute for K^+ in the Na^+/K^+-ATPase (Towle and Holleland 1987) and $Na^+/2Cl^-/K^+$ cotransporter (Good et al. 1984), and can penetrate bio-membranes through K^+ channels (Thomas 1984). The giant mudskipper, *Periphthalmodon schlosseri*, which can maintain constant internal $T_{amm}(150\ \mu M)$ and excretion rates in the face of high external pH and greatly elevated environmental ammonia concentrations (100 mM) provides the best example of this situation (Thomas 1984; Randall et al. 1999; Chew et al. 2003; 2007). Ammonia excretion in these animals is not sensitive to HEPES, indicating that diffusive acid trapping is not important under these conditions (Wilson et al. 2000). However, T_{amm} excretion has been shown to fall when fish were exposed to ouabain, an inhibitor of Na^+/K^+-ATPase or amiloride (Randall et al. 1999). Mitochondria-rich cells in *P. schlosseri* possess apical NHE2 and NHE3 (Wilson et al. 2000) and express high levels of basolateral Na^+/K^+-ATPase. The mechanism of ammonia excretion in these animals may be similar to that in mammalian renal proximal tubules (Evans et al. 2005), in which NH_4^+ is secreted across the basolateral membrane by substituting for K^+ on the Na^+/K^+-ATPase, thereby lowering intracellular Na^+ concentrations. These conditions stimulate NHE, and NH_4^+ is then excreted across the apical membrane via the NHE by substituting for H^+.

Another amphibious fish, the mangrove killifish *Kryptolebias marmoratus,* makes use of ammonia volatilization to survive long periods of air exposure (Frick and Wright 2002). Volatilization is made possible by greatly increasing the cutaneous NH_4^+ concentration and pH so as to favour gaseous NH_3 release (Litwiller et al. 2006). However, these same conditions will make NH_3 diffusion from the blood to the cutaneous boundary layer more difficult. How mangrove killifish

maintain T_{amm} excretion from plasma to skin is not currently known, but it may be that to maintain high NH_4^+ levels in the boundary layer fluid, *K. marmoratus* uses the same active excretion processes as *P. schlosseri*.

5.3 Problems with the Models

Most reviews invariably picture the gills as a single homogenous layer of cells that are universally permeable to ammonia. However, the gills are made up of several distinct cell layers that may be differentially permeable (or impermeable) to ammonia. Owing to its high water solubility and diffusivity (1,000 times that of CO_2; Wood 1993), ammonia is commonly assumed to move easily through cell membranes. However, ammonia is only moderately lipid-soluble (Wright 1995) and many lipid membranes are impermeable to NH_3, including those of the renal thick ascending limb (Kikeri et al. 1989) and *Xenopus* oocyte (Burckhardt and Frömter 1992). Among fish, the apical membrane of pavement cells from the gill of *Pleuronectes americanus* exhibited very low NH_3 permeability, while in *Squalus acanthias* the permeability of the basolateral membrane was twice that of the apical membrane (Hill et al. 2004). Even the assumption of NH_4^+ immobility is questionable, as a cultured gill epithelium from rainbow trout revealed greater permeability to NH_4^+ than NH_3 under conditions similar to those found in vivo (Kelly and Wood 2001). These results indicate that diffusion alone may not be sufficient for branchial ammonia excretion, i.e. that carrier-mediated transport may be required.

5.3.1 Rh Proteins

Ammonia transporters (Amt) in plants, methylammonium permeases (MEP) in yeast and rhesus (Rh) proteins in animals all serve to increase the flux of ammonia across the plasma membrane (Marini et al. 2006). In the last decade, several of the Rh proteins, long known to perform a structural role within RBCs, were discovered to be homologues to Amt proteins (Marini et al. 1997b, 2000). But while these intrinsic transmembrane proteins seem to function as CO_2 channels within green algae (Peng and Huang 2006), their main transport function in animal cells is for ammonia. When expressed in various heterologous expression systems (*Xenopus* oocytes, Nakhoul et al. 2006; HeLa, Benjelloun et al. 2005; yeast, Marini et al. 2000), mammalian orthologues always increased ammonia permeability. The members of the Rh family known to possess transport function include Rh A glycoprotein (Rhag), Rh B glycoprotein (Rhbg) and Rh C glycoprotein (Rhcg). In mammals, Rhag is found exclusively on RBCs, whereas Rhbg and Rhcg are found in a variety of tissues such as kidney, skin, liver, testes, ovary, and brain (Nakhoul and Hamm 2004). Interestingly, in liver and kidney, expression of Rhbg and Rhcg appears to be limited to the basolateral and apical cell membranes respectively (Eladari et al. 2002; Weiner et al. 2003; Quentin et al. 2003; Verlander et al. 2003).

Despite years of study of the Amt/MEP/Rh family, which have definitively demonstrated their ability to transfer ammonia across cell membranes (Kleiner 1985; Marini et al. 1994, 1997a; Ninnemann et al. 1994; von Wiren et al. 2000), whether ammonia is transferred as a gas (NH_3) (Peng and Huang 2006; Bostick and Brooks 2007) or as an ion (NH_4^+) (Verlander et al. 2003; Nakhoul et al. 2006) is less certain (Nakhoul and Hamm 2004; Mayer et al. 2006). Some of the confusion regarding the specific transport function of Rh proteins may arise from the widely varying experimental conditions used, but it is also possible that Rh proteins transport both the ionic and gaseous species of ammonia as suggested for the human orthologues of Rhag and Rhcg (Benjelloun et al. 2005; Bakouh et al. 2006).

Recently, the Rh proteins and their functional significance have been examined in fish. In an elegant study on the pufferfish *Takifugu rubripes*, Nakada et al. (2007a) identified four Rh protein homologues (fRhag, fRhbg, fRhcg1 and fRhcg2) that mediated methylammonium transport when expressed in *Xenopus* oocytes. In situ hybridization and immunohistochemistry clearly demonstrated that not only were the Rh proteins located on the gill, they possessed an orientation nearly identical to that found in mammalian kidneys (Fig. 2). While fRhag was localized to pillar cells, fRhbg and fRhcg2 were located on the basolateral and apical surfaces, respectively, of the pavement cells. Expression of fRhcg1 was detected only on the apical surface of mitochondria-rich cells, where it may be acting in concert with basolateral Na^+/K^+-ATPases to actively excrete ammonia (Nakada et al. 2007a). Rather than indiscriminate diffusion, ammonia may be following a specific pathway through the gill tissue from blood to water.

Rh proteins now have been found also in the gills of rainbow trout (Nawata et al. 2007), mangrove killifish (Hung et al. 2007), and zebrafish (*Danio rerio*) (Nakada et al. 2007b). Furthermore, their expression is inducible, and responsive to changes in the ammonia load. For example, the onset of ammonotely during development in zebrafish coincides with a marked increase in Rhcg expression (Fig. 3) (M. Braun, S. Steele and S.F. Perry, unpublished observations). Reported variation in Rh protein type and tissue location in these fish is not surprising, considering the wide range of habitats and lifestyles of these species. Nevertheless, Rh proteins appear to be a vital part of the ammonia excretion pathway in both marine and freshwater fish, and must be incorporated into existing models of ammonia excretion. Recent results reinforce the notion that acid trapping of NH_3 is crucial to the effective removal of ammonia. For example, Rhcg1 in zebrafish co-localizes with V-type H^+-ATPase (Nakada et al. 2007b), while in trout exposed to high environmental ammonia, increased expression of Rhcg2 and V-type H^+-ATPase occur simultaneously (Nawata et al. 2007). Active excretion of H^+ resulting in the conversion of NH_3 to NH_4^+ would increase the efficiency of NH_3 movement through Rhcg. Similar co-expression patterns of MEP and H^+-ATPase in fungi allow them to concentrate ammonia (Soupene et al. 2001), and this co-expression pattern in fish may allow ammonia excretion against a large gradient.

Answers to questions regarding regulatory mechanisms and reasons for the varied expression patterns remain elusive, and a larger number of species must be examined. For example, as yet only a single marine species has been investigated

Fig. 3 A comparison of Rhcg mRNA expression and ammonia excretion during development in zebrafish (*Danio rerio*) embryos. Expression levels were measured using real-time RT-PCR and have been calculated using the delta–delta Ct method relative to the expression of Rhcg at 1 day post-fertilization (dpf). Values are means ± 1 SEM with $N = 4$ (expression data) or $N = 8$ (excretion data). (M. Braun, S. Steele and S.F. Perry, unpublished observations)

and if, as suggested above, seawater interferes with acid trapping of NH_3, compensatory changes in the expression of Rh proteins may occur. The mangrove killifish demonstrated inducible expression of Rh proteins in both gills and skin when exposed to air (Hung et al. 2007); examination of other amphibious fish may provide insight into whether this expression pattern is broadly distributed in all these species or unique to killifish. Clearly, a full understanding of ammonia excretion in fish will only occur with a detailed examination of the functional significance of Rh proteins.

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Patterns of Acid–Base Regulation During Exposure to Hypercarbia in Fishes

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Abstract Acid–base regulation is one of the most tightly regulated physiological processes among vertebrates, and the specific mechanisms and patterns of acid–base regulation in fish have been investigated for decades, although primarily on a few species of teleosts and elasmobranchs. The most common response observed in fish during short-term (up to 96 h) exposure to hypercarbia (elevated environmental CO_2) is that of blood pH compensation for the induced respiratory acidosis by a net increase in plasma HCO_3^- in exchange for Cl^-, predominantly through processes at the gills. Studies on hagfish indicate that this pattern of pH compensation (i.e. net plasma HCO_3^-/Cl^- exchange, driving pH recovery) probably represents the ancestral state for fishes. Due to an apparent limit to this net HCO_3^-/Cl^- exchange, most fishes examined to date exhibit incomplete pH compensation for the acidosis, in both plasma and tissues associated with CO_2 tensions greater than 10–16 mmHg; in CO_2-sensitive fishes, this may be the basis for mortality during exposure to high CO_2. A few fish species, however, are capable of tolerating $PaCO_2$s well above 10–16 mmHg; in some of these species, this tolerance appears to be associated with the ability to completely regulate intracellular pH (preferential pHi regulation) of tissues, such as brain, muscle and liver, despite a large reduction in extracellular pH. We hypothesize that: (a) preferential pHi regulation in fish evolved in the ancestors of the pleisiomorphic freshwater (non-teleost) actinopterygiians, (b) is associated with high CO_2 tolerance, and (c)was an exaptation for air-breathing. A great deal of research remains to test these hypotheses, and to elucidate the origin and ubiquity of preferential pHi regulation among fishes and the cellular and molecular mechanisms involved.

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1 Introduction

Changes in pH can alter local charges on proteins, which will affect protein function through, for example, enzyme and membrane channel properties. These changes can in turn ultimately affect cellular processes such as cell-to-cell signalling, volume regulation and gene expression, as well as whole animal performance, including that relying on muscle contractility and metabolic pathways (see review by Putnam and Roos 1997). Consequently, pH homeostasis is central to survival in most vertebrates at both the cellular and extracellular level. All cells have some capacity for intracellular pH regulation (Putnam and Roos 1997), but the degree to which they defend cytoplasmic pH during an extracellular acid–base disturbance depends upon the origin and severity of the acidosis (i.e. environmental, respiratory or metabolic), the buffering capacity of the cell, and the degree to which the pH of the blood compartment can be actively altered during the disturbance (Truchot 1987). The specific mechanisms and general pattern of acid–base regulation in fishes have been studied extensively over the last several decades (for example, Heisler 1986, 1999; Goss et al. 1998; Claiborne et al. 2002; Evans et al. 2005; Perry and Gilmour 2006). While a great deal is known about this exciting field, much remains to be discovered at the molecular, cellular and organismal levels. While this chapter will first describe current models of acid–base regulation in fishes in a very brief summary of several recent excellent reviews (Claiborne et al. 2002; Evans et al. 2005; Perry and Gilmour, 2006), it will focus mainly upon new patterns of acid–base regulation observed in response to short-term (i.e. up to several days) hypercarbia (elevated environmental CO_2) in pleisiomorphic and air-breathing fishes.

2 Regulation of pH in the Blood and Extracellular Space

In vertebrates, an acid–base disturbance can be either minimized or compensated for by the following mechanisms: (a) physicochemical buffering with bicarbonate and non-bicarbonate buffers, (b) a change in ventilation to alter PCO_2 and thus pH, via the CO_2–HCO_3^- buffer system, or (c) net transport of acid–base equivalents between the cell and the blood compartment, and/or the blood compartment and the environment (See Evans et al. 2005). In terrestrial air breathers, bicarbonate buffering through ventilatory changes in blood PCO_2 plays a principle short-term role in acid–base regulation. In water breathers, blood PCO_2 levels are low relative to air-breathers due to the high ventilatory requirement for O_2 uptake and the high CO_2 capacitance of water relative to O_2 (Dejours 1988), and changes in ventilation can only slightly alter arterial CO_2 tensions. Thus, breathing can only have moderate effects on acid–base regulation in water-breathing fish. Clearly, more research is required to understand the role of breathing on acid–base status in fish (See Gilmour 2001; Perry and Gilmour 2006); however, the current consensus is that physicochemical buffering and net transport of acid–base equivalents (a and c

above) are the predominant pathways for respectively minimizing or compensating for pH disturbances in fish.

In general, the role of buffering is restricted to early periods of acid–base disturbance, and the buffer capacity of the blood and extracellular compartment is limited (Heisler 1986). Consequently, this mechanism cannot be heavily relied upon during an acidosis, and so net transport of acid–base equivalents between the fish and environment is of great importance, and the gills, kidney and intestine all play a role. The gills have traditionally been thought to account for approximately 90% or more of the net acid–base relevant ion transport in fish during compensation for an acid–base disturbance (See Heisler 1984; Claiborne et al. 2002; Evans et al. 2005), and as a result this organ has been investigated most intensively. Even so, exactly which molecular mechanisms are responsible for pH compensation remains largely uncertain (Evans et al. 2005; Perry and Gilmour 2006). Interest in the role of the kidney and intestine in whole animal acid–base regulation has increased of late (see Evans et al. 2005; Perry and Gilmour 2006 for reviews), and they may play a greater role than previously thought; however, it appears that quantitatively these organs remain secondary to the role of the gills. Many methods have been used to investigate acid–base regulation in fish (e.g. acid or base infusion, exercise, hypoxia, environmental acidification and alkalinization); however, this review will focus predominantly upon studies employing environmental hypercarbia. Hypercarbia is of current interest because of its environmental relevance associated with natural and anthropogenic processes, and historically it has been widely used because of its convenience as a tool to study acid–base regulation, as it can be used to generate a sustained acid–base challenge while PCO_2 is held constant. The following is a discussion of the sources of environmental hypercarbia followed by how fish compensate for the induced respiratory acidosis during short-term exposure (0–96 h) to elevated CO_2 tensions.

3 Environmental Hypercarbia

Environmental hypercarbia has been used extensively by physiologists as a tool to investigate the mechanisms and patterns of acid–base regulation in fish, and the relative contributions of the gills and kidney, in particular, to pH compensation. Consequently, a fair amount is known about fish responses to environmental CO_2 tensions of 7–14 mmHg. Hypercarbia is not only an experimental tool, however, as it also represents an acid–base challenge with great relevance both historically and currently. Hypercarbia occurs in both fresh and marine waters, and in tropical fresh water systems, for example, $PwCO_2$ levels of up to 60 mmHg (20- to 30-fold increase over the arterial PCO_2 of normocapnic fish) have been observed (Heisler et al. 1982; Ultsch 1996). Contributing factors can include thick surface vegetation, poor water mixing, thermostratification, high flora or fauna biomass, and anaerobic metabolism of micro-organisms (Heisler 1999; Ultsch 1996). While the degree of hypercarbia observed naturally in the marine environment is less, levels can still

reach 5–10 mmHg, for example, at depths of 200–500 m (Heisler 1986) or in tide pools (Burggren and Roberts 1991).

In addition to occurring naturally, hypercarbia can be induced through anthropogenic activities. Within aquaculture, an increasingly popular goal is to relocate fish-holding sites from, for example, open-sea pens to closed containment and thus re-circulating systems. While supplemental oxygenation within a re-circulating system permits increased biomass, it also results in hypercarbia (Sowerbutts and Forster 1981; Colt and Orwics 1991). As a result of other anthropogenic activities, globally projected increases in atmospheric CO_2 levels over the next several centuries are hypothesized to elevate surface-water CO_2 levels 5-fold, which may reduce the pH of these waters by 0.7 pH units (Caldeira and Wickett 2003). While this predicted level of hypercarbia is relatively low compared to the environmental and aquaculture-based levels described above, sequestering atmospheric CO_2 to deep ocean sites through high-pressure injection has been proposed as a means to prevent further increases in atmospheric CO_2. This procedure would potentially create a point source for CO_2 in the marine environment, which could result in CO_2 tensions greater than any naturally occurring levels, past or present, and consequently would create a severe challenge to marine organisms (Seibel and Walsh 2001). Given the anthropogenic potential for generating such high levels of hypercarbia, there is renewed interest in assessing CO_2 tolerance and understanding the compensatory physiological responses in fish during exposure to high CO_2 levels (10–50 mmHg, Hayashi et al. 2004; Ishimatsu et al. 2004, 2005; Kikkawa et al. 2004; Portner et al. 2004).

4 Acid–Base Compensation During Exposure to Hypercarbia

Studies on acid–base regulatory responses to hypercarbia in fishes are limited to a relatively small number of fish species, which are either teleosts or elasmobranchs (e.g. rainbow trout, *Oncoryhnchus mykiss*, Lloyd and White 1967; Wood and LeMoigne 1991; Larsen and Jensen 1997; Hyde and Perry 1989; Goss and Perry 1994; Bernier and Randall 1998; common carp, *Cyprinus carpio*, Claiborne and Heisler 1984; brown bullhead, *Ictalurus nebulosus*, Goss et al. 1992; *Anguilla anguilla*, McKenzie et al. 2002; *Conger Conger*, Toews et al. 1983; *Fundulus heteroclites*, Edwards et al. 2005; cod, *Gadus morhua*, Larsen et al. 1997; Tench, *Tinca tinca*, Jensen and Weber 1985; little skate, *Raja ocellata*, Graham et al. 1990; Wood et al. 1990; dogfish, *Scyliorhinus stellaris*, Heisler et al. 1988, *Squalus acanthias*, Claiborne and Evans 1992, Japanese amberjack, *Seriola quinqueradiata*, Ishimatsu et al. 2004; the bastard halibut, *Paralichthys olivaceus,* Hayashi et al. 2004). In general, the 'typical' acid–base regulatory response in these fishes consists of a respiratory acidosis followed by pH recovery over the following 24–96 h. This initial acidosis is rapid, where arterial PCO_2 equilibrates with water PCO_2 within minutes, and the pH of blood (pHe) and tissues (pHi) decreases as a function of both the newly-equilibrated CO_2 tension and non-bicarbonate (i.e. intrinsic) buffer value

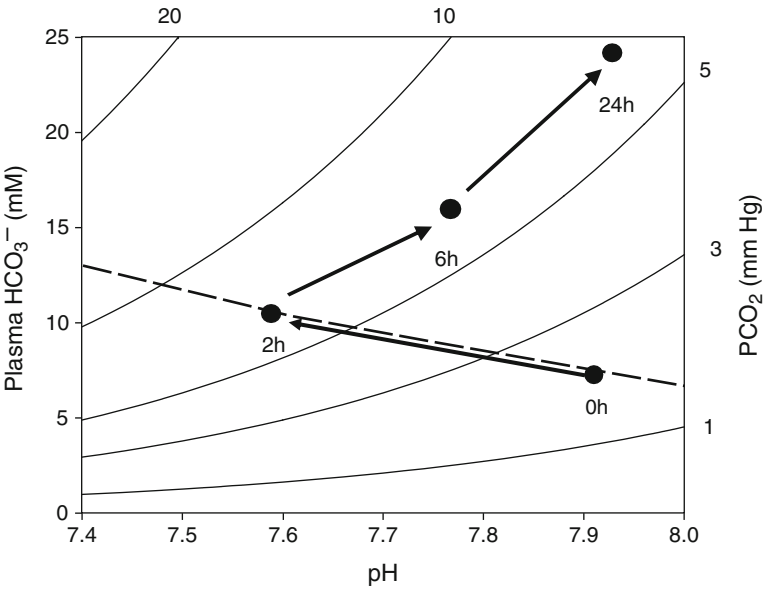

Fig. 1 The effect of sustained exposure to a $PwCO_2$ of 7 mm Hg on blood pH and plasma HCO_3^- in rainbow trout as represented on a $pH/HCO_3^-/CO_2$ plot. Isopleths are calculated based on previous pK' and solubility coefficients for CO_2 as reported by Boutilier and colleagues (1984). *Numbers* proximal to each data point represent exposure time; *arrows* indicate temporal pattern of change in blood pH and plasma HCO_3^-. The *dotted line* indicates the non-bicarbonate (i.e. intrinsic) buffer value of whole blood as reported by Wood and LeMoigne (1991) (see text for more details). Data from Larsen and Jensen (1997)

of the respective compartment. As a visual aid, this acidification is represented on a $pH/HCO_3^-/CO_2$ plot for rainbow trout, *O. mykiss*, in Fig. 1 (between 0 and 2 h, data are taken from Larsen and Jensen 1997). The dotted line on this and all subsequent $pH/HCO_3^-/CO_2$ plots (i.e. Figs. 1, 3, 4, and 6), represents the non-bicarbonate (i.e. intrinsic) buffering for whole blood. As tissues have greater intrinsic buffering than the blood, the initial intracellular acidosis during hypercarbia is less severe than in the blood, yielding a $\Delta pHi/\Delta pHe$ of 0.3–0.7 that is both tissue and species specific.

However, few studies have investigated this relationship between pHe and pHi in fish. In those few, pHi changes in a qualitatively similar pattern to pHe during a respiratory acidosis. This is the case for red blood cells, liver, and muscle in rainbow trout in vivo (although not the gill) (Wood and LeMoigne 1991; see Fig. 2). In vitro, isolated hepatocytes, when exposed to 1% CO_2 (7.5 mmHg), exhibited a decrease in pHi which stabilized within minutes, and did not change further over the following 60 min incubation. The resulting $\Delta pHi/\Delta pHe$ was 0.61 (Walsh et al. 1988) with a similar relationship observed in isolated hepatocytes during an isocapnic acidosis (Walsh et al. 1988). In primary hepatocyte isolations from the Antarctic eelpout (*Pachycara brachycephalum*) incubated in normoxia or 1% CO_2 (7.5 mmHg), depression of pHe through HCl addition at constant gas tension resulted in a depression in pHi, with a slope of $\Delta pHi/\Delta pHe$ of 0.4 after 50 min of

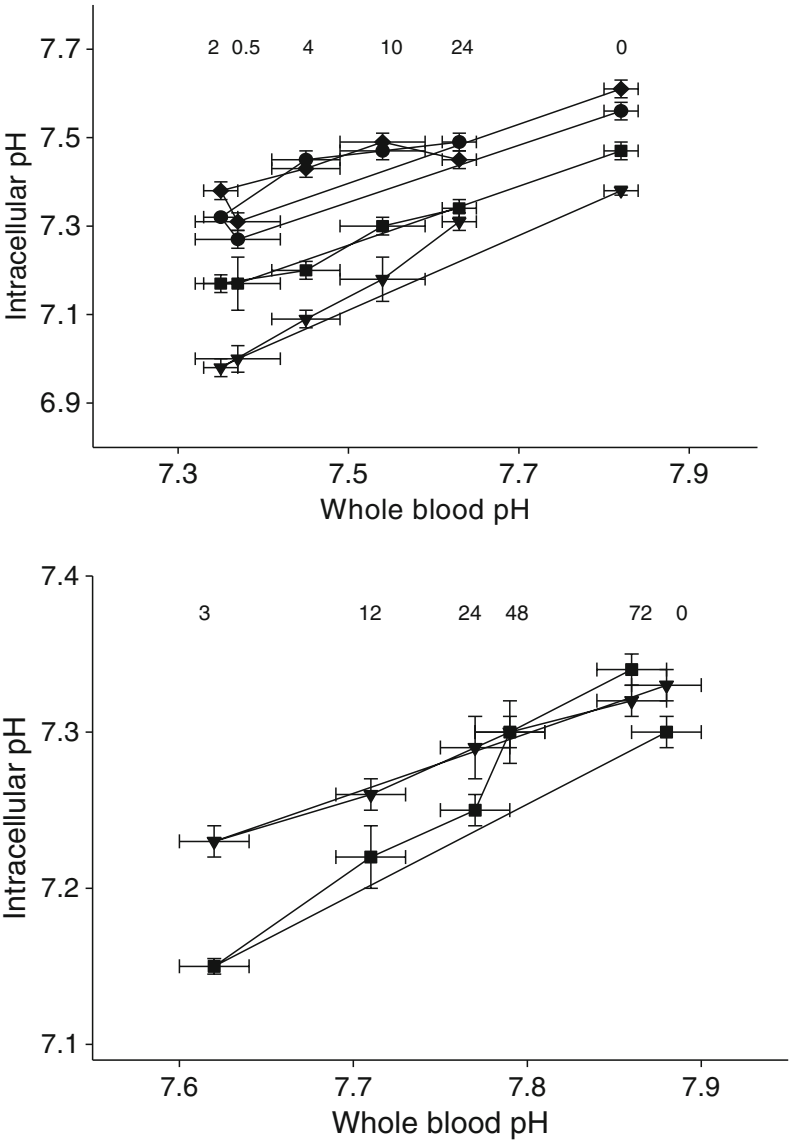

Fig. 2 The relationship between the pH of blood (pHe) and intracellular pH (pHi) of red blood cells (*inverted triangle*), brain (*circle*), heart (*diamond*), and white muscle (*square*) during exposure to short term hypercarbia in (*upper panel*) little skate, *R. ocellata*, and (*lower panel*) hyperoxia-induced hypercapnia in rainbow trout, *O. mykiss*. Note not all tissues are present in each panel. Time course (h) is indicated by *numbers* located directly above vertically oriented groupings. Note reversal of early time points (0.5 and 2 h) in *upper panel*. While pHi can recover more rapidly than pHe in tissues such as the brain and heart of skate, in all tissues presented here, pHi remains depressed if pHe does not recover. Mean values and SEM error bars approximated from Wood et al. (1990) for skate and Wood and LeMoigne (1991) for trout

incubation (Langenbuch and Portner 2003). In skate exposed to hypercarbia in vivo, a depression of pHe resulted in a reduction of pHi in red blood cell, heart, brain, and muscle consistent with that observed in trout; however, the brain and heart exhibited slightly more rapid pHi recovery than blood or other tissues (Fig. 2, data from Wood et al. 1990). Even so, the pattern of concurrent pH changes between pHe and pHi is a common pattern in the few studies published to date: despite this, some fish are able to defend pHi during large reductions in pHe (Heisler 1982; Brauner et al. 2004; Baker et al. in press) and these are described below.

Following a respiratory acidosis, pH recovery of the blood is associated with branchial acid–base relevant ion transfer, which over hours to days (Larsen and Jensen 1997), returns blood pH to normocapnic levels, through non-respiratory accumulation of HCO_3^-, which drives pH along the respective PCO_2 isopleth during sustained hypercarbia and is shown for rainbow trout in Fig. 1 (between 2 and 24 h). The elevation in plasma $[HCO_3^-]$ in most fishes studied to date is matched by an equimolar decrease in $[Cl^-]$ (Goss et al. 1998; Claiborne et al. 2002). While direct evidence for the specific cellular and molecular mechanisms associated with branchial acid–base relevant ion transport is largely lacking (Claiborne et al. 2002; Evans et al. 2005; Perry and Gilmour 2006), there are many studies that provide indirect evidence through, for example, changes in mRNA expression patterns and protein levels of putative transporters in the gills [i.e. Na^+/H^+ exchangers (NHE's), V-type H^+-ATPases coupled to apical membrane Na^+ channels (ENaC), HCO_3^-/Cl^- exchange via transporters belonging to the SLC26 and 24 multi-gene families] in response to acid- or base-loading events (Claiborne et al. 2002; Evans et al. 2005; Perry and Gilmour 2006). Further support for transport mechanisms comes from the experimentally observed morphological alterations of specific cell types in the gill epithelium (as described below) during exposure to hypercarbia and other acid–base disturbances (see Goss et al. 1998; Claiborne et al. 2002; Perry et al. 2003; Evans et al. 2005; Perry and Gilmour 2006 for excellent reviews).

While pH compensation for the respiratory acidosis during exposure to hypercarbia can be initiated quickly (within an hour), as indicated from the onset of increases in net H^+ efflux (for example, Wheatley et al. 1984; Edwards et al. 2005), a less rapid (hours to days) but extensive gill remodelling has been observed in a number of fish species, and is thought to play a role in pH recovery as well. The mitochondria rich cells (MRCS, also called chloride cells and most recently PNA+ MRCs) are thought to be the predominant site for Cl^- uptake and base secretion, while pavements cells are hypothesized to be the site of proton extrusion (see Perry 1997; Goss et al. 1998; Evans et al. 2005; Perry and Gilmour 2006). In rainbow trout and the brown bullhead, exposure to hypercarbia results in an increase in apical surface area of the acid-excreting PVCs (through proliferation of microridges) and a decrease in the fractional surface area of the base-secreting chloride cells (by physical covering by PVCs; Goss et al. 1994, 1995, 1998). By altering the cell surface area exposed to the environment, and thus sites for ion transport in the respective cell types, these morphological changes potentially aid in either increasing acid efflux or limiting base efflux during exposure to hypercarbia.

These responses described above (net HCO_3^-/Cl^- exchange and gill remodelling) together are effective in driving pH recovery during hypercarbia, and, albeit with modification, represent the paradigm for acid–base regulation during hypercarbia in water-breathing fish (Evans et al. 2005; Perry and Gilmour 2006). However, there are limitations to the use of this response to compensate for hypercarbia, which is the focus of the next section.

5 Limitations to Extracellular pH Compensation During Hypercapnia

As hypercapnia and the resulting acidosis increase in severity, the capacity of the fish to achieve complete pHe recovery through net HCO_3^-/Cl^- exchange becomes reduced. By extrapolation along the respective PCO_2 isopleth in Fig. 3 (see figure caption for more details), it is clear that a fish exposed to a PCO_2 of 30–50 mmHg would have insufficient Cl^- in the plasma to match the HCO_3^- accumulation necessary (i.e. $>$100–120 mmol l^{-1}) for complete pH compensation for the respiratory

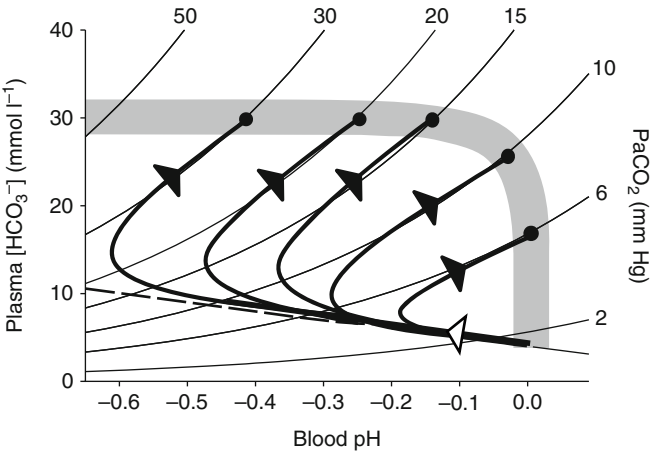

Fig. 3 A theoretical representation of the typical temporal response to short-term (less than 5 days) hypercarbia in fish. Upon transfer from normocarbia to hypercarbia, blood pH rapidly falls along the non-bicarbonate buffer line of the blood as indicated by *black open arrowhead*, and pH recovers along a given PCO_2 isopleth through a net increase in HCO_3^- in exchange for Cl^- as indicated by *black filled arrowheads*. *Black filled circles* represent final pHe values that would be achieved based upon limits to net HCO_3^- accumulation within 24–96 h of exposure to hypercarbia (see text for more details). *Shaded bar* indicates maximal pH compensation limited by the 'bicarbonate concentration threshold', as most fish do not increase plasma HCO_3^- beyond 25–35 mM (modified from Heisler 1986, 1999). Thus, compensation for a respiratory acidosis (within 48–96 h) during exposure to hypercarbia is incomplete above a PCO_2 of 10–15 mm Hg in most water-breathing fishes. Note that CO_2 isopleths are dependent on both temperature and osmolarity, and that the isopleths represented here are plotted for clarity purposes only

acidosis induced, and thus, ultimately, net HCO_3^-/Cl^- exchange has limits based on Cl^- availability; however, net HCO_3^- accumulation plateaus at HCO_3^- and corresponding PCO_2 levels much lower than this absolute limit. This 'apparent' limit has been referred to as 'the bicarbonate concentration threshold' (Heisler 1986, 1984). Because this bicarbonate concentration threshold (between approximately 27 and 33 mmol l^{-1}) is rarely surpassed (for the few exceptions, see Heisler 1986, 1999; Ishimatsu et al. 2005) during short-term exposure (<96 h) to hypercarbia, the acidosis induced by $PaCO_2$ tensions greater than 10–16 mmHg (resulting in an acidosis of approximately 0.25–0.4 pH units) cannot be fully compensated for in the blood of water-breathing fishes. This threshold was described over 20 years ago, yet the source of the limitation remains unresolved; however, there is evidence for the contribution of several factors.

For example, water conditions have been experimentally demonstrated to play a role in affecting the rate and degree of pH recovery during hypercarbia. Increasing water hardness (as measured by $CaCO_3$) increased the rate of normocapnic pH recovery in rainbow trout acclimated to the respective water types, so that compensation occurred earlier and was more complete than in softer water (Larsen and Jensen 1997). Carp acclimated to water with higher $[Ca^+]$ exhibited an increased rate of net bicarbonate influx into the plasma (Heisler 1999). Heisler proposed that increased calcium led to less permeable paracellular junctions, and reduced the rate of diffusive loss of HCO_3^- at the gill, allowing for greater net influx rates; this theory is yet to be verified experimentally.

It has also been suggested that increased salinity mediates faster pH recovery in fish exposed to short-term hypercarbia. Most support for this premise appears to be attributable to a perceived faster pH recovery in marine fishes (including elasmobranchs). Iwama and Heisler (1991) examined the rate of pH recovery during hypercarbia at two salinities (30 and 300 mOsm l^{-1}) in rainbow trout, and observed minor increases in the rate of pH recovery as environmental $[Na^+]$ and $[Cl^-]$ ions, (the only two ions manipulated in this study) increased. However, a review that examines pH recovery rates between or within a species has yet to be done, and attributing differences to environmental salinity is confounded by both the small sample sizes and species differences used in freshwater and seawater studies.

Interestingly, the effect of water pH on extracellular pH compensation during hypercarbia remains relatively unexplored, despite the fact that as CO_2 levels increase water pH decreases. To complicate the effect of water pH on net HCO_3^- accumulation, water $[HCO_3^-]$ is dependent on pH when PCO_2 is held constant: that is, more acidic water will have lower $[HCO_3^-]$ at the same $PwCO_2$ tensions. Furthermore, water pH may affect the rate of net H^+ efflux from branchial tissue; for example, the relative activity of V-type H^+ ATPase, which may play an important role in blood pH compensation during hypercapnia (Heisler 1999; Perry and Gilmour 2006), decreases as water pH decreases in rainbow trout (Lin and Randall 1990). In both dogfish and carp, pH recovery was severely compromised when water pH and $[HCO_3^-]$ were decreased at a constant $PwCO_2$ (Heisler 1986, 1999).

In summary, the rate and degree of pH compensation are influenced by ionic composition of the water. Furthermore, net HCO_3^- uptake in exchange for Cl^- for

complete pHe compensation appears limited as a viable response to CO_2 levels below 10–15 mmHg (see Fig. 3). Some species of fish do not survive CO_2 levels above this. For example, when exposed to $PaCO_2$ of 37.5 mmHg, the Japanese amberjack (*Seriola quinqueradiata*) died within 8 h. At the same tension, the bastard halibut (*Paralichthys olivaceus*) did not survive 48 h, and 17% had died by 8 h (Ishimatsu et al. 2004). Rainbow trout exposed to a PCO_2 of 30 mmHg did not survive even 12 h (Baker and Brauner, personal observation). The sensitivity of these species to hypercarbia is probably associated with exceeding the capacity to compensate for the respiratory acidosis. As environmental hypercarbia does occur at levels higher than 15 mmHg, it is no surprise that a few fish species have demonstrated the ability to tolerate CO_2 tensions well beyond the proposed limit of pH_e compensation. The strategies and mechanisms associated with this high CO_2 tolerance in these species will constitute the discussion of the remainder of this chapter; to facilitate conceptual ideas regarding the evolution of acid–base regulation, the following fish groups are discussed in order of phylogenetic progression.

6 Novel Patterns of pH Regulation in Response to Hypercarbia in Pleisiomorphic and Air-Breathing Fishes

6.1 Pacific Hagfish

Hagfish are the most basal extant craniate and many aspects of their physiology may be representative of the common ancestor of vertebrates (Holland and Chen 2001; Janvier 2007). Consequently, there is considerable interest in understanding their physiology. Hagfish are the only craniates that are ionoconformers; that is, they do not actively regulate Na^+ or Cl^- at levels much different from their environment (Morris 1965, Sardella et al. 2009); this strategy may represent the ancestral vertebrate state. Hagfish are burrowing animals, and this behaviour may include burrowing into mud or into a carcass during feeding for extended periods (Strahan 1963), probably severely limiting gill:water interaction and gas transfer. Therefore, it is likely that hagfish experience major disturbances to whole animal acid–base balance; however, this remains to be determined experimentally.

Hagfish possess MRCs in gills (Mallat and Paulsen 1986) which, it has been proposed, play a role in acid–base regulation, given that hagfish are ionoconformers (Evans 1984; Mallatt et al. 1987). In a few studies hagfish have been shown to be effective at whole animal acid–base regulation; both acid and base loads can be well-tolerated and compensated for (McDonald et al. 1991; Tresguerres et al. 2006). The gills of hagfish possess the ion-transporting proteins V–H^+-ATPase, Na^+, K^+-ATPase and NHE2, which are thought to be involved in acid–base regulation in teleosts (see above, and Evans et al. 2005; Perry and Gilmour 2006). Curiously, in hagfish all three transporters are localized to a single cell rather than distributed among two cell types, as seen in elasmobranchs and teleosts, where there are

different acid- and base-secreting cells (Tresguerres et al. 2006). Thus, hagfish represent the only water-breathing craniate with a single cell type for both acid and base excretion; no vertebrate studied to date exhibits this pattern. Concluding that the hagfish condition represents an ancestral state would be premature, but it remains an interesting and plausible possibility.

The CO_2 tolerance of Pacific hagfish (*Eptatratus stoutii*) is impressive in that they survive exposures of up to 50 mmHg for 96 h. This level of CO_2 induces a massive respiratory acidosis in the blood (greater than 1 pH unit within 3 h), which is reflected in the tissues. Remarkably, most of this pH decrease ($>70\%$ or 0.75 pH units) is compensated for within 96 h, representing the greatest extracellular pH compensatory capacity in a water-breathing animal studied to date (Baker, Sardella, Rummer and Brauner, unpublished). During the initial acidosis and recovery, the $\Delta pHi/\Delta pHe$ in the red blood cell was 0.65, and that in the muscle, liver and heart varied from 0.35–0.6, similar to that described above for trout and skate. Plasma pH compensation in hagfish is associated with net bicarbonate accumulation as high as 100 mmol l^{-1} (mean values of approximately 75 mmol l^{-1}; Baker, Sardella, Rummer and Brauner, unpublished data), well above the previously described 'bicarbonate concentration threshold' observed in other fishes (refer to Fig. 3). Plasma HCO_3^- accumulation is associated with an equimolar reduction in plasma Cl^-, indicating that pH compensatory ion exchange in hagfish is consistent with that of the typical response described above (i.e. net HCO_3^-/Cl^- exchange), but with an approximately threefold greater capacity. While the capacity for compensation in hagfish is exceptional relative to water-breathing fishes, net non-respiratory HCO_3^- accumulation expressed as a proportion of normocarbic plasma Cl^- is slightly greater than 15% (66–86 mM $[HCO_3^-]$/455 mM $[Cl^-]$), very close to the proportion observed in other fishes (about 20%, 25–30 mM $[HCO_3^-]$/120 mM $[Cl^-]$). While hagfish differ from water-breathing fishes in many ways (e.g. gill morphology), which could be the basis for their tremendous acid–base regulatory ability, it is intriguing to think that absolute plasma Cl^- levels may set the limits of net HCO_3^-/Cl^- exchange associated with pH compensation. While difficult to conclude or address, the tremendous increase in the capacity for acid–base regulation that this would confer to a seawater ionoconfomer may represent an important advantage to what is otherwise just thought of as the ancestral state (i.e. ionoconforming) in vertebrates.

6.2 Sturgeon

Sturgeons are another group of fishes that are very hypercarbia tolerant. They represent an ancient chondrostean family of fishes over 250 million years old, and have enormous value for studying vertebrate evolution, including physiological adaptations to the environment (Cech and Doroshov 2004). White sturgeon, *Acipenser transmontanus*, exhibit a biphasic response during exposure to hypercarbia. When exposed to CO_2 tensions below 14 mmHg, levels within the capacity for pHe compensation (see Figs. 1, 3), blood pH recovery is relatively rapid (within 24 h)

and similar to that described above as the typical response in fishes (Baker and Brauner, unpublished observations). Blood HCO_3^- is elevated to 25–30 mmol l^{-1} in exchange for Cl^-, and changes in gill morphology occur that are similar to those described for trout and catfish (Goss et al. 1994, 1995, 1998), that is, there is a reduction in apical surface area of mitochondria-rich cells and an increase in the apical surface area of pavement cell in the gills (Baker et al. in press). However, at higher CO_2 tensions, the response to hypercarbia is quite different. White sturgeon, *A. transmontanus*, can tolerate hypercarbia of 30 mm Hg for days, despite an extended acidosis in arterial blood (Crocker and Cech 1998) and both juveniles and adults can tolerate a PCO_2 of over 45 mm Hg CO_2 for days without mortality (Baker and Brauner, unpublished data). During exposure to a PCO_2 of 30 and 45 mmHg, net bicarbonate accumulation (and associated Cl^- loss) is almost absent, and consequently pHe compensation is minimal during exposure to these high CO_2 levels (Fig. 4). Although a depression in pHe of this magnitude would be expected to greatly reduce pHi in some fishes (Fig. 2 and discussion above) complete intracellular pH protection is achieved in heart, brain, liver and white muscle during both transient (i.e. 6 h, 11 mm Hg CO_2) and extended (i.e. 48 h, 23 and 45 mm Hg CO_2) pHe depression in sturgeon (Baker et al. in press). This tremendous capacity to protect intracellular pH, which will be referred to as preferential pHi regulation from this point forward (and is defined as a $\Delta pHi/\Delta pHe = 0$ within 3–6 h

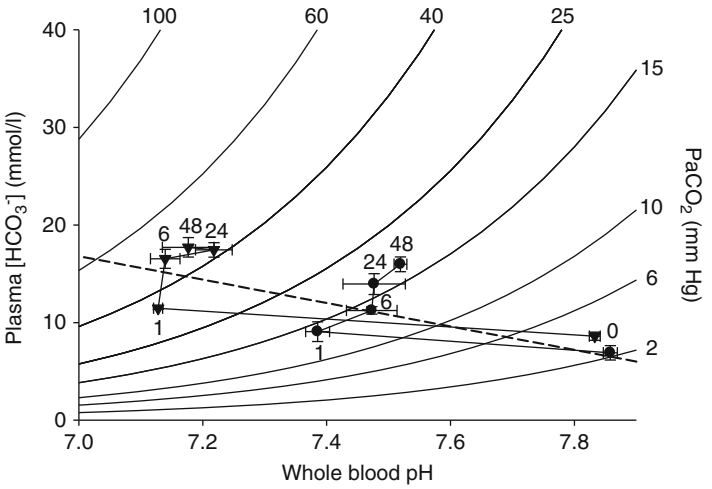

Fig. 4 The effect of sustained exposure at PCO_2 of 23 (*circles*) and 45 (*inverted triangles*) mm Hg on blood pH and plasma HCO_3^- in cannulated white sturgeon, *A. transmontanus*. Numbers proximal to data points indicate time (h) after transfer. The *dashed line* indicates the non-bicarbonate buffer value of whole blood as measured experimentally. Note that during exposure to both CO_2 tensions, blood pH drops below the blood buffer line, indicating preferential HCO_3^- transport into the tissues (Heisler 1982). Although sturgeon can accumulate up to 28 mM HCO_3^- during exposure to a PCO_2 of 11 mm Hg (see text), at higher CO_2 tensions net non-respiratory HCO_3^- accumulation does not exceed 5 mM in the plasma, and thus does not approach the proposed bicarbonate concentration threshold. Modified from Baker et al. in press

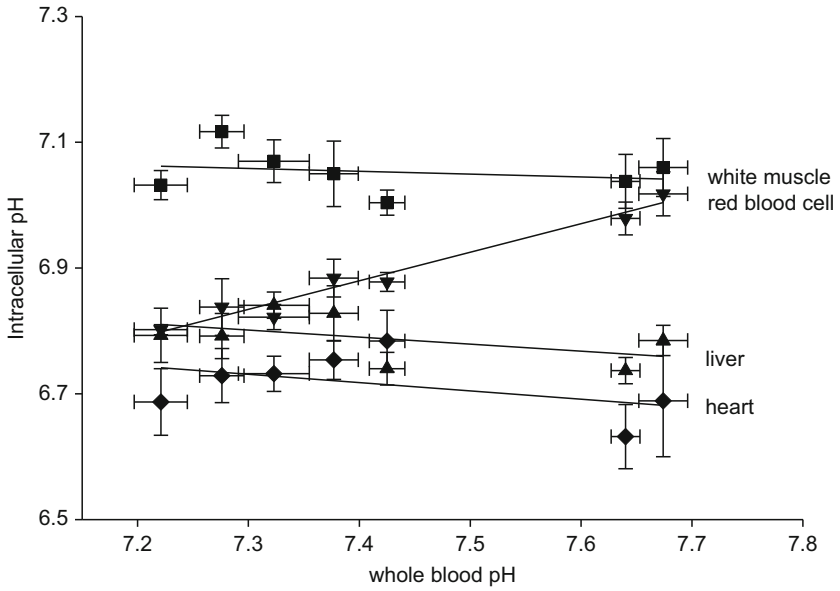

Fig. 5 The relationship between the pH of blood (pHe) and intracellular pH (pHi) of red blood cells (*inverted triangle*), heart (*diamond*), white muscle (*square*) and liver (*upright triangle*) following transfer to hypercarbia in the armoured catfish, *L. pardalis*. Armoured catfish were exposed to 14 or 28 mm Hg for up to 48 h (for a more detailed description of time course, see Brauner et al. 2004). Note that although pHe is greatly depressed, pHi is maintained at a constant value that did not differ between 6 and 48 h. Data for the armoured catfish is from Brauner et al. 2004

following exposure to hypercarbia) is not due to inherent buffering characteristics of the tissues, as they are not higher than that found in trout (Baker et al. in press), but probably reflect activation of cellular acid-extruding mechanisms, as indicated by the depression of blood HCO_3^- below the blood buffer line during exposure to hypercarbia (Fig. 4). The latter is indicative of HCO_3^- uptake into the tissues associated with maintenance of pHi (Heisler 1982). Preferential pHi regulation during hypercarbia has also been found in other fishes: however, all examples of this magnitude of pHi regulation to date have been observed in air-breathers. Consequently, this finding in white sturgeon is novel in that it is both the most basal vertebrate and the first non-air-breathing fish to exhibit this capacity for preferential pHi regulation. Furthermore, it seems entirely plausible that this may be the basis for the high tolerance of white sturgeon to hypercarbia and other acid-loading events.

6.3 Air-Breathing Fish

In bimodal breathers, the transition from water to air breathing is associated with a respiratory acidosis (Dejours 1981), and thus bimodal air-breathers may be quite tolerant to hypercarbia; this hypothesis has not been explicitly investigated. The

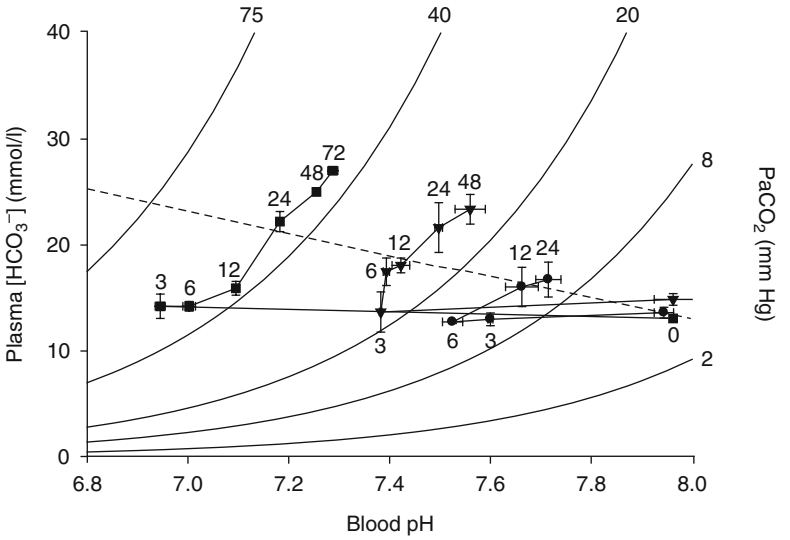

Fig. 6 The effect of sustained exposure to a PCO_2 of 11 (*circles*) and 23 (*inverted triangles*) or 45 (*squares*) mm Hg on blood pH and plasma HCO_3^- in the bowfin, *Amia calva*. *Numbers* proximal to data points indicate time (h) after transfer. The *dashed line* indicates the non-bicarbonate buffer value of whole blood. Note that up to 12 h following transfer to any of the hypercarbic treatments, blood pH is below the blood buffer line, suggesting preferential HCO_3^- transport into the tissues (Heisler 1986). Note that net non-respiratory HCO_3^- accumulation is almost negligible after 24 h at 11 mm Hg, and very similar between 23 and 45 mm Hg after 48 and 72 h respectively. Data are from Baker and Brauner, unpublished observations

pleisiomorphic facultative air-breather *Amia calva*, (common name bowfin) is very tolerant to hypercarbia. During exposure to a PCO_2 of 11, 23 and 45 mmHg, *A. calva* experience a respiratory acidosis in the blood that is largely uncompensated over 24 and 48 h respectively. Blood HCO_3^- levels during this time drop below the blood buffer line, indicating possible HCO_3^- transport into the tissues from the blood, as discussed for sturgeon above (Heisler 1982; Fig. 6). Intracellular pH of tissues were not measured in this study, but similarities to the patterns and tolerance of *A. transmontanus*, as discussed above, suggest that *A. calva* may use a similar strategy to sturgeon in tolerating hypercarbia, and are good candidates for preferential pHi regulation under these conditions.

In the facultative air-breathing teleost, the marbled swamp eel, *Synbranchus marmoratus*, aquatic hypoxia ($PO_2 = 16$ mmHg) induces air-breathing which results in a respiratory acidosis (Heisler 1982). Blood PCO_2 increased from 6 to 26 mmHg, and blood pH fell from 8.15 to 7.55 over 10 h, with little or no compensation in pHe over the following 4–5 days of maintained aquatic hypoxia. While air-breathing, the fish 'filled its buccal cavity with air and floated with its head on the water surface, with the rostral half of it in air'. During this time, there would not have been continuous contact between the gills and environmental water, and thus it is not surprising that it could not correct for the respiratory acidosis, given that the gills are the primary

site for acid–base compensation in other fishes. Despite the severe, uncompensated blood respiratory acidosis, pHi of skeletal and heart muscle following 4–5 days of air-breathing (the only time point measured) was not significantly different from water-breathing controls. This was the first observation of what appears to be preferential pHi regulation in tissues in fish. This regulation of pHi was associated with net transfer of HCO_3^- (presumably in exchange with Cl^-) from the plasma to the intracellular compartment, as indicated by blood HCO_3^- levels dropping below the blood buffer line, which was observed in white sturgeon and *A. calva* above. Thus, during an air-breathing-induced respiratory acidosis in *S. marmoratus*, pHi is preferentially regulated over pHe. Further investigation into the time course over which preferential pHi regulation may be occurring during air-breathing in *S. marmoratus* might prove extremely interesting, as might the acid–base regulatory response of these fish to aquatic hypercarbia; both of these remain to be investigated.

Another facultative air-breathing teleost, the armoured catfish, *Liposarcus pardalis*, is remarkably tolerant of aquatic hypercarbia. During exposure to a PCO_2 of 42 mmHg, blood pH dropped from 7.90 to 6.99 within 2 h, and there was minimal compensation over the following 96 h (Brauner et al. 2004). At 6, 24 and 72 h of exposure to hypercarbia (14 and 32 mmHg) there was no significant reduction in pHi of heart, liver, or white muscle, despite a largely uncompensated respiratory acidosis, again due to net intracellular uptake of HCO_3^- from the plasma (Fig. 5b). Thus, *L. pardalis* also has a tremendous ability to regulate intracellular pH in the presence of a large, almost completely uncompensated extracellular acidosis. In *L. pardalis* exposed to anoxia for 2 h, or following exhaustive exercise, a severe metabolic acidosis was observed in the blood (reduction in pHe of 0.4 and 0.7 pH units, respectively) but there was no significant effect on pHi of brain, heart or liver, indicating that intracellular pH is tightly regulated regardless of the source of the acidosis (Brauner, Baker, Hanson, Kuchel, Jackson and Val, unpublished).

It is not known whether preferential pHi regulation is a common characteristic of all facultative air-breathing fishes, as these are the only facultative air-breathing fishes in which it has been investigated to date. Clearly, more studies are needed to determine the ubiquity of preferential pHi regulation of this magnitude. Ultsch (1996) first pointed out that freshwater hypercarbia has been overlooked as a parameter influencing the transition of life from water to land. Adjustment of net plasma HCO_3^-/Cl^- exchange appears to be effective only in compensating for a respiratory acidosis at a PCO_2 below 10–16 mmHg, far lower than naturally occurring levels in tropical freshwaters, which, as mentioned earlier, can reach up to 60 mmHg (Heisler et al. 1982; Ultsch 1996). Preferential pHi regulation may have evolved as an adaptation to these high CO_2 tensions; and if it evolved in freshwater pleisiomorphic actinopterygiians, at a time when average global water temperatures, eutrophication and water CO_2 tensions were considerably higher than today (Clack 2007), preferential pHi regulation may have been an exaptation to air-breathing by providing a mechanism for dealing with the air-breathing-induced respiratory acidosis. Hypoxia would still represent the driving force for air-breathing in fishes (Graham 1997), but preferential pHi regulation would minimize the acid–base disturbances at the cellular level associated

with air-breathing. This intriguing and speculative hypothesis remains in need of experimental and conceptual support through further study.

7 Conclusions and Speculations

Acid–base regulation is one of the most tightly regulated physiological processes among vertebrates (Boron and De Weer 1976; Heisler 1986), and the specific mechanisms and patterns of acid–base regulation in fish (predominantly teleosts and a few elasmobranchs) have been investigated for decades (Heisler 1984, 1993, 1999; Claiborne et al. 2002; Evans et al. 2005; Perry and Gilmour 2006). The most generally described response in fish during short-term exposure to hypercarbia is to compensate for the induced respiratory acidosis by a net increase in plasma HCO_3^- in exchange for Cl^-, mediated through processes at the gills (Perry and Gilmour 2006). Most fishes investigated to date cannot completely compensate for the blood acidosis induced by CO_2 tensions greater than 10–16 mmHg, due to some apparent limit for net HCO_3^-/Cl^- exchange. Exposure to CO_2 tensions greater than this results in an uncompensated respiratory acidosis in the plasma, which in CO_2 sensitive fishes may be the basis for mortality. However, several fish species are capable of tolerating CO_2 tensions well beyond this limit, and studies on pleisiomorphic and air-breathing fishes are providing interesting insight into mechanisms associated with CO_2 tolerance.

The Pacific hagfish can tolerate direct transfer to a PCO_2 of greater than 50 mmHg, during which time blood HCO_3^- levels can reach almost 100 mM within 96 h, with an equivalent decrease in plasma Cl^-. Whether this exceptional ability to accumulate HCO_3^- in the plasma is associated with high Cl^- levels, the result of being a marine osmo- and ionoconformer, is unknown, but remains an interesting possibility. The hagfish is the most pleisiomorphic extant craniate; if its physiology is assumed to represent the ancestral state, then compensation for an acid–base disturbance through net HCO_3^-/Cl^- exchange at the gills is probably the ancestral condition in vertebrates. If the ancestral vertebrate which evolved in seawater (Bray 1985; Halstead 1985) was an osmoconformer, this may have conferred a tremendous capacity for acid–base regulation by virtue of high plasma Cl^- and potential for net HCO_3^-/Cl^- exchange. An addendum to this possibility is that the reduction in plasma ion levels in all other fish, including elasmobranchs to some degree, may have severely limited acid–base regulation when relying on net plasma HCO_3^-/Cl^- exchange at the gills.

In the more derived CO_2 -tolerant fishes studied to date, exposure to, and tolerance of, high CO_2 tensions appear to be associated with a combination of net HCO_3^-/Cl^- exchange (in sturgeon and *A. calva*) and tight pHi regulation, despite a large reduction in pHe. Sturgeon exposed to lower CO_2 tensions (10 mmHg) exhibit the typical response to hypercarbia, whereby they compensate for a respiratory acidosis with similar physiological and anatomical changes to those seen in teleosts. However, at CO_2 tensions of approximately 23 and 45 mmHg, which, as

mentioned above, precludes complete pH recovery by net HCO_3^-/Cl^- exchange at the gills (Fig. 3), this strategy is abandoned (i.e. there is virtually no pHe compensation), but there is complete regulation of pHi of heart, liver, brain and muscle. The exceptional CO_2 tolerance of sturgeon may be associated with the ability to regulate pHi tightly and preferentially despite a large reduction in pHe. To date, the only other fishes which have been demonstrated to regulate pHi preferentially despite a large and predominantly uncompensated respiratory acidosis are, possibly, *A. calva* (another pleisiomorphic actinopterygiian) and two teleost facultative air-breathers, *S. marmoratus* and *L. pardalis*. The other extant non-teleost actinopterygiian groups (reedfish and bichirs, paddlefish and osteoglossomorphs) are in general extremely hardy, largely facultative air-breathers (Brauner and Berenbrink 2007; Ilves and Randall 2007), and are promising candidates for preferential pHi regulation, however, this remains to be determined.

We hypothesize that preferential pHi regulation in vertebrates evolved in the pleisiomorphic (non-teleost) actinopterygiians, as it does not exist in hagfish or elasmobranchs (but remains to be investigated in lampreys), may be associated with high CO_2 tolerance, and may have been an exaptation to air-breathing. Clearly, a great deal of work remains to test these hypotheses; however, if validated, these ideas may support Ultsch's (1996) proposal that freshwater hypercarbia has been overlooked as a parameter influencing the transition of life from water to land. Finally, almost nothing is known about the cellular/molecular basis for preferential pHi regulation, and consequently, it remains an exciting area for further research.

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Buoyancy Control in Aquatic Vertebrates

B. Pelster

Abstract Water-living animals typically face a buoyancy problem because the density of body tissues often exceeds the density of the water replaced by the body. Depending on the particular ecological niche, aquatic vertebrates and especially fish have adopted various behavioral strategies and/or special buoyancy devices to compensate for these density differences, in order to reduce overall energy expenditure. Lipid accumulation or gas cavities such as the swimbladder or the lung, for example, may significantly contribute to a reduction in overall body density. For some species swimming activity may be more efficient than special low-density tissues in order to stay at a certain water depth.

1 Introduction

Compared to terrestrial animals organisms living in aquatic environments are exposed to a high-density medium. The density of water is usually given as $1.00\,\mathrm{kg\,l^{-1}}$ for fresh water and about $1.026\,\mathrm{kg\,l^{-1}}$ for sea water. Water-living animals, therefore, are experiencing a buoyancy problem not known from terrestrial animals. Depending on the overall density of the animal's body, the animal may be neutrally buoyant, if it has the same density as the environmental water; positively buoyant, if the density is lower than that of the water; or negatively buoyant if it is higher. In fact, most animal tissues are denser than water. Accordingly, a large number of marine animals are benthic, i.e. they live at the bottom and their overall density is higher than that of the water.

To stay in the open water space is a problem, however, if the animal has a higher density than the surrounding water. Therefore, organisms that have successfully invaded the pelagic space show special adaptations to compensate, or at least

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partially compensate, for the higher density of most animal tissues, in order to a achieve a comfortable equilibrium between weight and volume of the displaced water body, and overall body mass and volume of the animal displacing it. Basically, two different strategies have been developed. To compensate for the higher density of most tissues, special tissues with a particularly low density may be used. With only few exceptions the density of lipids or fat is lower than 1.00 kg l^{-1}, making them effective buoyancy aids. An even more effective buoyancy aid is a gas cavity trapping air within body structures, because the density of gas is negligible at low or moderate hydrostatic pressures. The accumulation of fluid with a lower salinity than the surrounding water will also provide upthrust, as water density increases with increasing salinity. Combined with the use of special low-density tissues, some species also attempted to reduce the density of typically 'high-density tissues'. A reduction in skeleton mineralization, for example, will not provide upthrust, but it will significantly reduce the density of the skeleton, and thus increase overall buoyancy. Another strategy is the use of fins as hydrofoils, providing hydrodynamic lift during swimming.

This chapter will demonstrate the advantages and possible disadvantages of these strategies, all of which can be found in the vertebrate kingdom. All of the pelagic species use either a single strategy or structure as a buoyancy device, or have adopted several strategies at the same time. It should be mentioned, however, that compared to the number of benthic species the percentage of truly pelagic marine vertebrates is low. Benthic species take advantage of the high density of body tissues, simply abandon any buoyancy device, and dwell on the bottom of the ocean. An even smaller number of species adopted a semi-aquatic lifestyle, i.e. they are mainly terrestrial, but forage in the water. Given the bulk of literature available on this topic this chapter does not attempt to provide a complete account. The focus is on the various strategies and problems encountered, and for these different strategies typical examples are presented.

2 The Problem of Buoyancy

2.1 Hydrostatic Pressure

Organisms living in water are exposed to hydrostatic pressure, which increases by 1 atm for each 10 m of water depth. Animals living at the water surface experience a hydrostatic pressure of 1 atm, while at a depth of 100 m it increases to 11 atm. At physiological pressures the water itself is almost incompressible. With a small margin of error, the same appears to be true for lipids (Corner et al. 1969), although there may be small but significant changes in density induced by temperature-dependent changes in lipid fluidity encountered during vertical migrations (Clarke 1978a, 1970). Nevertheless, animals without a swimbladder typically experience only small changes in density with changes in depth.

Gas-filled cavities like the fish swimbladder, however, will change volume in proportion to changes in hydrostatic pressure according to the Gas law. Any change in volume of a gas cavity in turn will reduce the volume of the organism, but not reduce overall mass, and therefore change the buoyancy of the organism. The compression of gas increases its density, and therefore the difference between gas density and water density decreases with increasing water depth. This is certainly relevant for deep sea fish. Based on a report of the National Research Council (1928), Alexander (Alexander 1966b) calculated the specific gravity of oxygen as 0.6 kg l^{-1} at a pressure of 500 atm, equivalent to a depth of about 5,000 m.

2.2 Volume of Low Density Buoyancy Devices

The density of body tissues is quite variable (Table 1). Tissues with high water content can be expected to have a density close to water, but accumulation of heavy ions such as Ca^{2+}, for example, or tissue mineralization, significantly increases tissue densities. Thus, most tissues have a density higher than water, and bones usually have the highest density at about 1.3–1.5 kg l^{-1}, followed by cartilage, skin and muscle tissue. Average body density typically is in the range of 1.1–1.3 kg l^{-1}.

Tissue density (ρ) and volume (V) determine the specific weight of an animal. If overall density (ρ_a) and volume (V_a) of the animal are equal to the volume and density of the displaced water (V_w, ρ_w), the specimen has no weight, and neutral buoyancy is achieved

$$V_a \rho_a = V_w \rho_w. \tag{1}$$

The animal can float or hover in the water at a certain water depth and no movement is required. Total animal volume can be considered as the sum of the volume of the buoyancy organ plus the volume of all the other tissues

$$V_a = V_b + V_s, \tag{2}$$

where V_b is the volume of the buoyancy organ, and V_s represents the volume of all other tissues together. In order to identify the volume of a specific buoyancy device,

Table 1 Density of fish tissues in kg l^{-1}

Tissue	*Pleuronectes platessa*[a]	*Myoxocephalus scorpius*[a]	*Scyliorhinus canicula*[b]
Skin	1.054	1.070	1.128
Fins	1.092	1.151	–
Muscle	1.048	1.062	1.071
Liver	1.040	1.062	1.072
Head	1.300	1.530	1.165
Axial Skeleton	1.299	1.532	1.128

[a]Webb 1990;
[b]Bone and Roberts 1969

the equation can be written as

$$V_s\rho_s + V_b\rho_b = V_w\rho_w. \tag{3}$$

This gives the volume of the buoyancy organ as a fraction of the fish volume without this organ:

$$V_b/(V_s + V_b) = (\rho_s - \rho_w)/(\rho_s - \rho_b). \tag{4}$$

It becomes intuitively clear that the smaller the difference in specific density of the water and the buoyancy device, the larger the volume required for this organ in order to compensate for the higher density of the other tissues. For a gas-filled swimbladder a volume of about 5% of total body volume is required in seawater animals, and about 8% is necessary for freshwater animals. The situation is quite different for a lipid filled structure. Assuming a density of $0.86\,\mathrm{kg\ l^{-1}}$, it can be calculated that — depending on the density of the other tissues — the fractional volume of the buoyancy structure must be about 20–25% of the total volume in order to achieve the status of neutral buoyancy. Furthermore, if the animal grows this percentage of total volume has to be added to the buoyancy organ in order not to lose neutral buoyancy. This also means, however, that the additional 20–25% of body volume has to be transported during swimming, thus increasing drag.

2.3 Energy Expenditure

Although one intuitively would expect that being neutrally buoyant is energetically much cheaper than continuous swimming, a quantitative energetical description comparing the various strategies of achieving neutral buoyancy remains incomplete, or at least is based on a number of assumptions that are difficult to verify (Alexander 1990, 1972). The energy expenditure is, for example, dependent on body mass, which in turn is dependent on the buoyancy device, and on swimming speed. Nevertheless, some important conclusions are obvious: to remain at a certain depth it is generally desirable to use material of the lowest possible density as a buoyancy device. This would be a gas cavity, but a flexible-walled gas cavity changes volume with changing water depth. This means during descent gas must constantly be secreted into the gas cavity, and gas must be resorbed during ascent. Thus, descending in a state of neutral buoyancy requires energy expenditure for gas secretion. The energy required for gas secretion and to compress gas can be calculated according to the Gas law, but it is clearly dependent on the efficiency of the process of gas deposition and on the diffusive loss of gas out of the gas chamber (Alexander 1972, 1971).

A fluid- or lipid-filled buoyancy device, on the other hand, provides neutral buoyancy irrespective of depth, but due to the much lower difference in specific density requires a much larger volume. The additional power necessary to swim with a larger volume due to a buoyancy organ increases with the ratio of V_b/V_s. Accordingly, the larger the volume of the buoyancy organ the more expensive swimming will be.

If a fish swims very fast it may be more economical to use the fins as hydrofoils instead of introducing a special organ as a buoyancy aid. Alexander (Alexander 1990) calculated that for a fish with a body mass of 1 kg it becomes more economical to use hydrodynamic lift instead of a swimbladder if it swims more than 0.75 m s^{-1}. If squalene is accumulated, it becomes more economical at a speed of 0.45 m s^{-1}. The speed, however, at which using fins as hydrofoils is more economical than introducing a special organ as a buoyancy device increases with body mass.

3 Buoyancy Devices in Teleosts and Elasmobranchs

3.1 The Swimbladder

At least at moderate water depth gas has a density almost negligible compared to the density of water. Therefore, a very effective way to achieve neutral buoyancy is a gas-filled cavity such as the flexible walled swimbladder of the teleosts. The swimbladder originates as an unpaired dorsal outgrowth of the foregut (Fig. 1). In so-called physostome fishes during development the connection to the gut may

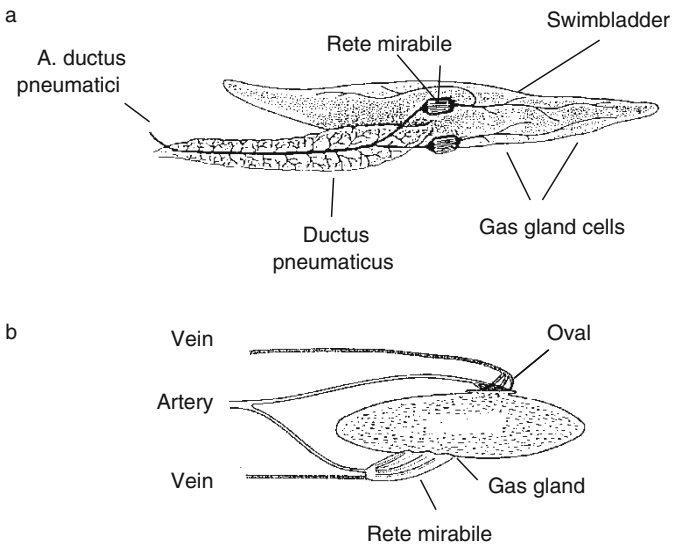

Fig. 1 **a** Swimbladder structure of a physostome fish (*Anguilla anguilla*) with a persisting ductus pneumaticus. In the eel, the ductus pneumaticus is converted to a resorbing section of the swimbladder. The rete mirabile is bipolar (modified after Dorn 1961). **b** Swimbladder structure of a physoclist fish (*Perca fluviatilis*). In perch, the rete mirabile is unipolar and in intimate contact to the gas gland. The oval represents the resorbing section of the swimbladder (modified after Denton 1960)

persist as the pneumatic duct. In the great majority of teleosts, however, this connection is completely lost at an early stage during development, and the swimbladder is a closed gas cavity (physoclist fishes). Deep sea fish (usually) are physoclist.

The structural diversity in general swimbladder morphology is remarkable and has been reviewed by Fänge (1953) and Steen (1970). In Cyprinids, for example, the swimbladder consists of two chambers, a thick-walled section in which gas can be deposited, and a thin-walled chamber in which gas can be resorbed. In other fish, a special section of the secretory bladder, the oval, can be closed off by muscular activity and is used as the resorbing part of the swimbladder (gadoid fishes). The eel is a physostome fish, but in the adult fish the pneumatic duct is transformed to a resorbing part of the swimbladder and functionally closed (Fig. 1).

The wall of the secretory bladder consists of a number of thin tissue layers, the inner epithelium, muscularis mucosae, sub-mucosa and tunica externa (Fänge 1953). The tunica externa represents a dense connective tissue capsule. The sub-mucosa is usually impregnated with guanine crystals (Lapennas and Schmidt-Nielsen 1977; Kleckner 1980a) or may include layered lipid membranes (Brown and Copeland 1978), rendering the swimbladder wall quite impermeable to gases in order to reduce diffusional loss of gases (Kutchai and Steen 1971; Denton et al. 1972; Lapennas and Schmidt-Nielsen 1977; Kleckner 1980a). The muscularis mucosa mainly consists of smooth muscle cells.

Crucial for the secretion of gas into the swimbladder are gas-gland cells in the swimbladder epithelium. Gas-gland cells are specialized for the production of acidic metabolites. In the eel (*Anguilla*), a model species for the analysis of swimbladder function, gas-gland cells are spread over the whole internal epithelium of the secretory bladder (Zwerger et al. 2002). In *Perca*, *Gadus* and many other species gas-gland cells are clustered together, forming a massive complex of several cell layers. In some fish a compact gas gland is formed by an extensive secondary folding of a single layer of epithelium (*Gobius*, *Syngnathus*) (Woodland 1911; Fänge 1983). For the functioning of gas-gland cells, an intimate contact with an extensive capillary vascular system is essential.

Gas-gland cells are cubical or cylindrical, with a size ranging from 10 to 25 µm to giant cells of 50–100 µm or even more (Fänge 1953; Marshall 1960). Gas-gland cells are polarized with some small microvilli on the luminal side, while the basal side is often more densely vacuolated, and shows a large number of infoldings known from other secretory or resorbing tissues (Dorn 1961; Copeland 1969; Morris and Albright 1975;Würtz et al. 1999). Gas-gland cells are characterized by the presence of only few filamentous or elongated mitochondria with few tubular cristae (Dorn 1961; Copeland 1969; Morris and Albright 1975; Jasinski and Kilarski 1969).

Crucial tor the functioning of the swimbladder is the blood supply. Blood supply to the secretory section of the swimbladder is characterized typically by the presence of a countercurrent system, a rete mirabile. The rete mirabile consists of several ten thousand arterial and venous capillaries arranged so that each arterial capillary is surrounded by several venous capillaries, and vice versa. The length of the capillaries in a rete can be several millimeters, and correlates with depth of occurrence of the species (Marshall 1972). In the macrourid fish, *Coryphaenoides*

acrolepis, a physoclist fish which can maintain neutral buoyancy between 830 and 1,668 m water depth, the length of the rete mirabile showed a clear correlation with water depth, but not with body length. Interestingly, the length of the rete mirabile of smaller specimens with a pre-anal fin length of less than 15 cm was not affected by depth of occurrence, but increased with fish size (Suetsugu and Ohta 2004).

The diffusion distance between arterial and venous vessels is about 1–2 µm (Stray-Pedersen and Nicolaysen 1975). After leaving the rete mirabile in an unipolar rete the capillaries almost directly supply a special area of the swimbladder epithelium, in which so-called gas-gland cells are located. From there they return as venous vessels to the rete mirabile. In a bipolar rete the capillaries at the swimbladder pole reconvene to only a few larger arterial vessels, which then give rise to an additional capillary system supplying the swimbladder epithelium. Although the rete mirabile is a crucial structure for the generation of high gas partial pressures, it is not present in all fish with a swimbladder (Berenbrink et al. 2005). Salmonids, for example, lack a rete mirabile.

3.1.1 Mechanisms of Gas Deposition

Although the term secretion typically implies an active process, either primary or secondary, 'gas secretion' into the fish swimbladder is passive, and is achieved by diffusion of gas molecules from the blood or from gas-gland cells into the swimbladder along a partial pressure gradient. Therefore the term 'gas deposition' may in fact be more appropriate. The high gas partial pressures necessary to establish a diffusion gradient between the blood and the swimbladder lumen are established by two mechanisms: the reduction of the effective gas-carrying capacity of blood in swimbladder vessels, and subsequent countercurrent concentration of gases in the countercurrent system, the rete mirabile.

The reduction of the effective gas-carrying capacity of swimbladder blood is brought about by the metabolic and secretory activity of the epithelial gas-gland cells. Although the oxygen partial pressure usually is high in the swimbladder epithelium, gas-gland cells are specialized for the anaerobic production of acidic metabolites (for review see Pelster 1995c). Glucose, the fuel for the production of anaerobic metabolites, is mainly removed from the blood, whereas internal glycogen stores do not appear to be important. Gas-gland tissue of various species incubated in vitro or artificially perfused with saline has been shown to produce large amounts of lactate (Pelster 1995a; Ewart and Driedzic 1990). Even under hyperbaric oxygen partial pressures, gas-gland tissue of *Sebastodes miniatus* continued to produce lactic acid, indicating the absence of a Pasteur effect (D'Aoust 1970). In vivo lactate formation has been demonstrated in only two species, namely the barracuda *Sphyraena barracuda* and the European eel *Anguilla anguilla* (Steen 1963b; Enns et al. 1967; Kobayashi et al. 1989b). A quantitative analysis of lactate and glucose metabolism of the active, gas-depositing swimbladder of the European eel revealed that about 75–80% of glucose taken up from the blood is converted into lactate (Pelster and Scheid 1993).

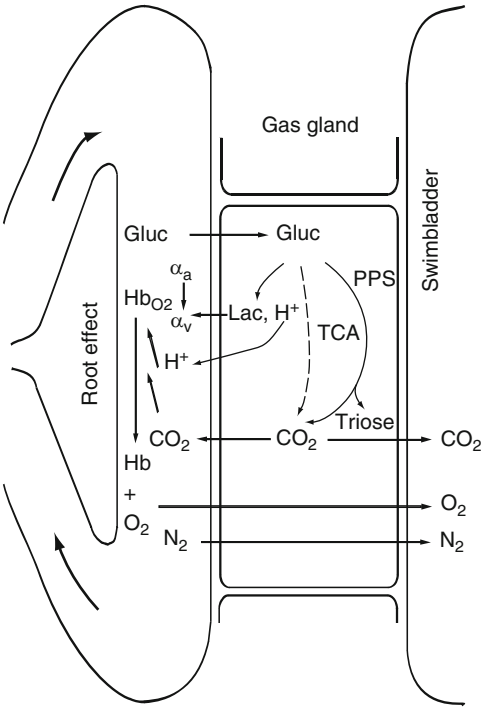

Fig. 2 Schematic diagram showing the metabolic pathways of glucose metabolism in swimbladder gas-gland cells. Lactic acid and CO_2 secretion reduce the effective gas-carrying capacity of the blood, and cause an increase in gas partial pressures in blood. *Arrows* indicate the direction of gas diffusion. Hb, hemoglobin, PPS, pentose phosphate shunt, TCA, tricarboxylic acid cycle, α, physical solubility of gases (modified after Pelster and Randall 1998)

A comparison of glucose metabolism combined with measurements of O_2 uptake and CO_2 production in the swimbladder of the European eel suggested that only 1% of the glucose removed from the blood was oxidized to CO_2 and water (Pelster and Scheid 1992, 1993). Nevertheless, the gas-gland cells produced significant amounts of CO_2 in the pentose phosphate shunt, i.e. without concomitant consumption of oxygen (Pelster et al. 1994;Walsh and Milligan 1993). Thus, results obtained from the European and the American eel demonstrate that glucose is mainly metabolized to lactic acid in the anaerobic glycolytic pathway, but there are significant amounts of CO_2 produced in the pentose phosphate pathway, while the contribution of the aerobic metabolism is almost negligible (Fig. 2). Because CO_2 is produced in the gas-gland cells, the highest partial pressure for CO_2 must be in these cells. Along the partial pressure gradient CO_2 diffuses into the blood, but also into the swimbladder lumen. In blood the acidic character of CO_2 causes an acidification:

$$H_2O + CO_2 \leftrightarrow H^+ + HCO_3^- .$$

The equilibrium of this reaction is rapidly established due to the presence of carbonic anhydrase activity. Inhibition of carbonic anhydrase has been shown to reduce

the rate of acid release from cultured gas-gland cells, and there appears to be cytoplasmatic as well as membrane-bound carbonic anhydrase activity (Pelster 1995b; Würtz et al. 1999).

Lactate and the accompanying proton are also released into the blood. Pathways for the release of protons from gas-gland cells include Na^+-dependent carriers, such as Na^+/H^+ exchange and Na^+-dependent anion exchange, and a proton ATPase (Pelster 1995b). Gas-gland cells must release acid over a wide range in pH. At the onset of gas deposition pH values can be expected to be in the alkaline range, while the fully active gas gland pH values must be highly acidic. In fact, pH values between 7.8 and 6.6 have been measured in swimbladder blood after passage through the gas gland (Kobayashi et al. 1990). It therefore is quite possible that the various mechanisms for proton secretion are characterized by differences in their pH dependence.

The acidification of the blood and also the increase in the salt concentration (osmolarity) of the blood induce a reduction in the effective gas-carrying capacity of the blood, which in turn results in an increase in gas partial pressures in the blood (Fig. 3), the so-called single concentrating effect (Kuhn et al. 1963).

The largest increase in gas partial pressure has to be expected for oxygen (Fig. 3b). Hemoglobin of many fishes is characterized by the presence of a Root effect (Brittain 2005; Pelster and Randall 1998; Pelster 2001), i.e. by a decrease in hemoglobin oxygen-carrying capacity with increasing acidification of the blood. While allosteric effectors typically modify the stability of the deoxygenated state of the hemoglobin, studies on the Root-effect hemoglobin of the spot *Leiostomus xanthurus* suggest that substitution of several amino acids results in a stabilization of the oxygenated R-state of the Root hemoglobin at low pH, promoting the transition to the deoxygenated T-state (Mylvaganam et al. 1996). In fact, several evolutionary pathways may have resulted in the generation of highly pH-dependent hemoglobins or Root effect hemoglobins, and it appears that protonation of Root-effect hemoglobins either destabilizes the R-state or stabilizes the T-state of the tetrameric protein (Bonaventura et al. 2004; Brittain 2005). pH dependent conformational changes may occur, and the primary structure of the hemoglobin alone may not necessarily be the sole criterion to make a hemoglobin a Root hemoglobin (Yokoyama et al. 2004). Fishes equipped with a swimbladder typically possess Root-effect hemoglobins, and the acidification of the blood during passage through the gas gland in these hemoglobins induces the transition from the oxygenated R-state to the deoxygenated T-state. Depending on the species between 2 and 80% of the hemoglobin may be deoxygenized (Berenbrink et al. 2005). Given a hemoglobin content of several $\mathrm{mmol\,l^{-1}}$ this allows for a large increase in P_{O_2} in swimbladder blood.

For CO_2 the situation is different. As already discussed, CO_2 diffuses from the gas-gland cells into the swimbladder lumen. In addition, the acidification of the blood during passage through the gas gland induces a marked increase in P_{CO_2}. Therefore, for CO_2 a large increase in gas partial pressure can be expected (Fig. 3c). Indeed, Kobayashi et al. (1990) observed an increase in P_{CO_2} from $4.1 \pm 0.9\,\mathrm{kPa}$ to $8.3 \pm 2.4\,\mathrm{kPa}$ during passage of the gas-gland cells in the European eel.

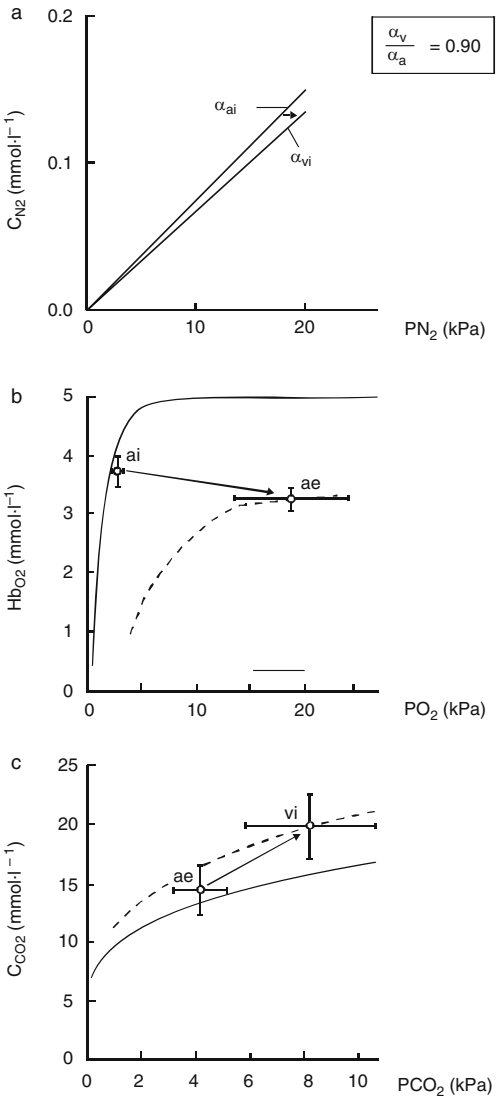

Fig. 3 Mechanisms that reduce the effective gas-carrying capacity in swimbladder blood (single concentrating effect). Metabolic end products of glucose metabolism mainly are lactic acid and CO_2, and both metabolites are released into the blood, initiating the single concentrating effect. **a** The increase in blood lactate concentration reduces the physical solubility of gas according to the salting out effect. Based on our present knowledge a salting out effect of about 1% can be expected, resulting in an increase in gas partial pressure of 1%. The figure shows a 10% decrease for clarification of the principle. **b** Acidification induces a severe increase in PO_2 via the Root effect. **c** Acidification shifts the equilibrium of the HCO_3^-/CO_2 reaction towards formation of CO_2, and CO_2 is produced in the metabolism, both contribute to a marked increase in P_{CO_2} (modified after Pelster et al. 1990). In **b** arterial influx (ai) PO_2 into the rete and arterial efflux (ae) PO_2 out of the rete mirabile of the eel is included to illustrate the effect of acid back diffusion in the rete on the onset of the Root effect. In **c** P_{CO_2} values of arterial efflux out of the rete and venous influx (vi) into the mirabile of the eel are included to illustrate the CO_2 release by swimbladder gas gland cells (data from Kobayashi et al. 1990)

The increase in lactate concentration causes an increase in the osmolarity of the blood, which in turn results in a decrease in the physical solubility of any gas, due to the salting-out effect (Fig. 3a). The increase in blood lactate concentration measured during passage of gas-gland cells in the eel is in the range of 5–10 mmol l^{-1} or even lower. This allows only for a minor decrease in physical gas solubility of no more than 1 or 2%, with a concomitant increase in gas partial pressures of the same magnitude (Pelster et al. 1988). Nevertheless, according to our present knowledge, the increase in gas partial pressures for inert gases such as nitrogen and argon can be induced only via the salting-out effect.

The release of lactic acid and of CO_2 from gas-gland cells thus causes an increase in the gas partial pressure of all gases in blood, and, following partial pressure gradients, gases will enter the swimbladder by diffusion. If the increase in gas partial pressures is not totally abolished by gas deposition into the swimbladder, the gas partial pressures in venous blood returning to the countercurrent system are higher than in the arterial blood supplying the swimbladder epithelium. Thus, back-diffusion of gas from the venous to the arterial capillaries of the rete mirabile results in a countercurrent concentration of gases in the swimbladder.

The basic principle of countercurrent concentration has been outlined by Kuhn et al. 1963). Functional analysis of the rete mirabile of the eel combined with model calculations revealed that the capillaries of the rete are permeable not only to gases but also to metabolites such as lactate, and that the countercurrent concentration of lactate enhances the salting-out effect (Kobayashi et al. 1989a, b). Figure 4 shows a theoretical plot of the concentrating ability of a countercurrent system for an inert gas, i.e. without chemical binding of gas. Important for the concentrating ability of a countercurrent system are the diffusing capacity and the permeability of the barrier between venous and arterial capillaries, blood perfusion and the magnitude of the salting-out effect. The enhancement in arterial inert gas partial pressure in the rete clearly increases with the magnitude of the salting-out effect, the larger the decrease in solubility and thus the initial increase in gas partial pressure (the single concentrating effect), the higher the maximal gas partial pressure achieved in the rete. Accordingly, very high gas partial pressures for CO_2 and O_2 can be generated in the rete, and these two gases are mainly deposited into the swimbladder (Kobayashi et al. 1990; Fänge 1983).

CO_2 plays a pivotal role in the concentration of gases in the swimbladder. Arterial P_{O_2} in the rete mirabile is enhanced not only by back-diffusion of oxygen, but also by back-diffusion of CO_2, because back-diffusion of CO_2 in the rete acidifies the arterial blood and increases arterial P_{O_2} by initiating the Root effect (Kobayashi et al. 1990).

Model calculations (Kobayashi et al. 1989a; Kuhn et al. 1963; Enns et al. 1967; Sund 1977), typically based on parameters taken from the eel with a capillary length of about 4–6 mm, clearly show that in the swimbladder gas partial pressures can be generated sufficiently high to explain the occurrence of fish with a gas-filled swimbladder at a depth of several thousand meters. The length of the capillaries of the rete mirabile increases with increasing depth of occurrence (Marshall 1972).

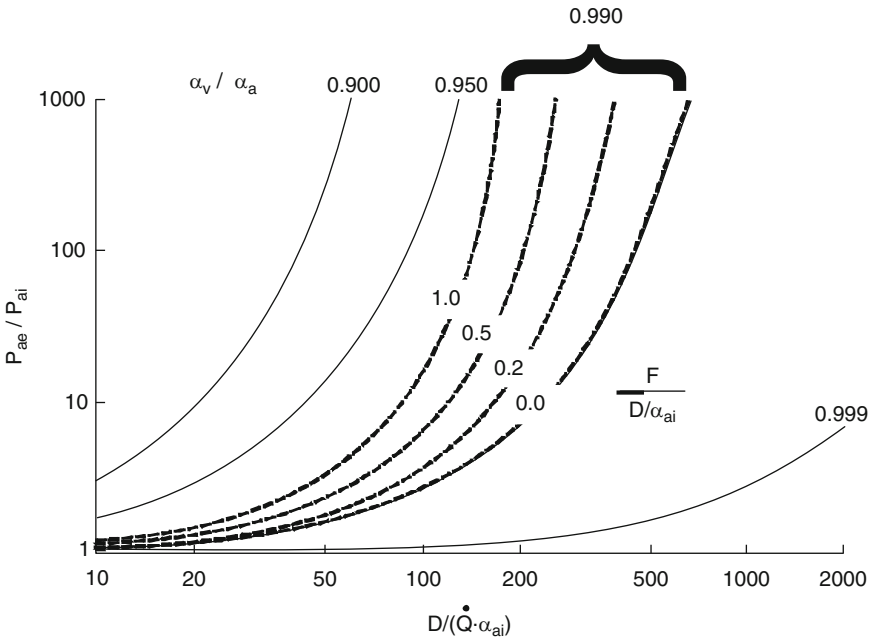

Fig. 4 Efficiency of the rete in enhancing inert gas partial pressure, calculated as the ratio of partial pressure in the arterial efflux and influx (P_{ae}/P_{ai}). The efficiency is given by the conductance ratio ($D/Q \cdot \alpha$), the magnitude of the salting out effect (α_v/α_a), and the permeability ratio of the rete ($F/D/\alpha$); (adapted from Kobayashi et al. 1989a)

Therefore, in deep-sea fish the concentrating ability of the countercurrent system should be even higher (Fig. 4).

The specific gravity of gas increases with gas pressure, so that the difference between swimbladder gas density and water density decreases with increasing water depth. Therefore, the effectiveness of a gas-filled swimbladder decreases at great depth. The maximum depth at which fish with a gas-filled swimbladder have been found is about 5,000–7,000 m (Marshall 1960; Nielsen and Munk 1964; Nybelin 1957). According to the calculation of Alexander (Alexander 1966b) the density of oxygen, representing the main swimbladder gas at this depth, is about 0.6–0.65 kg l^{-1}. Although this value is much higher than oxygen density at moderate water depth, it is still below lipid density. Therefore, even at a depth of 5,000–6,000 m, a swimbladder appears to be more effective in terms of achieving neutral buoyancy than lipid accumulation.

3.1.2 Resorption of Gas

In the same way as gas deposition gas resorption is driven by diffusion. Total gas partial pressure in arterial blood after passage of the gills is close to ambient

(i.e. close to one atmosphere or even below), and gas partial pressures in the swimbladder are therefore higher than in blood. In the oval or in the resorbing section of the swimbladder gases diffuse into the richly vascularized epithelia lining of this section of the bladder (Denton 1961; Steen 1963a). The quantity of gas resorbed per unit of time is dependent on the gas solubility in blood. As a result, the more soluble gases CO_2 and O_2 are preferentially resorbed, leaving behind the less soluble inert gases such as nitrogen (Piiper 1965). Consequently, CO_2 makes up some 20–30% of newly deposited gas (Meesters and Nagel 1935; Wittenberg et al. 1964; Kobayashi et al. 1990), but, under steady state conditions, contributes no more than 1 or 2% to swimbladder gas.

Gases are not only resorbed from the swimbladder in the special resorbing section, they are also lost from the swimbladder by diffusion through the swimbladder wall. Although the swimbladder wall is characterized by low gas permeability, it is never impermeable to gases. Consequently, with increasing water depth the diffusion gradient between the swimbladder lumen and the surrounding water increases, and therefore the diffusional loss of gas through the swimbladder wall increases as well. This may in part be balanced by a reduced gas permeability of the swimbladder wall at great depth (Denton et al. 1972; Kleckner 1980b). Nevertheless, the advantage of a gas-filled cavity as a buoyancy organ is clearly diminished at great depth; the energy expenditure to retain a gas-filled bladder increases with increasing water depth.

3.1.3 Control of Swimbladder Inflation

With a flexible walled swimbladder the status of neutral buoyancy can be maintained only if swimbladder volume can be kept constant in the face of changing hydrostatic pressures. This means that during descent gas has to be deposited, and during ascent gas has to be resorbed. Although the regulation of gas deposition is not really well understood, two parameters appear to be crucial for adjusting gas deposition. The first parameter is the metabolic activity of the gas-gland cells. The secretory activity of gas gland cells represents a critical determinant for the magnitude of the single concentrating effect in the blood. The second parameter appears to be blood flow through the swimbladder tissue and the rete mirabile, which is crucial for countercurrent concentration. Both parameters impinge on the extent of gas partial pressure enhancement in the countercurrent system, and thus the rate of gas deposition into the swimbladder of the European eel has been shown to correlate with the rate of proton secretion by gas-gland cells, as well as with the rate of blood flow through the swimbladder (Pelster and Scheid 1992). Other parameters influencing the effectiveness of the countercurrent system are, for example, the diffusing characteristics of the rete mirabile or the magnitude of the Root effect, but these parameters cannot be modified in the short run, and therefore can be neglected for short-term control of gas deposition.

In adult fish the swimbladder tissue is autonomically innervated, mainly by the splanchnic nerve, originating from the celiac ganglion, and the vagosympathetic

trunk. Vagotomy and also cholinergic blocking agents have been shown to impair gas deposition (Fänge et al. 1976; Lundin and Holmgren 1991). Near gas-gland cells adrenergic nerves could not yet be demonstrated, so that gas-gland cells appear to be under cholinergic, parasympathetic control (McLean and Nilsson 1981).

For the control of blood flow, however, adrenergic effects have been shown to be crucial. Electrical stimulation of the splanchnic nerve of the vagosympathetic trunk elicits vasoconstriction, and both nerves stain for catecholamines (Nilsson 1972; McLean and Nilsson 1981; Wahlqvist 1985). Perfusing the eel or the cod swimbladder with adrenergic agonists and antagonists clearly demonstrated the presence of adrenergic vasoreactivity. In a blood-perfused swimbladder preparation of the European eel the presence of α-adrenergic vasoconstriction close to the rete mirabile could be demonstrated (Pelster et al. 1994).

In addition to adrenergic vasoreactivity, VIP and substance P responsiveness has been demonstrated (Lundin and Holmgren 1991). Muscle cells within the swimbladder wall also appear to be under autonomic control, with contribution of α- and β-adrenergic reactivity (Stray-Pedersen 1970; Fänge et al. 1976). These muscle cells are responsible for shifting gas between secretory and resorbing sections of the bladder. Similarly, the oval appears to be under adrenergic control (Nilsson 1971; Ross 1978; Wahlqvist 1985).

Autonomic innervation of the zebrafish swimbladder is established only in later developmental stages. The zebrafish swimbladder is first formed as a single chamber inflated 1–3 days post hatching (dph), i.e. at the time of first feeding (the posterior chamber). Cranially this chamber gives rise to a second chamber, the anterior chamber (at 18 dph). Lateral nerves were present at first inflation of the chamber, but no autonomic innervation has been detected. The ductus communicans forms a constriction between the two chambers. At 27–28 dph it narrows significantly and can separate the two chambers. The anterior chamber appears to be involved in sound detection, while the posterior appears to be a buoyancy chamber. Initial innervation included peptidergic neurons with NPY- and VIP-like immunoreactivity. Tyrosine hydroxylase, indicating sympathetic innervation, was detected only at 22–25 dph, and no choline acetyltransferase-like immunoreactivity was found at this stage. Smooth muscle cells can be detected 22–25 dph. Structure and innervation suggest that the swimbladder volume cannot be actively controlled before 25–28 dph (Robertson et al. 2007).

3.1.4 Swimbladder Function During Vertical Migrations

Analysis of sound-scattering layers revealed that larval, and also some adult, fish routinely perform vertical migrations, spanning several tens or maybe hundreds of meters in water depth on a daily basis. Moving up and down in the water column requires constant adaptations in the rate of gas deposition or gas resorption, if a fish wants to retain neutral buoyancy. Because especially gas deposition is a slow process, the buoyancy status of fish during vertical migrations has attracted attention for a long time. Energetically it appears to be most efficient if fish performing

extensive vertical migrations use hydrodynamic lift to compensate for the change in hydrostatic pressure and keep the swimbladder volume constant, adjusted to neutral buoyancy at the upper water level (Alexander 1972; Gee 1983). Myctophids are well known for their migratory behavior. Acoustical analysis of sound-scattering layers suggests that small fish perform the migrations with constant swimbladder mass, and larger fish with a constant swimbladder volume, but at a volume that results in slightly negative buoyancy (Vent and Pickwell 1977; Kalish et al. 1986). On the other hand, some myctophids have been found with gas-filled swimbladders only in surface water during the night, while in deeper water the occurrence of inflated swimbladders decreased significantly (Neighbors 1992).

The ability to control vertical position has been correlated with diurnal rhythms, diversification of diet, or predation avoidance (Piet and Guruge 1997; Suetsugu and Ohta 2004; Trotter et al. 2005). Depending on the species (sea bream *Pagrus major*, for example), the larvae may inhabit deeper water during the day in a status of negative buoyancy, and migrate upwards by swimming at dusk. By increasing swimbladder volume the larvae achieve neutral buoyancy during the night (Trotter et al. 2005). The striped trumpeter (*Latris lineata*) spends daytime in the lower water layers with an almost neutral buoyancy status. During daytime body density was measured was 1.0260 g cm^{-3} in 1.0265 g cm^{-3} seawater, while at night time the body density decreased to 1.0245 g cm^{-3} and, being positively buoyant, the larvae rise to surface water layers. Thus, dual buoyancy control is achieved by diurnal changes in swimbladder volume, and the photoperiod appears to be a crucial stimulus for this behavior. Other species show the opposite behavior by moving downwards at dusk and returning to the upper water layers during daytime (Neilson and Perry 1990; Trotter et al. 2005).

Obviously, different strategies have been developed with respect to the buoyancy status, and each of the strategies may have its advantages. Being neutrally buoyant or close to neutrally buoyant at night time has been related to energy conservation, but also to a reduction of predation, because the larvae may hover motionless in the water (Uotani et al. 2000; Trotter et al. 2005). On the other hand, being neutrally buoyant at daytime, typically the feeding time for fish larvae, means energy conservation, because swimming and hunting in a status of neutral buoyancy requires less energy than swimming in a status of negative buoyancy (Trotter et al. 2005).

3.2 Lipid Accumulation

The density of lipids is generally lower than the density of water. Accumulation of lipids thus reduces the overall density of a body, and thus may serve as a buoyancy aid. The most widespread lipids are triacylglycerol, alkyldiacylglycerol, wax ester and squalene. Table 2 presents density values for the most common lipids. Density of lipids hardly changes with hydrostatic pressure (1–2% for a pressure change from 1 to 200 atm) (Clarke 1978b) and thus is often neglected. Density of lipids does change, however, with changes in temperature. Density of the spermaceti oil

Table 2 Specific gravities of lipids accumulated in vertebrates as a buoyancy aid (g cm^{-1}, $T = 21°C$). The gravity of wax esters, alkyldiacylglycerol and triacylglycerol varies slightly with chain length of the fatty acids and alcohol and with the degree of unsaturation (Sargent 1989; Phleger 1991). Spermaceti oil is a mixture of wax esters and triglycerides (Clarke 1978b)

Lipid	*Density*
Triacylglycerol	0.93
Alkyldiacylglycerol	0.91
Wax ester	0.86
Squalene	0.86
Cholesterol	1.065
Pristane	0.78
Spermaceti oil	0.86 (30°C, fluid)
Spermaceti oil	0.89 (20°C, crystalline)

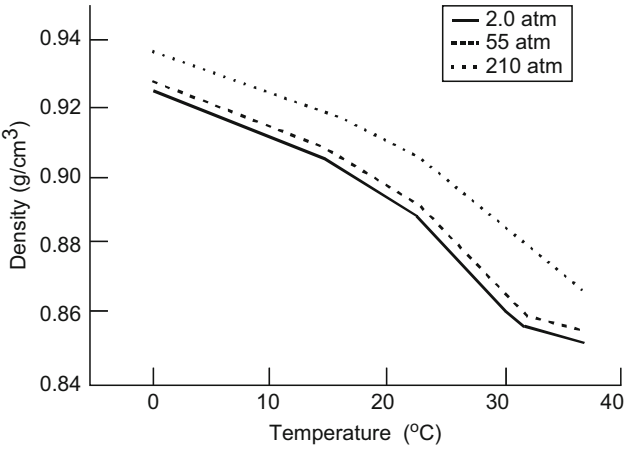

Fig. 5 Increase in spermaceti oil density, mainly composed of wax ester and triglyceride, with decreasing temperature (data taken from Clarke 1978b)

of the sperm whale, for example, composed mainly of wax ester and triglycerides, increases from 0.853 kg l^{-1} at 37°C to 0.925 kg l^{-1} at 0°C (Fig. 5) (Clarke 1978b). Temperature effects on the density of lipids may also apply to teleost fish, as reported for the orange roughy *Hoplostethus atlanticus* (Phleger and Grigor 1990). The lipid mainly accumulated in this species is wax ester (about 95%). At the surface (14°C) the fish was positively buoyant, but at the depth of occurrence — typically it is caught at 1,000 m, where the temperature is down to 6°C — the lipid is expected to be partly (17%) solid. This change in fluidity would increase the density of the lipid and give neutral buoyancy to the fish.

Lipid accumulation is a widespread phenomenon and, depending on the species, various organs may be involved.

Metabolically these lipids can be synthesized in various tissues (Nevenzel 1970, 1989; Grigor et al. 1990) but they can also be taken up from the diet. Lipids represent

a common fuel for aerobic energy metabolism. Therefore lipid stores may be useful not only as a buoyancy aid, but also as an energy reserve. Starvation has indeed been shown to cause a reduction in lipid stores (Benson and Lee 1975; Phleger 1987, 1988b; Phleger and Laub 1989). Triacylglycerols have an especially high turnover rate, whereas the metabolic turnover rate of alkyldiacylglycerol and wax ester, synthesized by reaction of a fatty acid with a long chain alcohol, is much slower. Burning the lipids will, however, increase overall density of the organisms, because it reduces the fractional contribution of the low-density components to the total body mass. In this case, to retain neutral buoyancy the fish must increase hydrodynamic lift by expending more energy for swimming activity. Lipid accumulation may also be influenced by changes in the diet. As a consequence, the composition as well as the amount of lipids accumulated, may be dependent on the nutritional status as well as on the actual metabolic activity of the animal.

3.2.1 Lipid-Filled Swimbladders

In midwater and deep-sea fishes, quite often large amounts of lipid may be present in the swimbladder instead of gas. Basically, there are two strategies for the accumulation of lipid in the swimbladder, fat-investment of regressed swimbladders, and fat-filled swimbladders, which are still fully functional in terms of gas deposition (Phleger 1991).

In fat-invested swimbladders fat mainly consists of wax esters and is deposited extracellularly. Fat-invested swimbladders are found in myctophids (Butler and Pearcy 1972), in the coelacanth *Latimeria chalumnae* (Nevenzel et al. 1966) or in the orange roughy *H. atlanticus* (Phleger and Grigor 1990). Regressed, fat-invested swimbladders appear to be quite common among midwater fishes which show extended vertical migrations that are difficult to perform in a status of neutral buoyancy, if a gas-filled swimbladder is present. Interestingly, histological analysis of gas-gland cells and the rete mirabile did not reveal any degeneration of these tissues in *Myctophum punctatum*, although the swimbladder was much smaller and the swimbladder wall was thickened (Kleckner and Gibbs 1972; Kleckner 1974, cited in Neighbors 1992). The extracellular localization of fat suggests that the lipid is not available for intermediary metabolism, but deposited purely to reduce the overall density of the fish.

Among deep sea fishes a fat-filled swimbladder is quite common (Morris and Culkin 1989; Phleger 1991). These swimbladders are fully functional in terms of gas deposition and are partially gas-filled. Because the oxygen content of swimbladders increases with increasing water depth, these swimbladders mainly contain oxygen. The lipid accumulated in these swimbladders mainly consists of cholesterol and phospholipid, often with unsaturated fatty acids (Phleger and Benson 1971; Phleger et al. 1978). Ultrastructural analysis of the membranous lipids of *Coryphaenoides* and *Parabassogigas* revealed that they exist as sheets of bilayered membranes (Phleger and Holtz 1973). Accordingly, large quantities of membranes can be isolated from these swimbladders (Josephson et al. 1975; Phleger et al. 1978).

In the swimbladder of many deep sea fish significant amounts of cholesterol are found. Cholesterol density is close to seawater density and therefore accumulation of cholesterol does not provide any lift. In phospholipid membranes, however, addition of cholesterol reduces the diffusion constant for gases approximately tenfold (Finkelstein 1976; Wittenberg et al. 1980). These considerations suggest that presence of cholesterol in the swimbladder of deep sea fishes would render the swimbladder wall more impermeable to gases and thus reduce diffusional loss of gas.

In addition, the swimbladder lipids may — depending on the species — be stored in various tissues, ranging from subcutaneous stores to stores in liver and bone tissue; occasionally it even appears to be in extracellular lipid sacs.

3.2.2 Lipid Accumulation in the Liver

While the liver usually makes up about 2–4% of the body weight, in sharks it may contribute up to 20–25%. The relative size of the liver has been shown to correlate inversely with the density of the liver tissue (Bone and Roberts 1969; Baldridge 1970). If the liver makes up more than 10% of the total body weight, the species is usually close to neutral buoyancy (Bone and Roberts 1969), and the liver may comprise up to 95% of the total lipid content of a specimen (Phleger 1988a; Van Vleet et al. 1984).

High squalene contents in liver lipid stores have been found in several families of sharks (Nevenzel 1989; Corner et al. 1969; Sargent et al. 1973; Hayashi and Takagi 1981; Van Vleet et al. 1984). Squalene is an intermediate of the cholesterol synthesis pathway. Metabolically, it is quite inert compared to the other lipids and can only be converted to cholesterol. Accumulation of squalene, therefore, is connected primarily to buoyancy adjustment.

In rays and in chimaeras, liver lipid consists mainly of diacylglyceryl ether, with only traces of squalene. Occasionally triglycerides and wax esters may also be accumulated in liver tissue. The accumulation of lipid in the liver apparently has been invented several times, independent of the systematic position (Nevenzel 1989; Bone and Roberts 1969; Baldridge 1970).

As already mentioned, the relative volume of accumulated lipid is quite large, due to the small differences in specific density between lipid and seawater. In addition, growing tissues must be compensated for by an appropriate accumulation of lipid. Malins and Barone 1969 artificially disturbed the equilibrium between body mass and the buoyancy devices in the dogfish *Squalus acanthias*. The liver of *S. acanthias* contains 62–76% lipid, mostly triglycerides (TG) and diacylglyceryl ethers (DAGE). While in control animals the ratio DAGE/TG was 0.73 ± 0.20, in a group of dogfish in which the body weight was artificially increased for 2 days with lead weights the ratio significantly increased to 1.29 ± 0.23. This suggests presence of regulatory mechanisms that selectively affect the metabolism of DAGE and TG and thus allow for buoyancy control in dogfish.

The use of the liver as a storage of lipids is not only found in elasmobranchs. In the coelacanth *Latimeria chalumnae* the liver contains 67.7% lipid with 8.2%

wax ester, and contributes significantly to the buoyancy status of this species (Nevenzel et al. 1966). Even a few teleosts, such as the larvae of the redlip blenny *Ophioblennius atlanticus*, or *Laemonema longipes*, accumulate lipid in their livers, and rely on this strategy to reduce whole-body density (Nursall 1989; Hayashi and Kashiki 1988).

3.2.3 Lipids in Bones and Other Tissues

The skeleton usually represents the tissue with the highest density. Members of several teleost families, however, use their bones to store lipids and thus significantly reduce their density (Phleger et al. 1976; Phleger 1987, 1988a). Occasionally, the lipid content even reduces the skeleton density below seawater density (Phleger 1975). In general triacylglycerol is the main bone lipid, with minor contributions by cholesterol and phospholipids (Phleger 1991). Typical locations for the storage of bone lipids are spine and skull, but lipids may also be found in other locations. In the hawkfish *Cirrhitus pinnulatus* on a dry weight basis the skull contains 90% lipid and floats in seawater (Phleger 1975). Other examples of a high lipid content present in the skull are the giant hawkfish *Cirrhitus rivulatus* (Phleger 1987), *Peprilus simillimus* and *Anoplopoma fimbria* (Lee et al. 1975). Well known for the high lipid content is the castor oil fish *Ruvettus pretiosus* (Bone 1972). Interestingly, in this species the lipid mainly consists of wax esters of cetyl- and oleyl-alcohols. In this species some of the dermal roofing bones and the skull are little other than girder systems enclosing oil sacs. Bones of the orange roughy *Hoplostethus atlanticus* also contain wax esters (Phleger and Laub 1989).

In some fish bone lipids may even be the main lipid store of the organism. In sheepshead wrasse *Pimelometopon pulchrum* and in sablefish *Anoplopoma fimbria*, bone lipid comprises 79–93% and 52–82% of total body lipid respectively (Phleger et al. 1976). *Acanthurus chirurgus* stores 81% of the total body lipid in bones, while in land mammals bone lipid usually is less than 1% of dry weight (Phleger 1988b).

Apart from liver and bones, lipids may be stored in several other body tissues, e.g. muscle, intestine or subcutaneously. Typically the lipid is stored in adipocytes, but examples of the extracellular storage of lipids in oil sacs are also found. Lipid stored in adipocytes is readily available for metabolism. The availability of extracellular lipid stores for energy metabolism remains questionable.

In the eulachon *Thaleichthys pacificus*, both whole body and liver contain about 20% wet weight lipid. The lipid consists mainly of triglyceride. Because of the high lipid content after drying, the fish is suitable for burning as ‘candle fish’ (Ackman et al. 1968). The Antarctic fishes *Pleurogramma antarcticum*, *Dissostichus mawsoni* and *Aethotaxis mitopteryx* have achieved neutral buoyancy by reducing the mineralization of the skeleton and by accumulation of lipid (Eastman 1985). While *Pleurogramma antarcticum* accumulates lipids in special lipid sacs, *Dissostichus mawsoni* and *A. mitopteryx* possess a subcutaneous layer of adipose tissue. In addition, white muscle tissue contains 23% lipid, mainly triglycerides (Eastman 1985, 1988; Eastman and DeVries 1981; Nevenzel et al. 1966; Clarke et al. 1984). A few

species appear to rely on extracellular lipid stores. For example, juvenile *Lumpenus maculatus* have large oil sacs, mainly consisting of triacylglycerol, situated on the ventral part of the fish from the pectoral fins to the anus (Falk-Petersen et al. 1986).

3.2.4 Lipid Droplets in Eggs and Larvae

Compared to adult fishes, eggs and larvae have the advantage that skeletal elements are not yet developed or are only starting to develop. Nevertheless, a planktonic lifestyle demands neutral buoyancy in order to retain a certain water depth, and eggs and larvae of deep sea fish typically are pelagic. Oil droplets or oil globules are present in the plasma of many eggs, such as in eggs of ling, turbot and grenadier. Nevertheless, lipid accumulation does not appear to be the prime reason for the achievement of neutral buoyancy in fish eggs, and Tocher and Sargent (1984) did not find differences in the lipid content of pelagic and demersal marine eggs. Pelagic marine eggs are usually near neutrally buoyant for most of the development, while dead or dying pelagic eggs will tend to sink, supporting the idea that osmotic and ionic regulation plays a crucial role in achieving neutral buoyancy in these eggs (Coombs et al. 1985). The water content in pelagic eggs is in the range of 90–92%, the lipid content usually varies between 10–15% of dry weight (Craik and Harvey 1987), while in demersal eggs the water content tends to be lower (Yin and Blaxter 1987).

The situation is different in fresh water. Due to the higher osmolarity of the body fluids eggs are denser than the environmental water and the importance of oil droplets to achieve neutral buoyancy increases. Eggs of the Amur snakehead *Ophiocephalus argus warpachowskii*, the macropod *Marcropodus opercularis* or the gourami *Colisia lalia*, for example, achieve neutral buoyancy by means of an enormous oil droplet (Craik and Harvey 1987).

3.3 Watery Tissues

Plasma osmolarity of most vertebrates, including freshwater and marine vertebrates, is about 300 mOsm except for elasmobranches, in which plasma osmolarity is adjusted to values close to seawater osmolarity by accumulation of urea. Water density increases with increasing salinity, and fluids of lower salinity than the surrounding water therefore provide lift. In consequence, plasma of freshwater living vertebrates is denser than water density, but plasma of most marine vertebrates is less dense than seawater and provides lift. The density differences, however, are very small and therefore the contribution of body fluids to overall buoyancy is small, or almost negligible (Denton et al. 1969).

Pelagic marine eggs are usually near neutrally buoyant for most of the development because of the high water content (Craik and Harvey 1987; Yin and Blaxter 1987). During post-vitellogenic meiotic maturation of the eggs a massive water

influx takes place (factor 4–5), leading to the characteristic high water content (Wallace and Selman 1981). Increases in K^+ content and sometimes in Na^+ content, as well as proteolysis, are the driving forces for the osmotic water uptake (Craik and Harvey 1987).

Water can be accumulated not only in fluids, but also in tissues. The water content of tissues averages between 60 and 80%. Some species of fish have much higher water contents in the tissues; they have so-called watery tissues. Typically, the density of watery tissues is still higher than water density, but significantly lower than the density of normal tissue. Thus, although water accumulation often does not result in neutral buoyancy, it significantly reduces the density of the fish in water.

Watery muscle tissue is much softer than muscle tissue of normal water content, and the contractility is reduced. Accordingly, the swimming ability of fish with watery muscles is typically low. Lumpsuckers have water accumulated in muscle tissues, and the density of the muscle tissue is as low as $1.024\,kg\,l^{-1}$. The large dorsal muscle is especially loose-fibred, watery and low in osmolarity, giving a density of only $1.019\,kg\,l^{-1}$ (Davenport and Kjorsvik 1986). Deep-sea fish are often characterized by a high water content in tissues, coinciding with a low mobility (Horn et al. 1978).

Gelatinous or jelly-like material mainly consists of glycosaminoglycan, which is hygroscopic and has a very low density. It may contribute to the low density of the pelagic larvae, but is also well known from various deep sea fish (Yancey et al. 1989; Davenport and Kjorsvik 1986). The material may be located subcutaneously, between the muscle cells, along the dorsal midline and surrounding the spine.

Gelatinous masses are also found in elasmobranchs. A gelatinous layer of watery, jelly-like tissue floating in seawater, is present in the nose of the sharks *Cetorhinus* and *Prionace*; it is also found underneath the skin of the skate *Torpedo nobiliana* (Bone and Roberts 1969).

A reduction in skeleton weight can be achieved by reducing size and thickness of the bones, and it can also be achieved by reducing the mineral content of the bones. The high density of the skeleton is related to the high content in heavy ions such as Ca^{2+} and phosphate or sulfate, and reducing their content in bones will significantly decrease bone density. Deep sea fish and Antarctic fish are examples for both strategies. Depending on the species, the size of the vertebrae and of the spines is reduced, and the mineral content of skeletal elements is reduced (DeVries and Eastman 1978; Eastman 1985; Eastman and DeVries 1982). Another well-known example for a reduced skeleton is the lumpsucker *Cyclopterus lumpus* with a cartilaginous and almost uncalcified skeleton (Davenport and Kjorsvik 1986). The density of the vertebral column is about $1.05\,kg\,l^{-1}$, compared to $1.229\,kg\,l^{-1}$ in plaice, for example.

3.4 Hydrodynamic Lift

Not only low-density structures are suitable for achieving neutral buoyancy, a high tissue density can also be compensated by hydrodynamic lift. Small plankton can be

kept in suspension by eddies, and this is particularly facilitated by parachute devices like long antennae. Ciliary activity also allows small organisms to remain suspended. Larvae of the lancelet *Branchiostoma lanceolatum* (Cephalochordata) hover almost motionless in midwater by means of beating epidermal cilia, metachronal waves that pass from anterior to posterior at about $0.3\,\mathrm{m\ s^{-1}}$ (Stokes and Holland 1995).

Swimming animals produce hydrodynamic lift mainly by using their fins as hydrofoils. As with an airplane, a certain speed is required to generate sufficient lift in order to compensate for the higher overall body density, and to stay at a certain water depth. Accordingly, hydrodynamic lift is important for fast-swimming teleosts, elasmobranches or marine mammals. The metabolic power (E_m) needed to propel the hydrofoils through the water can be calculated from drag on the hydrofoils and speed

$$E_\mathrm{m} = 4L^2/\eta\pi\rho_\mathrm{w}U\lambda^2,$$

where η is the efficiency coefficient for the conversion of metabolic energy to mechanical power, ρ_w is the water density, U is the speed, and λ is the span of the hydrofoil (Alexander 1990). Thus, the additional power needed to produce the lift necessary to remain at a certain water depth decreases with increasing swimming speed.

During swimming, lift is primarily produced by the pectoral fins (Alexander 1990; Magnuson 1978). Analogous to wings on an airplane, water flows faster over the upper surface of the pectorals than over the lower surface, creating a higher pressure on the lower surface (Fig. 6a, b). The pressure difference produces a net lift, directly proportional to the area of the hydrofoils, and proportional to the swimming speed. Vortices induced at the tip of the fins generate drag. Therefore, long fins are especially economical because, in relation to the drag produced at the tips, they produce more lift. The aerodynamic design of the pectoral fins is compromised by their structural strength and the ability of the fish body to carry them (Magnuson 1970).

Sharks, sturgeons, scombrid fishes (tuna, bonito and mackerel) and some marine mammals are well known to swim more or less continuously at high speed. The fins of selachians and sturgeons cannot be folded, and project permanently from the body. Pectoral fins of scombridae, however, are not fixed, and lift produced by the pectorals varies with their extension. Accordingly, at high speed the pectoral fins are extended less, because less hydrodynamic lift is required for hydrostatic equilibrium (Magnuson 1970).

Additional lift is produced by the peduncular keel and heterocercal tails (Alexander 1965, 1966a; Simons 1970). Water flows diagonally across the keel, and the keel with its sinusoid movement travels faster than the fish (Magnuson 1970). Hydrodynamic lift produced by the tail acts behind the center of gravity, which appears necessary for longitudinal stability, to avoid a continuous rising of the swimming fish. In fish with heterocercal tails the larger dorsal section of the tail generates lift, and the smaller ventral section reduces lift. If the tail is equipped with radial muscles, as in some sharks, the tail can become a ‘horizontal trim’ (Simons 1970).

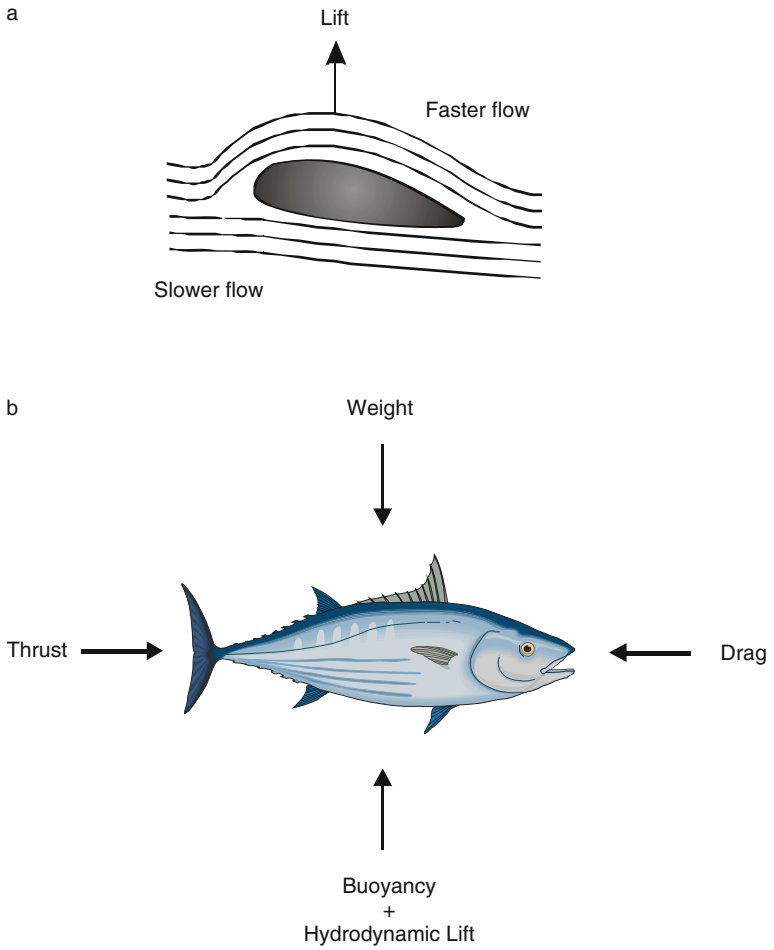

Fig. 6 **a** Like wings of an aeroplane, fins may generate hydrodynamic lift during swimming. Due to the structure of the fins, the water flows faster over the upper surface than over the lower surface, generating hydrodynamic lift. **b** Forces acting on a fish during swimming (Magnuson 1970)

4 Diving Reptiles and Birds

Loggerhead turtles (*Caretta caretta*) perform dives during which they remain at a particular water depth without actively swimming (Minamikawa et al. 2000). At this particular depth the turtles are neutrally buoyant, and this depth is clearly dependent on the amount of air remaining in their lungs. But this volume and thus the depth of neutral buoyancy do not appear to be actively determined by the turtles. Application of additional weight to the turtle resulted in a change in diving depth. This observation suggests that the turtles do not actively select a diving depth, but adjust the depth according to lung volume. The typical diving profile of *Caretta caretta* revealed a gradual ascent without any movement, i.e. a gliding type of movement.

The authors suppose that this type of movement is buoyancy-driven and related to a status of neutral buoyancy (Minamikawa et al. 2000, 1997). This appears quite possible, although a number of uncertainties remain. Turtles use fat during diving, resulting in an RQ of 0.7. Accordingly, lung volume should decrease during the dive, and this may be enhanced by transient storage of CO_2 in the blood due to a shift in acid–base equilibrium. On the other hand, during ascent the hydrostatic pressure decreases, resulting in an increase in lung volume. In addition, the authors assume a behavioral compensation to permit a status of neutral buoyancy to be maintained (Minamikawa et al. 2000). Application of artificial weight to the shell of green turtles also revealed that this species usually selects resting depth within the range over which they can use the lungs to achieve close to neutral buoyancy (Hays et al. 2004). Freshwater turtles (*Pseudemys scripta elegans*) also appear to adjust the buoyancy status. In these animals buoyancy is corrected by reciprocally changing the volumes of lung air and of water stored in the cloacal bursae (Jackson 1969).

The peculiar lung structure, presence of air sacs and also the amount of air trapped in the plumage result in a low specific gravity of birds, which is advantageous during flight. Some birds, however, have returned to a semi-aquatic life style. Positive buoyancy creates a problem when foraging under water. A comparative analysis demonstrated that diving birds significantly reduce the amount of air trapped in the feathers in order to reduce upthrust (Wilson et al. 1992). Nevertheless, birds typically remain positively buoyant, and cessation of active swimming in most diving water birds results in passive surfacing. Cormorants counteract positive buoyancy by tilting the body at a certain angle to the swimming direction, and this tilting is controlled by the tail (Ribak et al. 2004).

Diving king and adelie penguins, *Aptenodytes patagonicus* and *Pygoscelis adeliae* respectively, appear to anticipate the duration and depth of the dive ahead, and adjust their lung volume appropriately in order to optimize cost benefits of the dive (Sato et al. 2002). A strong correlation was observed between the volume of the final breath before the dive, and dive duration. While the penguins flapped continuously during descent, during ascent they partially stopped movements and used a gliding type of movement to return to the surface, which is related to positive buoyancy. The return was typically slowed down by changing the angle at which the penguins approached the water surface. It was speculated that this delay may contribute to avoidance of getting the bends (Sato et al. 2002).

5 Buoyancy in Mammals

Based on molecular and morphological data it is currently assumed that terrestrial mammals from seven different lineages secondarily invaded the aquatic habitat (Uhen 2007). The largely different mechanical constraints of locomotion in air and water resulted in dramatic structural transformations. Many marine mammals have modified their external shape, developing new propulsion systems for locomotion in water; the breathing system has been redesigned, and the fur has had to be adjusted

(Reidenberg 2007). A recent account of these changes has been presented as a special volume of the Anatomical Record (Vol. 290, 2007).

Significant changes can be observed in the fur, providing thermal insulation to terrestrial mammals. Some marine mammals have lost the fur altogether and 'replaced' it with a thick, waterproof epidermis with a remarkable layer of fat underneath, known as the blubber (e.g. whales, dolphins, walruses or hippopotami). The thick layer of fat significantly reduces heat loss to the water, which has a much higher thermal conductance than air. In others, and in so-called semi-aquatic mammals, the properties of the fur show clear adaptations to the aquatic environment. A waterproof, oily fur is found in polar bears, otters, seals, sea lions and beavers. This waterproof fur may even confer protection against heat loss to the water (Reidenberg 2007). An increased hair density combined with a large number of kinks and interlocking cuticular scales of adjoining hairs may trap air, and minimize compression and water infiltration as a result of hydrostatic pressure. Accordingly, the fur contributes significantly to overall buoyancy of these animals. Buoyancy force increases with increasing hair density of the fur (Fig. 7) (Fish et al. 2002). In muskrats the air

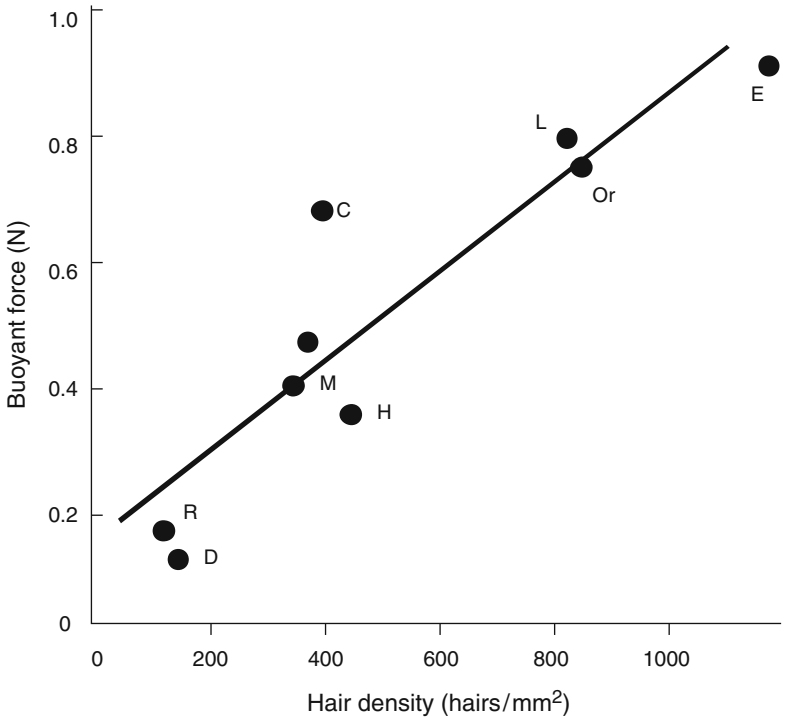

Fig. 7 Buoyant forces (*N*) in relation to hair density in a number of semi-aquatic mammals. *C*, *Castor canadensis*; *D*, *Didelphis virginiana*; *E*, *Enhydra lutris*; *H*, *Hydromys chrysogaster*; *L*, *Lutra canadensis*; *M*, *Mustela vison*; *Or*, *Ornithorhynchus anatinus*; *R*, *Rattus norwegicus*, (after Fish et al. 2002 with permission)

layer within the fur contributes about 21.5% to total body volume, resulting in an average specific weight of 0.79 (Johansen 1962).

Due to the higher density of water, the constraints of terrestrial weightbearing are significantly reduced in water-living mammals, and the skeleton has undergone a severe reorganization in order to meet the new requirements. For an adequate locomotion, skeletal elements have also been reorganized at the microstructural level. Bone density of marine mammals appears to be a means of adjusting buoyancy. While the bones of bottom-dwelling mammals typically are characterized by a very high density, in more pelagic species osteopetrosis and osteoporosis has resulted in the formation of bones with a particularly low density (Gray et al. 2007).

The blubber represents another important component contributing to buoyancy in marine mammals. The density of lipid is lower than the density of seawater, and therefore the blubber is important not only with respect to thermoregulation, but also with respect to buoyancy. It contributes a defined volume to the total volume of the animal and therefore provides a constant contribution to buoyancy, irrespective of the water depth, but its density changes with changes in water temperature. Elephant seal pups *Mirounga leonina* perform drift dives, i.e. they passively drift through the water column, a gliding type of movement. The drift dives require the status of neutral buoyancy and are related to the amount of fat on board (Biuw et al. 2003).

For the sperm whale, it has been discussed that the fluidity of the lipid stored in the head varies with depth (Clarke 1978b), and these changes are related to changes in water temperature. A decrease in temperature will transfer the spermaceti oil, a complex mixture of wax ester and triglycerides, from the fluid to the crystalline state, and this will increase density (Clarke 1978b). It has been proposed that heat transfer and heat exchange is achieved by an intense capillary supply or by intake of seawater into the nasal opening (Clarke 1978a; Clarke 1970). This hypothesis appears quite intriguing, but it did not remain unequivocal. Presence of a dense capillary supply could not be confirmed, and taking seawater into the nasal cavity may indeed cause osmotic problems, because the seawater is hyperosmotic to the body fluids of marine mammals (Cranford 1999).

The lung as a gas-filled structure has also to be considered as a possible buoyancy aid. However, pinnipeds exhale before diving, and therefore the contribution of the lung to buoyancy appears to be questionable. In dolphins and elephant seals, for example, the contribution of the lung to buoyancy changes with water depth. While dolphins are positively buoyant at the water surface, the gradual collapse of the alveoli with increasing water depth causes a decrease in lung volume. Finally, at a certain water depth complete collapse is observed. Accordingly, the residual lung volume influences the overall buoyancy of the diving mammals (Biuw et al. 2003). The decrease in lung volume at constant body mass decreases buoyancy at descent, and the dolphins become negatively buoyant (Williams et al. 2000; Biuw et al. 2003). This decrease in buoyancy is apparently used by these animals to switch to a gliding type of movement. For the northern elephant seal a significant correlation between buoyancy and the rate of descent was observed. The elephant seals often used a gliding type of descent, and a more negative buoyancy status coincided with a greater rate of descent (Webb et al. 1998). Using submersible cameras attached to

the animals, it could be shown that Weddell seal, dolphins and blue whale frequently refer to this type of movement during descent, and that this significantly reduces energy expenditure during diving (Skrovan et al. 1999; Williams et al. 2000).

6 Conclusions

In water-living vertebrates the high density of most body tissues can be compensated by various strategies, ranging from muscular activity to accumulation of low density material such as water, lipid or gas. For bottom-dwelling fish it is not 'useful' to be neutrally buoyant; for a fish foraging in the open water column it might be 'useful'. Pelagic species hunting at high swimming speed typically make use of hydrodynamic lift, while for a fish travelling at slow speed or hovering at a reef it might be an energetical advantage to have a swimbladder. For air-breathing vertebrates foraging under water it appears almost impossible to achieve neutral buoyancy irrespective of water depth. Nevertheless, at some point during descent or ascent these species make use of their buoyancy status, and switch to an energetically advantageous drifting type of movement. Thus, the various strategies to achieve neutral buoyancy have been adopted irrespective of the systematic position of a species. The energetical advantage of neutral buoyancy is clearly demonstrated by the extent to which vertebrates from different classes have adopted all these strategies, suitable to the individual way of life or adjusted to the constraints of the biotop.

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Gas Exchange and Control of Respiration in Air-Breathing Teleost Fish

M.L. Glass and F.T. Rantin

Abstract Atmospheric air at sea level contains 30 times more O_2, when compared to fully O_2-saturated water and, in addition, the O_2 content of water is ever-changing. The gill systems of fish are highly efficient for O_2 extraction, but this cannot save the animal if its O_2 supply is insufficient. This explains why air-breathing in fish has evolved in at least 60 independent lines. Lungfish and bichirs (Polypteriidae) possess true lungs, whereas other air-breathing organs (ABOs) can be derived from the swimbladder as in the gar pike (*Lepidosteus*) and the bowfin (*Amia*). In *Hypostomus sp*. (Loricariidae) the ABO is a modified part of the digestive system. The functions of gills, ABOs and lungs all depend on surfactants. Aerial breathing increases with activity and/or reduced O_2 availability in the water. In addition, increases of temperature result in larger air-breathing efforts. These responses are adjusted by O_2 receptors, located in the gills, whereas the role CO_2/H^+-receptors is minor in actinopterygian fish.

1 Introduction

1.1 Air and Water as Respiratory Media

Compared to air, water is an oxygen poor environment, because the O_2 solubility in water is low. At 20°C, the O_2 concentration in the fully saturated water is $0.007\,(l^{-1}l^{-1})$ against $0.2095\,(l^{-1}l^{-1})$ in air, which implies that air-breathers have 30 times more O_2 available when compared to aquatic breathers in fully saturated water. The relative density and the viscosity of water and air should also be taken

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into account. The water density is no less then 800 times higher compared to the density of air (water: $1.00\,kg\,l^{-1}$; air: $0.0013\,kg\,l^{-1}$) and the water viscosity is 50-fold higher than atmospheric air at sea level. The joint analysis of these variables gives an impression of the difficulties faced by water-breathers to maintain an aerobic metabolism. The low O_2 availability in the aquatic environment forces water-breathers to ventilate the respiratory surface area a much higher volume of inert mass with high density and viscosity, which leads to a high metabolic cost of gill ventilation. Gills are absent in most aquatic invertebrates, but they may ventilate some specific part of the body. Fish possess a highly sophisticated countercurrent system, in which the blood passes the gills in the opposite direction to the inspired and expired water flow. It should also be pointed out that the O_2 content in water depends on photosynthesis, which changes on a daily and seasonal basis. Further determinants of O_2 levels are water movement, currents, changes in temperature, and salinity (Dejours 1981).

The metabolic cost of gill ventilation in water-breathing fish is species-specific. It ranges between 3.7 and 5.7% of the total oxygen uptake ($\dot{V}O_2$) in the sharksucker, *Echeneis naucrates*, which is an unusually low value (Steffensen and Lomholt 1983), while the highest value (43%) was measured in the tench, *Tinca tinca* (Schumann and Piiper 1966). Gill ventilation consumed about 13–22% of the total $\dot{V}O_2$ in carp, *Cyprinus carpio*, 10–25% in trout, *Onchorhynchus mykiss* (Hughes and Shelton 1962; Hughes and Saunders 1970), and 22–28% in plaice, *Pleuronectes platessa* (Edwards 1971). The cost of ventilation may increase in response to hypoxia, since hyperventilation is an attempt to compensate for the reduced O_2 level. The metabolic cost of gill ventilation in normoxic Nile tilapia, *Oreochromis niloticus*, is about 3% in normoxia, increasing to 20% when the inspired PO_2 falls to 35 mmHg (Fernandes and Rantin 1994). Likewise, the hypoxia-tolerant and sedentary erythrinid fish traíra, *Hoplias malabaricus*, spends 3% of its total $\dot{V}O_2$ to ventilate its gills when in normoxia, but hypoxia ($PwO_2 = 25\,mmHg$) increases the cost of ventilation to no less than 13% (Rantin et al. 1992).

Air-breathing organs (ABOs) have evolved in at least 60 independent lines of teleost and holeost fish (Graham 1997). Fish of bimodal respiration have large advantages when exposed to hypoxic water. Transition to air-breathing will alleviate the hypoxic conditions, which obviously increases the chances for survival. In addition, air-breathing assures access to a steady O_2 supply at a much higher concentration.

1.2 Evolution of the Atmosphere

The early atmosphere contained hydrogen and helium, which the solar wind gradually removed, after which CO_2 became the prevalent gas. This prevalence of CO_2 is still predominant in the atmospheres of Venus and Mars (Stearns and Hoekstra 2005). Geochemical research indicates that evolution of photosynthesis occurred in precursors to the cyanobacteria, which suggests that the catalyzing

reaction of the photosystem II oxidizing complex emerged some 3 billion years ago (Dismukes et al. 2001), and this event would gradually lead to an ozone protection resulting from the photosynthesis. In addition, Dismukes et al. (2001) point out that oxidation of water is one of the most challenging multielectron reactions in biology.

Estimates for the atmospheric O_2 levels are available from the beginning of the Silurian period to the end of the Permian period, which ranges from 444 to 250 million years ago. There is evidence that atmospheric O_2 levels in the early Devonian could have reached up to 25%, exceeding our actual level of 20.95%. The middle and late Devonian periods were characterized by reduced O_2 levels (13% O_2), but then between 340 and 250 million years ago, the O_2 levels rose and might have reach up to 35% (Berner and Canfield 1989; Scott and Glasspool 2006). As pointed out by Dudley (1998), it is unlikely that 35% O_2 was exceeded, because this value is an approximate threshold for spontaneous combustion of the atmosphere. Recent studies agree with an O_2 peak of up to 25% in the early Devonian, followed by low O_2 levels of about 15% during the beginning of the Late Devonian (Frasnian) period (Clack 2007). Based on Dehadrai and Tripathi (1976), Daniels et al. (2004) proposed that Devonian bony fish may have developed aerial respiration due to low oxygen levels. The message of this short section is that ambient O_2 availability is a principal determinant of the levels of metabolism and of new strategies for respiratory function; one of these is bimodal respiration.

1.3 Hypoxia and Hypercarbia

Based on Junk (1984), Nelson et al. (2007) listed some adverse conditions for O_2 uptake, which are particularly common in tropical and subtropical regions, and these are: (1) respiratory rates may become larger than the production of O_2 by photosynthesis, (2) a stagnant air/water interface impedes an adequate distribution of O_2, and (3) a poor light penetration decreases photosynthesis.

By comparison, the atmosphere above us seems a stable source of O_2. At sea level and 20°C, the PO_2 would be about 155 mmHg with 20.95% O_2. The atmospheric source of O_2 will obviously be advantageous for fish exposed to frequent reductions of ambient O_2 levels. Hypercarbia is the condition of elevated CO_2 levels in the environment, whereas hypercapnia is elevated CO_2 levels within the body. Temperature is also an important factor, since higher temperatures increase the metabolic demands, and, the higher the temperature, the larger are the increases of gill ventilation in response to hypoxia (Glass et al. 1990). An ever-changing O_2 availability is a challenge to the O_2-oriented respiratory control of teleost and holeost fish (cf. Dejours 1981), and each species has a critical PO_2 threshold (PcO_2) below which the normal $\dot{V}O_2$ can no longer be maintained. Holeosts and teleosts compensate for an excess of CO_2 (hypercarbia) by active increases of plasma [HCO_3^-] levels, which returns pHa towards the normal (normocarbic) value. About 90% of acid–base

relevant ions are mediated by specialized cells within the gill epithelia, and the kidney contributes the rest (Heisler and Claiborne 1986).

The PcO_2 of an exclusively water-breathing fish often reflects the O_2^- availability of the habitat. As a typical example, carp (*Cyprinus carpio*) is very resistant to hypoxic conditions, and its Hb–O_2 dissociation curve (ODC) has a very high Hb–O_2 affinity, which implies that a high O_2 saturation is preserved until PaO_2 falls to extremely low pressures ($P_{50} = 9.4$ mmHg at 20°C) (Albers et al. 1983), which explains its survival under severely hypoxic conditions (Soncini and Glass 2007). Conversely, dourado (*Salminus maxillosos*) is a river fish capable of a high swimming speed, and it is distributed in regions of Southern Brazil. At pHa 7.7, its P_{50} is high (21 mmHg at 25°C.) This leaves dourado vulnerable to reductions of O_2, because moderately hypoxic conditions will reduce its Hb–O_2 saturation and, thereby, decrease its swimming performance (Salvo-Souza et al. 2001).

1.4 Gill Function and O_2 Extraction

The O_2 extraction of a gas exchanger can be defined as:

$$EO_2 = \dot{V}O_2 \cdot (\dot{V}_G \cdot C_IO_2)^{-1},$$

An alternative expression is:

$$EO_2 = (C_IO_2 – C_{EO2}) \cdot C_{IO_2}^{-1},$$

where $\dot{V}O_2 = O_2$ uptake, $\dot{V}_G =$ the water flow irrigating the gill, C_IO_2 and $C_EO_2 = O_2$ contents of the inspired and expired water (Dejours 1981). The difference $(C_IO_2 – C_EO_2) \cdot C_IO_2^{-1}$ is the amount of O_2 extracted by the blood flow and C_IO_2 is the total inspired amount of O_2. The degree of O_2 extraction by fish gills is high and often about 85% (Lomholt and Johansen 1979). This is very high compared to the extraction of a human lung, which is only about 30%. Evidently, the gas exchange is very effective, because the blood flows in the opposite direction to the water movement, and the gas exchange takes place within the blood lacunae of the secondary lamellae (cf. Piiper 1992).

A cardio-vascular shunt is defined as a quantity of incompletely saturated blood that bypasses the gas exchanges surfaces. As the second step, the shunted blood fraction mixes into the saturated blood from the gas exchanger, and the larger this shunt, the lower Hb–O_2 saturation. Such shunts are apparently small or absent in fish gills, although there is some evidence for shunted blood within the basal channels of the secondary lamellae of rainbow trout (*Oncorhynchus mykiss*) (Tuurala et al. 1984). On the other hand, convincing evidence against major shunts was provided by Eddy (1974), who constructed an in vivo Hb–O_2 dissociation curve for tench (*T. tinca*). The saturation of the oxygen dissociation curve (ODC) ranged from 75 to 95%, but the in vivo arterial point was in some cases located on the upper portion of the oxygen dissociation curve (ODC), which indicates that shunts

are minimal. Most teleost fish have almost hyperbolic ODCs (n_{Hill}∼1.0 to 1.3), which implies that PO_2, even when high, cannot saturate the blood. Therefore, the incomplete saturation can be due to the shape of the ODC and not to cardiovascular shunt (Glass and Soncini 1997). Application of hyperoxia permits calculation of the shunt fraction based on the mixed venous and arterial points. Such measurements are available for pacu (*Piaractus mesopotamicus*), in which the shunt fraction was less than 10%. The equation cannot distinguish between diffusion limitation and ventilation–perfusion mismatching, which leaves 10% shunt as an overestimated value (Soncini and Glass 1997). Consistent data were obtained by Gilmour and Perry (1994), when they exposed trout (*O. mykiss*) to an aquatic PO_2 of 548 mmHg, which increased PaO_2 to a range within 352–502 mmHg. The characteristics of teleost gills are impressive, but in spite of this the fish may not survive severely hypoxic conditions. This explains why air-breathing in fish has evolved within many different and, mainly, tropical taxonomic groups.

Some fish avoid hypoxia by skimming the air/water interface, which has the highest O_2 level. This respiratory behavior is known as aquatic surface respiration (ASR) and is common to a number of tropical fish that inhabit lakes with frequent hypoxic conditions (Kramer and McClure 1982). An example is pacu (*P. mesopotamicus*). To escape from hypoxia, this species performs ASR, which is facilitated by a gradual swelling and extension of the lower lip (Saint-Paul and Bernardino 1988; Val and Almeida-Val 1995). Pacu immediately initiates ASR even in moderate hypoxia (50–70 mmHg), while the full development of swelling may take no less than 3 h or more (Rantin et al. 1998).

1.5 Air-Breathing Organs and Their Function

Respiration by a lung or an ABO requires surfactant to reduce surface tension. Daniels et al. (2004) addressed the role of surfactant in fish in two air breathers: the pirarucu (*Arapaima gigas*) and the tarpon (*Megalops cyprionoides*), along with the exclusively water-breathing snapper (*Pagrus auratus*). They concluded that the fish surfactants share basic features with the alveolar Type II cells of tetrapod lungs, and proposed that surfactant in lungs or swimbladders are homologous. The swimbladder and the lungs are of separate ontogenetic origin (Perry 2007), and Daniels et al. (2003) proposed that surfactant systems already existed before lungs and swimbladders evolved. It also turns out that the surfactant of Osteichthyes is uniform, and may represent the vertebrate prototype.

Adequate O_2 uptake and CO_2 elimination can be obtained via modifications of structure and function, which often leads to surprising solutions. In this section, we present a selection of ABOs and their function, beginning from the most ancient forms. The text takes us from real lungs to respiration using modified stomach tissue.

1.6 Polypterus senegalus (Polypteridae)

The genera *Polypterus sp*. and *Erpetoichthys* [earlier *Calamoichthys*] form a prominent group of ancient fish. It has been argued that they are a phylogenetically isolated group and that they are the most original extant representatives of actinoterygians (Carroll 1987), and their habitats are tropical waters in Africa. Land vertebrates, lungfish and Polypteriformes possess lungs of a ventral, posterior pharyngeal origin and these are pleisiomorphic paired structures (Perry 2007), i.e. they are ancestral trait and are not apomorphic structures.

There is little information available on the respiratory physiology of Polypteriformes. Studying a specimen of *P. senegalus*, Babiker (1984) reported that the fish in aerated water would surface only once or twice between 11p.m. and 3a.m., which would classify the specimen as a marginal obligatory air-breather. Juvenile specimens (10–30 g) were exclusively gill breathers, which proved fatal during aquatic hypoxia. Larger specimens (290–460 g) survived due to air-breathing, and Magid et al. (1970) measured their lung gas PO_2, which increased from about 30 to 100 mmHg during inhalation, with a concomitant reduction of $PaCO_2$ from about 14 to 9 mmHg. In this context, it should be mentioned that aerial respiration in fish increases $PaCO_2$ and lowers pHa and, according to Dejours (1981): "the more an animal depends on pulmonary breathing, the higher its PCO_2". The reason for this is the 30-fold difference of O_2 content in water and in atmospheric air. To compensate for this difference, an exclusively gill-breathing fish must ventilate a 30-fold higher volume, when compared to an air breather. A high degree of gill ventilation leaves a carp with a $PaCO_2$ of 2–3 mmHg (Glass et al. 1990), while the $PaCO_2$ *P. senegalus* would be about 3-fold higher due to additional aerial ventilation. Reductions of PwO_2 markedly increased pulmonary ventilation of *P. senegalus*. As a second defense strategy, the O_2 loss to near anoxic water was minimal, due to a shutdown of gas exchange by the gills (Lomholt and Glass 1987). This is consistent with Babiker (1984), who reported that small specimens (10–30 g) would not develop air-breathing, whereas larger individuals (>100 g) could survive in near-anoxic water by lung ventilation.

1.7 The gars (Lepisosteus sp.) and the bowfins (Amia calva) Amiidae, Amiiformes

The gar pikes and the bowfins form a sister group which is ramified with the teleosts (Filleul and Lavoué 2001). The gar and the bowfins are restricted to North America, and their ABOs are gas bladders (Hedrick and Jones 1999). The ventilation of an ABO may seem irregular, but this is not the case for *Amia*. Hedrick et al. (1994) applied a spectral analysis to evaluate air-breathing behavior, and two distinct types of breaths were recorded. The first type began with an expiration followed by inhalation. A second type of breath consisted of an inhalation and the authors very

reasonably suggested that this behavior represented an adjustment related to buoyancy. Upon removal of this component, a very regular pattern emerged, with an interval of 30 min between breaths at the surface (temperature 20–24°C), which indicated the presence of a central rhythm generator and/or peripheral O_2-receptor input.

The effects of activity on air-breathing in *Amia* and *L. oculatus* were evaluated by Farmer and Jackson (1998), who first studied the animal at rest and then at stepwise increases of swimming speed (temp.19–23°C). It turned out that *L. oculatus* at rest obtained less than 2% of total O_2 uptake from aerial respiration, but it turned out that swimming activity increased the contribution of O_2 from air-breathing to no less than 53% of total $\dot{V}O_2$. The corresponding numbers for aerial respiration in *Amia* were 10% at rest and no less than 66% of total $\dot{V}O_2$. As the authors pointed out, the capacity to increase ventilation of the ABO is important for activity, and not only for survival in hypoxic waters.

As expected, the long-nosed gar increased ventilation of the ABO, when exposed to hypoxic water. In addition, it reduced gill ventilation, which minimized O_2 losses to the water (Smatresk et al. 1986). Similar responses have been reported for *Polypterus* in near-anoxic water (Lomholt and Glass 1987).

There is evidence for membrane-bound carbonic anhydrase located in the ABO of *Amia*, and it resembles mammalian carbonic anhydrase IV as regards inhibition characteristics and membrane attachment (Gervais and Tufs 1998). Curiously, glucose is a major source of fuel in the Florida gar (*L. plathyrhinchus*), and the enzyme activities of its ABO resembles those of a mammalian lung, and are different from the enzymes of fish swimbladders (Frick et al. 2007). Evidently, the function of the ABO can be sophisticated, and it turned out that mechanoreceptors are present in *L. oculatus* (Smatresk and Azizi 1987). Two types of receptors were identified. A rapidly adapting type was identified, while a slowly adapting type turned out to be CO_2-sensitive. Application of hypercarbia (6–10% CO_2) decreased firing rate of the slowly adapting receptors to the slowly adapting receptors. Very similar responses have been reported for lungfish (DeLaney et al. 1983) and land vertebrates (Milsom 2002), which would suggest a very ancient origin, if the receptors turn out to be identical (Smatresk et al. 1987).

1.8 Channa argus (Channidae)

The snakehead fish *Channa argus* is carnivorous and widely distributed in temperate zone of East Asia. Ventilation of the ABO is obligatory during the summer season, whereas survival by gill ventilation alone is possible during the winter. The ABO of *Channa* is placed within the gill system. The first and second gill arches are perfused by the anterior ventral aorta, after which the blood traverses the bilaterally and dorsally positioned ABO. Differently, the posterior ventral aorta supplies blood to the third and fourth gill arches, bypassing the ABO (Andresen et al. 1987). Animals weighing 1–2 kg were studied at 15–25°C to evaluate the combined effects of

hypoxia and temperature on aerial ventilation. Glass et al. (1986) exposed C*hanna* to normoxia (∼155 mmHg) and hypoxia (75 and 35 mmHg). Aquatic hypoxia was tested and the responses were weak but significant at 25°C, while the response at 15°C failed to reach statistical significance. On the other hand, aerial hypoxia had a large effect on ventilation of the ABO. At 15°C, the ventilation of the ABO rose 2.8-fold with reduction of gas phase PO_2 from 155 to 35 mmHg, and at 25°C the same reduction increased aerial ventilation 3.4-fold. Curiously, this response to hypoxia is also amplified by high temperature in the South American lungfish (*Lepidosiren paradoxa*) and in ectothermic land vertebrates, including the toad *Chaunus scheideri* (earlier *Bufo paracnemis*) (Kruhøffer et al. 1987) and the turtle *Chrysemys picta bellii* (Glass et al. 1983); see Fig. 1.

In addition, *Channa* had a weak increase of aerial ventilation in response to aquatic hypoxia, while the South American lungfish (*L. paradoxa*) had no increase of pulmonary ventilation in response to aquatic O_2 levels. This raises questions about the positions and roles of the involved O_2 chemoreceptors.

Figure 1 clearly shows that ventilatory responses to hypoxia in air breathers become strongly reduced when temperature decreases. The dependence of exclusive water breathers may be different. Carp (*Cyprinus carbio*) increased gill ventilation in response to light hypoxia at 10, 20 and 25°C. These responses were significant both at 10, 20 and 25°C. Moreover, the gain of the responses could be expressed as:

$$(\dot{V}_G \text{ hypoxia}/\dot{V}_G \text{ normoxia}) \cdot 100\%.$$

Expressed in this way, it became clear that the percentage increase was independent of temperature. In other words, the control value and ventilation at a level of hypoxia were scaled up or down by the same factor, which differs from responses to gas-phase hypoxia, which fade away in response to low temperature (Glass et al. 1990; Soncini and Glass 2000).

Channa seemed to respond to hypercarbia (range 0–8%), but this effect did not reach significant levels. Likewise, Graham and Baird (1982) reported that *Hypostomus* would elicit ABO breathing more easily, if hypercarbia (∼10 mmHg) was added. Moreover, juvenile specimens of the bichir (*P. senegalus*) increased gill ventilation in response to 0.8%CO_2 in the water. Therefore, it seems that a weak CO_2-related component is present, and this topic will be taken up later.

1.9 Hypostomus sp. (Loricariidae)

Facultative air-breathers ventilate exclusively use gills when in normoxic water, whereas the ABO is ventilated, when the water turns hypoxic. This applies to the armored catfish *Ancistrus chagresi*, *Hypostomus plecostomus* and *Hypostomus regain*, which have species-specific thresholds for the onset of aerial ventilation. Thus, *A. chagresi* initiated ventilation of the ABO when PwO_2 fell to 33 mmHg, whereas *H. plecostomus* already ventilated the ABO, when PwO_2 dropped to

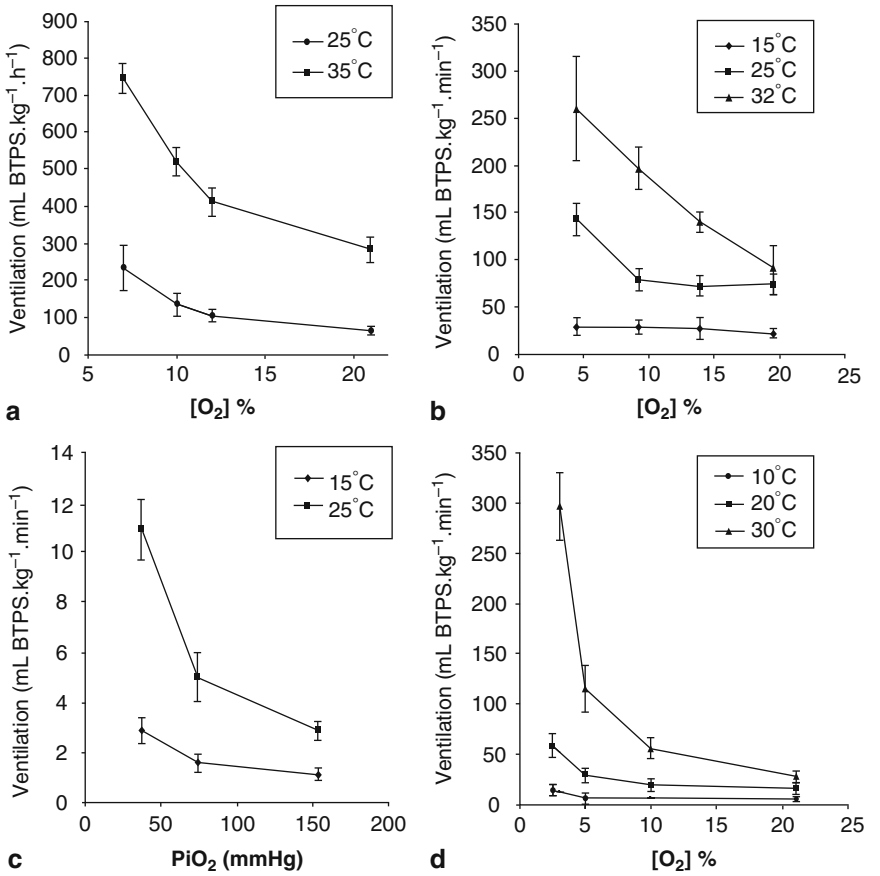

Fig. 1 This figure compares the effects of hypoxia combined with increases of temperature. **a** The South American lungfish *Lepidosiren paradoxa* (da Silva et al., in preparation). **b** The toad *Chaunus schneideri* (Kruhøffer et al. 1986). **c** The teleost fish *Channa argus*, a facultative air-breather equipped with an air-breathing organ. (Glass et al.). **d** The turtle *Chrysemys picta bellii* modified from Glass et al. (1983). These responses are similar, although they are obtained from very distant groups

the higher threshold of 60 mmHg. Application of light hypercarbia (∼10 mmHg) changed both thresholds to the higher values 64 mmHg and 79 mmHg, which was in agreement with the relative sensibility of the two species (Graham 1982). Their ABO is modified tissue located within the stomach. The stomach tissue of the catfish *H. plecostomus* is quite different from that of typical fish, since the wall is transparent, and the mucosal layer is smooth, and capillaries are abundant in the arterial part of the stomach. Part of the epithelium contains respiratory epithelial cells, where the air–blood is thin (0.25–2.02 µm) (Podkowa and Goniakowska-Witalinska 2003).

Rhinelepis strigosa (cascudo preto) has been studied in some detail. It will not surface when in well-oxygenated water at 25°C, but air-breathing was initiated

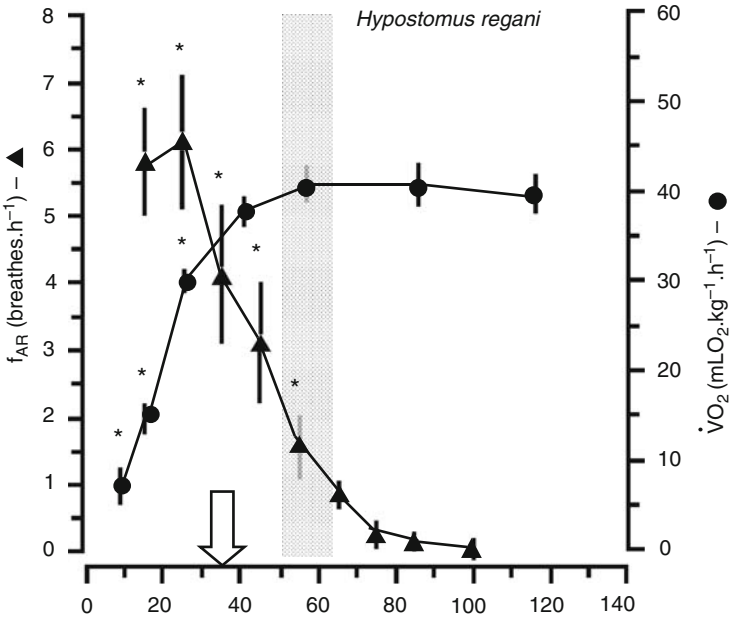

Fig. 2 The threshold O_2 tension for air breathing (*shaded area*) and air-breathing frequency (*filled triangle*) of *H. regani* with free access to the water surface and aquatic $\dot{V}O_2$ (*filled circle*) when the access to the water surface was denied during progressive hypoxia. *Arrow* indicates the PcO_2. *Asterisks* indicate significant difference compared to the normoxic values. Values are mean ± SEM. (Modified from Mattias et al. 1998)

below a PwO_2 of 30 mmHg and it peaked at 7 breaths $\cdot$ h^{-1} at 10 mmHg (Takasusuki et al. 1998), which certainly proves the efficiency of respiration by an ABO. In this context, it should be remembered that temperature and O_2 levels interact, and behavioral responses may include dislocation to a lower temperature and/or a higher O_2 level (Schurmann and Steffensen 1994).

Mattias et al. (1998) measured $\dot{V}O_2$ and gill respiration during progressive hypoxia in the facultative air-breather *H. regani*. Air-breathing was absent in aerated water, but *H. regani* maintained aquatic $\dot{V}O_2$ at 31 ml O_2 kg^{-1} h^{-1} down to the critical oxygen tension (PcO_2) of 34 mmHg (temperature 25°C). Gill ventilation and ventilation of the ABO reached their peak values within the P_IO_2 range from 56 to 25 mmHg (Fig. 2).

1.10 Clarias sp. (Clariidae)

Members of the genus *Clarias* ventilate the ABO in a regular manner, and some species maintain a low level of air-breathing, when in normoxia. In spite of this, some studies question which species are obligatory or facultative air breathers.

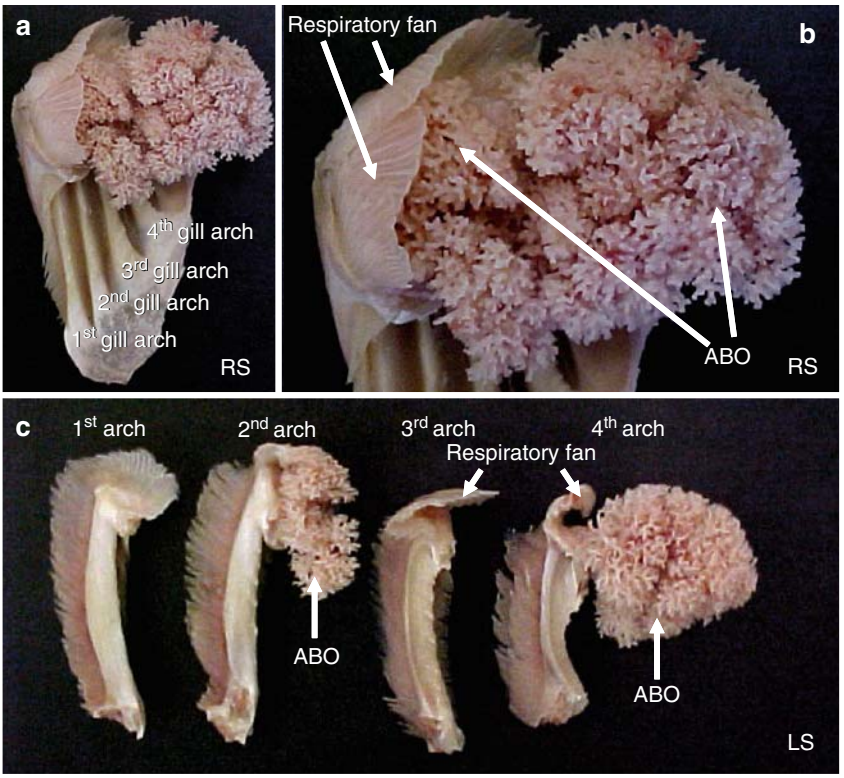

Fig. 3 Gill structures of the African catfish, *Clarias gariepinus*, showing: (**a)** general view of the four gill arches with the ABO on top (*RS* – right side), (**b)** closer look at the ABO showing the respiratory fan and the arborescent organ, which is the gas-exchange unit (*RS* – right side), and (**c**) Details of the four gill arches showing the branchial system with lamellae, ventilatory fan in the 3rd and 4th gill arches and the arborescent organ (ABO) in the 2nd and 4th gill arches (*LS* – left side)

Most of the studies on *C batractus*, *C. lazera, C. gariepinus* and *C. macrocephalus* concluded that they are facultative air breathers (Hora 1935; Magid 1971; Donnelly 1973; Jordan 1976; Bevan and Kramer 1987), but some authors consider the first two species as obligatory air-breathers (Greenwood 1961; Singh and Hughes 1971).

The ABO in this genus (Fig. 3) consists of two chambers in superbranchial position and located within the posterior–dorsal part of the opercular cavity. This space is nearly filled up with arborescent gill organs, which are extensions of the second and fourth gill arches. In addition, the openings to each chamber are equipped with valves to prevent water to enter. These valves are fan shaped extensions and are located on the last gill lamellae of the interior gill arch. Aerial respiration involves a modified gill epithelium, including the inner surface of these openings, its base, the

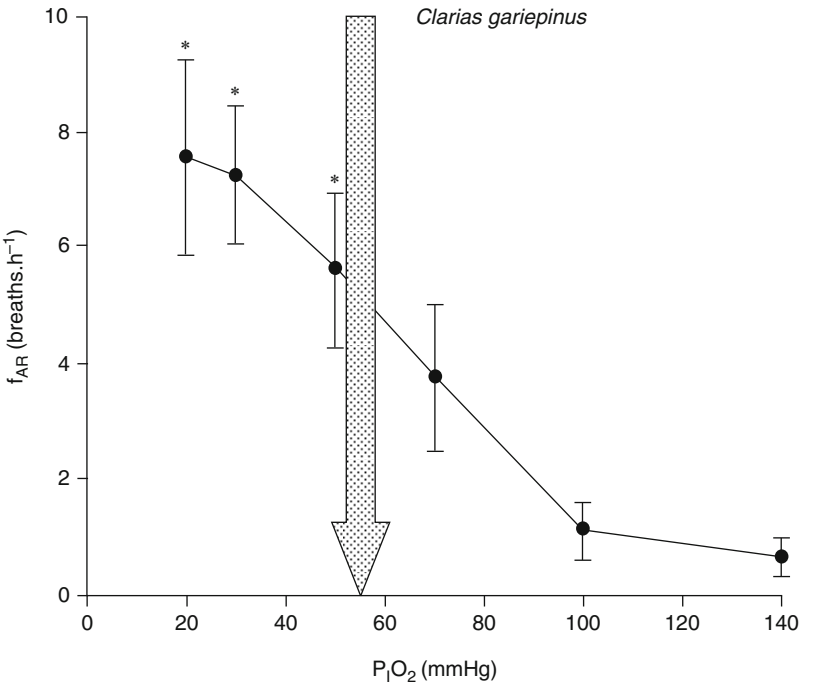

Fig. 4 Air-breathing frequency of the African catfish, *Clarias gariepinus*, as a function of the inspired PO_2. The *arrow* indicates the critical oxygen tension ($PcO_2 = 55$ mmHg) for this species. Notice that the f_{AR} increased significantly near the PcO_2 to reach maximum values (10-fold in relation to the initial values) at a P_IO_2 of 20 mmHg. *Asterisks* represent statistical significance in relation to the normoxic condition (unpublished data)

walls of the interior chamber, and the end of the arborescent surfaces (Munshi 1976; Singh et al. 1982).

The African catfish *Clarias gariepinus* is a facultative air-breather, which can maintain an adequate $\dot{V}O_2$ down to a critical PO_2 of about 55 mmHg. Below this tension, the fish increases the $\dot{V}_G$ considerably, due to a larger V_T. At this point the species develops hypoxic bradycardia before the air-breaths, followed by tachycardia after the surface episode (unpublished data). Figure 4 shows the frequency of air breathing (f_{AR}) of *C. gariepinus* in response to graded hypoxia.

1.11 Oxygen and CO_2/H^+ Receptors

The ever-changing conditions in water are matched by a highly O_2-oriented respiratory control in holeost and teleost fish. Oxygen receptors are located within the gill system to monitor blood gases, or alternatively, the inspired water (Burleson and Milsom 1995a, b, 1990). These external receptors are important for homeostasis

of the blood. As an example, carp (*Cyprinus carbio*) was exposed to light hypoxia ($PwO_2 = 97$ mmHg), but PaO_2 remained at ~30 mmHg during both normoxia and hypoxia. Simultaneously, gill ventilation increased from 296 to 470 ml kg^{-1} min^{-1}, due to stimulation by the external O_2 receptors. In addition, there is evidence for $[O_2]$ receptors in carp, since gill ventilation increased 50%, after reduction of $[O_2]$a from 6.8 to 4.4 vol% by application of CO. Carp were also exposed to hypercarbia (normoxia, 7 mmHg and 14 mmHg, which increased gill ventilation twofold. Unfortunately, the increases of gill ventilation correlated with significant reductions of $[O_2]$a due to hypercarbia-induced Root and Bohr-shifts (Soncini and Glass 2000). Therefore, it was not possible to pin down the exact modality of the underlying receptors.

Gilmour et al. (2005) studied cardiac and respiratory responses to hypercarbia in tambaqui (*Colossama macroporum*). Stepwise increase of $PaCO_2$ augmented the amplitude and frequency of ventilatory movements, which suggested the presence of CO_2 receptors. The tested range was $PCO_2 = 7$–25 mmHg, but information on conditions in the habitat would have been useful. The responses to CO_2 predominated whereas the effects of pH changes were weaker. As a problem, it is difficult to distinguish between specific CO_2/H^+ receptors and $[O_2]$ receptors. The latter type can be stimulated by reduction of $[O_2]$ due to Bohr shifts and Root effects (cf. Soncini and Glass 2000).

Perry et al. (2004) addressed the exposure of tropical fish to large fluctuations of ambient $[O_2]$ levels. One choice was the air-breathing erythrinid jeju (*Hoplerythrinus unitaeniatus*), an active freshwater predator, widely distributed in tropical and subtropical regions of South America (Kramer 1978; Rantin and Johansen 1984). The air-breathing organ (ABO) of jeju is the swimbladder, which is subdivided into an anterior and a posterior chamber (Fig. 5), which is subdivided into an anterior richly vascularized respiratory portion, and a nonrespiratory caudally oriented sac. As a facultative air-breather, jeju relies primarily on its gills for gas exchange as long as the water remains normoxic or moderately hypoxic, but it ventilates its ABO as a facultative option, when exposed to severe aquatic hypoxia (Kramer 1978; Stevens and Holeton 1978; Graham 1997). It turned out that this species had no release of plasma catacholamines, regardless of levels of aquatic hypoxia. With reductions of PwO_2 the ventilation of the ABO increased in a hyperbolic manner down to $PwO_2 = 10$ mmHg. On the other hand, its hypoxia-tolerant relative traira (*Hoplias malabaricus*) depends completely on gill ventilation, and its catacholamine levels increased no less than 18-fold, which clearly documents the advantages of an ABO. Catacholamines have been considered as a stimulus to gill ventilation (Randall and Taylor 1991), but their release failed to increase ventilation of the ABO in jeju. Oliveiro et al. (2004) evaluated the effects of aquatic hypoxia on jeju, and it turned out that the critical PO_2 was $PwO_2 = 40$ mmHg, and 50% of its time was spent at the surface, when P_IO_2 was reduced to 20 mmHg (Fig. 6). In this species, air-breathing was totally abolished after complete branchial denervation (cranial nerve IX to first gill arch and all branches of cranial nerve X innervating the four gill arches). This indicates that the control of air-breathing in jeju involves O_2 chemoreceptors distributed on all gill arches (Lopes 2003).

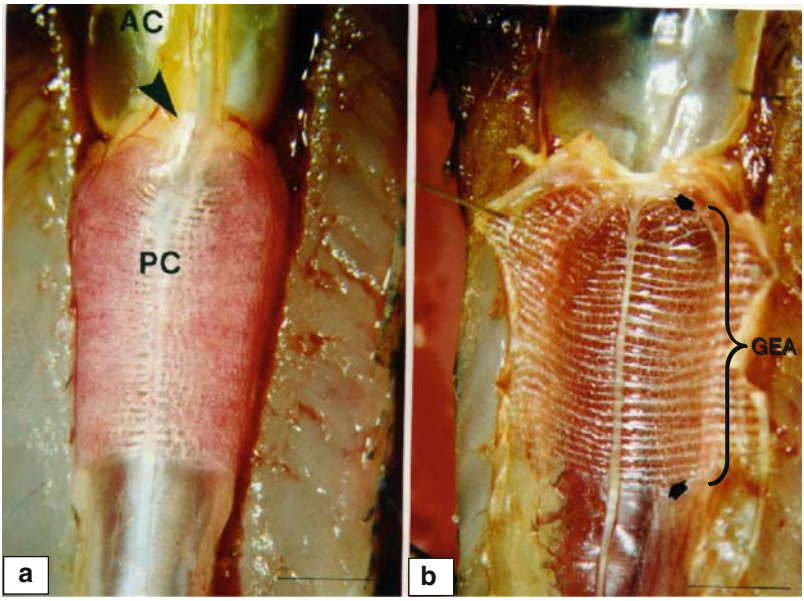

Fig. 5 Swimbladder of jeju, *Hoplerythrinus unitaeniatus*. **a** Intact organ showing the anterior membranous and nonvascularized portion (*AC* – anterior chamber) and the gas exchange unit (*PC* – posterior chamber with the vascularized anterior segment). **b** Opened bladder showing the gas exchange area (*GEA*). Courtesy from Dr. Marisa Narciso Fernandes (UFSCar, São Carlos, SP, Brazil)

Several studies report that hypercarbia stimulates ventilation of the gills and/or an ABO, but these effects are usually weak and, therefore, hard to pin down. By contrast, the responses to hypoxia are large and predominant. The existence of central acid–base receptors in holeost and teleost fish is still a disputed topic. Hedrick et al. (1991) applied central superfusion of mock CSF to the ventricular system of *Amia calva*, but this failed to stimulate ventilation of the ABO.

The long-nosed gar (*L. osseus*) gave a different and partly controversial result. Wilson et al. (2000) reported that hypercarbia increased the frequency of the air-breathing motor output from the in vitro brain stem. Smatresk and Cameron 1982) had earlier reported a modest increase of aerial ventilation in the gar pike (*Amia calva*) exposed to hypercarbia. It is not a surprise that an isolated preparation can change firing characteristics, if it is isolated from a possible frequency modulator, and the discovery of central respiratory neurons is highly important.

1.12 The Air-Breathing Descendents of the Sarcopterygians

Sarcopterygians (lobe-finned fish) are in a key position in vertebrate evolution, because their descendants were the land vertebrates (Tetrapoda), the lungfish

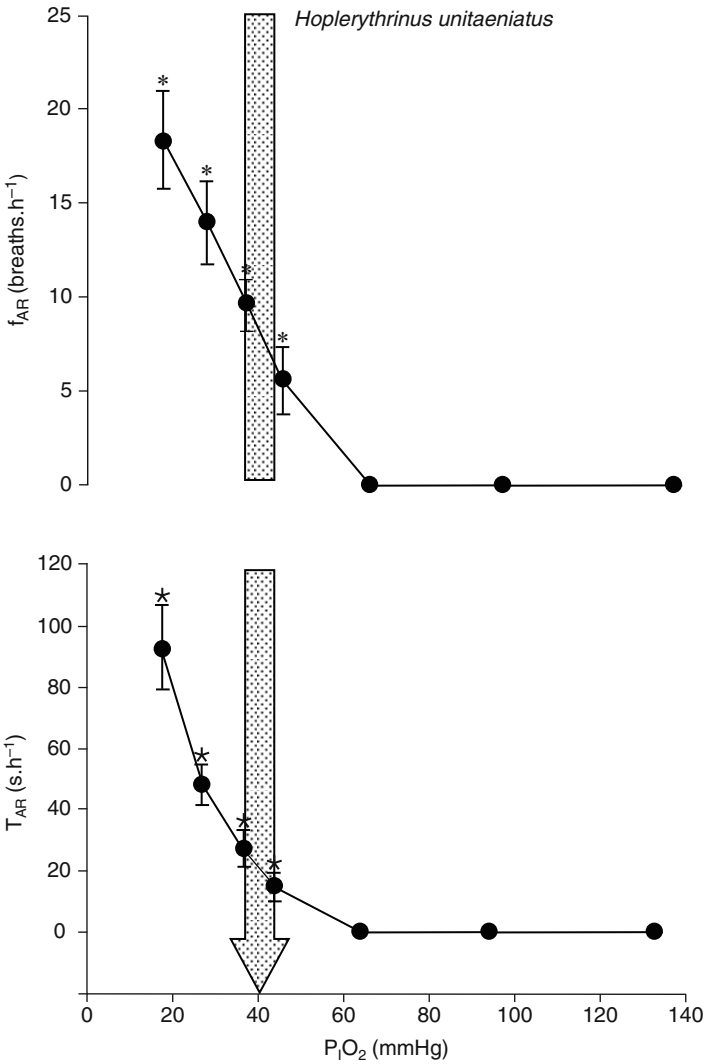

Fig. 6 Air breathing frequency (f_{AR}) and time spent at the surface (T_{AR}) of jeju, *Hoplerythrinus unitaeniatus* as a function of the inspired PO_2. The *arrow* indicates the critical oxygen tension ($PcO_2 = 40$ mmHg) for this species. *Asterisks* represent statistical significance in relation to the normoxic condition (modified from Oliveiro et al. 2004)

(Dipnoi) and the coelacanth (Actinistia). Currently, the most likely sister group constellation is lungfish with the tetrapods (Toyama et al. 2000; Brinkmann et al. 2004), and a fossil (*Styloichthys*) estimated to be no less than 417 million years old fossil, which may be their last common ancestor (Zhu and Yu 2002). The South American lungfish *Lepidosiren paradoxa* inhabits the Amazon and Paraná–Paraguai regions, while the African lungfish *Protopterus* inhabits West and South Africa with four

species. The Australian lungfish (*Neoceratodus forsteri*) inhabits rivers within the Queensland region, and it has well-developed gills combined with a simple lung (Kind et al. 2002). The gills of *Protopterus* and *L. paradoxa* are highly reduced, while the lungs are well developed, and the O_2 uptake from the water is only 5% at 25°C and nil at 35°C, whereas the CO_2 outputs were 50 (25°C) and 25% (35°C) (Amin-Naves et al. 2004). Lungfish have true lungs with a diffusing capacity (D_LO_2) is on level with that of a bullfrog (Bassi et al. 2005; Moraes et al. 2005), but the capacity for a similar-sized mammal is 16-fold higher (Takezawa et al. 1980). Just like land vertebrates, *L. paradoxa* and *P. annectens* have central chemoreceptors involved in acid–base regulation (Sanchez et al. 2001; Gilmour et al. 2007) and can be stimulated by CO_2 and H^+ (Amin-Naves et al. 2007a). The central chemoreceptors of *L. paradoxa* provide 80% of acid–base-related drive to ventilation, while 20% of the drive is provided by peripheral receptors. These relative contributions of peripheral and central CO_2/H^+ drives are largely identical to those of tetrapods (Amin-Naves et al. 2007b). Intrapulmonary receptors that are stimulated by stretch and inhibited by high CO_2 levels are found in many land vertebrates (Milsom 2002), and are also present in *L. paradoxa* and *Protopterus* (Delaney et al. 1983). Many highly specific physiological mechanisms are evidently common to tetrapods and lungfish, which certainly back up the idea of a sister group.

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Effects of Temperature on Cardiac Function in Teleost Fish

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Abstract Changes of environmental temperatures immediately and substantially influence cardio-vascular function in teleost fish. In most species, the body temperature closely parallels that of the environment. Some species live at a nearly constant temperature, such as deep seas and polar oceans. On the other hand, subtropical and tropical environments expose fish to ever-changing temperatures, which provokes large adjustments of physiological and biochemical rate processes and behavioral patterns. Teleost fish display large differences in cardiac function, depending on adaptations to temperature, mode of life, and activity levels. Seasonal and acute environmental temperature fluctuations, and the temperature-dependent regulation of cardiac performance become crucial for ectothermic fish. This motivates a growing concern about the possible impacts of global warming, since it is associated with ever-changing temperatures and oxygen levels in aquatic ecosystems. Currently, there is a growing need to understand the physiological basis of biodiversity, and to evaluate the resistance of distinct species to cope with changing environments. On this background, this chapter reviews how ambient temperature changes cardiac function in teleost fish, emphasizing both in vivo (heart rate, stroke volume and cardiac output) and in vitro components (cardiac contractility).

1 Introduction

Teleost fish are by far the largest number of species, when compared to other groups of vertebrates. In the oceans, they are dominant and invade an amazing number of different marine habitats, and the freshwater habitats are also dominated by teleosts (Nelson 1994). The evolutionary adaptation to different habits, modes of life and activity levels has resulted in an impressive interspecific variation

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with regard to cardiac anatomy and physiology which, in particular, concerns excitation–contraction coupling (E–C coupling).

Living in a variety of vulnerable habitats, teleosts are increasingly threatened by global warming. Greenhouse gases released mainly by human activities such as burning of coal, oil, and natural gases have recently caused a considerable increase in mean global temperatures. Against this background, most studies predict that ambient temperatures will increase by 1.4–5.8°C from now until the end of the century, increasing the temperatures of oceans and inland waters. Increased water temperatures, as well as changes in rainfall, oceanic currents, and sea levels are already affecting the world's fish and fisheries (Combes 2005).

In most species of fish, the body temperature closely parallels that of their environment. Some species live at a nearly constant temperature, and these include deep seas or polar oceans, while other species are exposed to ever-changing temperatures, which provokes major changes in their physiological and biochemical rate processes and behavioral patterns. Depending on adaptations to temperature, life styles, and activity levels, teleost fish display large differences in cardiac function. When exposed to seasonal and acute environmental temperature fluctuations, the temperature-dependent regulation of cardiac contractility becomes crucial for ectothermic fish. For example, vertical movement of fish in the water column across a thermocline can cause significant and rapid oscillations of body temperature. Since these changes are, largely, unpredictable, the fish heart must have some intrinsic mechanism to protect against acute temperature changes (Tiitu 2002; Vornanen et al. 2002; Rocha et al. 2007a, b).

There is a growing concern about the possible impacts of global warming on fish, because it is associated with changes in temperature and oxygen levels in all aquatic ecosystems. Currently, there is a growing need to understand the physiological basis of biodiversity, and to evaluate the resistance of distinct species to withstand changes of the environment. Against this background, this chapter reviews current information on the effects of ambient temperature changes, with a special emphasis on cardiac function in teleost fish.

1.1 Temperature as a Controlling and Limiting Factor

Temperature range is one of the most important features to describe any water body, because the temperature influences the development and distribution of the species. In addition, the degree of circulation will disperse nutrients and pollutants within the water column. Moreover, temperature affects virtually all levels of biological organization, from molecular diffusion to biochemical reactions, membrane function and organ function, which constitute the whole organism.

According to Guderley and St-Pierre (2002), ectothermic vertebrates have two choices when exposed to unfavorable temperatures. They may move to a compatible temperature or, alternatively, remain within their habitat. In this latter case, they will be exposed to the constraints imposed by the law of Arrhenius, which reflects

the impact of temperature upon the frequency of molecular collisions and, in addition, formally describes the thermal dependence of rate processes. When subjected to environmental temperature changes, organisms can: (a) submit to the effects of Q_{10} by slowing down or, alternatively, accelerating their physiological processes, (b) enhance the Q_{10} effects on rate processes (hibernation, torpor), or (c) offset the Q_{10} effects by triggering compensatory mechanisms to maintain functions and capacities. Additionally, a combination of high temperatures and unfavorable conditions, such as hypoxia and decreased food availability, may lead to aestivation, which is a period of torpor.

Body temperatures of small animals can not deviate much from the temperature of the aquatic environment. This is due to the high thermal conductivity and large heat capacity of water (Schmidt-Nielsen et al. 1975; Stevens and Sutterlin 1976). Most teleost fish must cope with ambient temperature changes, which is an important aspect of their physiology. Nevertheless, highly active species of large aerobic scope such as blue fin tuna, skipjack tuna and bigeyed tuna are able to maintain an elevated body temperature in relation to their environment (Stevens et al. 1974; Stevens and Neill 1978).

A preference for a specific temperature range is a common feature among teleosts. This preferred temperature range generally coincides with an optimal growth rate (Jobling 1982) and/or with a high aerobic capacity (Kelsch and Neill 1990). Seasonal changes, diurnal rhythms and growth can, however, alter the preferred temperature in a species-specific manner (Reynolds and Casterlin 1979; Roberts 1979). Further, the preferred temperature is influenced by the O_2 availability, because fish faced with hypoxic conditions prefer lower temperatures, which decreases metabolic demands. Obviously, this response increases their survival rate, when in hypoxia (Schurmann and Christiansen 1994). In addition, animals inhabiting small water bodies such as shallow and small lakes, streams, ponds, canal and drains are subjected to diurnal or seasonal temperature changes without any major temperature gradient from surface to bottom, which sets very narrow limits for any efficient behavioral thermoregulation (Harder et al. 1999).

Cardiac function in eurythermal fish is subject to large temperature changes, and the effects of temperature acclimation on cardiac contractility vary according to the strategy of environmental adaptation (Aho and Vornanen 1999; Tiitu and Vornanen 2001, 2002a). Conversely, stenothermal species have acquired special characteristics, which are highly adapted to habitats with specific thermal characteristics. These specific evolutionary adaptations occurred, however, at the expense of reduced plasticity for temperature change (Tiitu and Vornanen 2002b). For instance, Egginton et al. (2006) pointed out that most of the organisms in the Antarctic marine environment are stenothermal and, therefore, will die at temperatures in the range +5–7°C, and such Antarctic species will be at high risk when faced with even moderate increases of ambient temperature, which may be triggered by global warming.

For the reasons mentioned, temperature can be classified as both a controlling and limiting environmental factor for fish, as earlier described by Fry (1948), which requires physiological and behavioral adjustments. In this review we discuss what

happens to cardiac function in teleost fish exposed to unavoidable changes of body temperature.

2 Cardiac Output, Heart Rate, and Stroke Volume

The function of the heart is to supply blood to the tissues, and generate and sustain the systolic blood pressure needed for adequate perfusion of the tissues. The contraction of the heart by its muscular walls must generate sufficient pressure to propel blood from the cardiac chamber (e.g., ventricle), into the ventral aorta. For each beat, a volume of blood is ejected. This stroke volume (S_V–ml kg^{-1}), times the number of beats per minute (heart rate, f_H–bpm), equals the cardiac output (Q), which is the total volume of blood delivered by the ventricle each minute (ml min^{-1} kg^{-1}). In this context, all changes in either f_H or S_V will influence the other components. In addition, Q is determined not only by activity of the heart but also by peripheral resistance (Klabunde 2004). Cardiac function is well established for mammals, whereas data on fish are more controversial, which results from a lack of standard methods (e.g., estimated or direct measured) and large interspecific differences (Table 1).

Changes of environmental temperatures immediately and substantially influence cardio-vascular function in teleost fish. For the majority of teleosts, f_H is typically between 10 and 60 bpm and will not exceed 120 bpm (Farrell 1991; Anelli-Jr et al. 2004). Nevertheless, even higher values for f_H were described by Brill (1987) who studied scombrid species, including yellowfin tuna, *Thunnus albacares* (90–160 bpm), kawakawa, *Euthynnus affinis* (175–253 bpm), and skipjack tuna, *Katsuwonus pelamis* (191–237 bpm), where high f_H and high swimming performance are matched. In this context, the frillfin goby, *Bathygobius soporator*, a resident intertidal species, is abundant in Brazilian rocky tide pools, where the temperature can reach more than 40°C during low tide. When exposed to acute temperature transitions from 25 to 35°C, this species increased f_H progressively and significantly, reaching a maximum value of about 225 bpm (Rantin et al. 1998).

The temperature coefficient Q_{10} provides a measure of the rate of change of a biological or chemical system, which increases temperature by 10°C. According to Webber et al. (1998), a Q_{10} of 1.0 would imply that metabolism is independent of temperature. The expected value for an increase is $Q_{10} = 2.0$, which implies that metabolism increases twofold for each increase by 10°C.

In most temperate fish, acute changes of temperature typically increase f_H with an average Q_{10} of about 2.0 (Priede 1974; Butler and Taylor 1975; Cech et al. 1976; Graham and Farrell 1985; Blank et al. 2002; Tiitu and Vornanen 2002a). In addition, Rantin et al. (1998) suggested that these Q_{10} values also apply to tropical species. Nevertheless, some tropical species present Q_{10} values for f_H of about 3.0 during acute temperature transitions. This applied to pacu, *Piaractus mesopotamicus*, an active migratory serrasalmid fish which increased f_H from 37 to 115 bpm (Anelli-Jr et al. 2004). This is compatible with values for trahira, *Hoplias*

Table 1 Cardiac output (Q), heart rate (f_H) and stroke volume (S_V) of different teleosts species at different temperatures and measurement techniques

Species	Temp (°C)	Wt (g)	Q ($ml\,kg^{-1}\,min^{-1}$)	f_H (bpm)	S_v ($ml\,kg^{-1}$)	Method	Reference
	6	~1,110	9.5	7	1.22	In vivo – Resting	
Cyprinus carpio	10	~1,230	19	12	1.58	In vivo – Resting	Stecyk and Farrell (2002)
	15	~790	15	17	0.77	In vivo – Resting	
	18	250–320	91	65–70	1.38	In situ perfused heart	Farrell et al. (2007)
Dicentrarchus labrax	22	250–320	95	95–100	1.05	In situ perfused heart	
	5	~1,910	9	16	0.52	In vivo – Resting	
	5	~1,910	31	28	1.12	In vivo – Swimming	Webber et al. (1998)
	10	~1,910	12	28	0.45	In vivo – Resting	
Gadus morhua	10	~1,910	35	40	0.88	In vivo – Swimming	
	10	861–1,335	22	36	0.60	In vivo – Swimming	Gollock et al. (2006)
	20	861–1,335	47	73	0.67	In vivo – Swimming	Gollock et al. (2006)
	25	1,600	138[b]	125.9	1.1	Spinally blocked	Bushnell and Brill (1992)
Katsuwonus pelamis	25	1,300	105[b]	154	0.68	In vitro perfused heart	Farrell et al. (1992)
	4	~1,120	5	38	0.14	In vivo – Resting	
	4	~1,120	14	44	0.25	In vivo – Swimming	
	11	~822	21	52	0.53	In vivo – Resting	
	11	~822	73	59	1.35	In vivo – Swimming	Taylor et al. (1996)
	18	~810	20	91	0.25	In vivo – Resting	
Oncorhynchus mykiss	18	~810	41	95	0.50	In vivo – Swimming	
	10	~500	15	55	0.27	In situ perfused heart	Overgaard et al. (2004)
	10	290–1,060	100[a]	47	100[a]	In vivo – Resting	
	13	290–1,060	120[a]	62	91[a]	In vivo –Resting	Sandblom and Axelsson (2007)
	16	290–1,060	131[a]	76	80[a]	In vivo – Resting	

(*Continued*)

Table 1 (Continued)

Species	Temp (°C)	Wt (g)	Q ($ml\,kg^{-1}\,min^{-1}$)	f_H (bpm)	S_v ($ml\,kg^{-1}$)	Method	Reference
	4	453–843	9,8	21	0.5	In vivo – Resting	
	4	453–843	25.4	31.6	0.80	In vivo – Swimming	Joaquim et al. (2004)
Pleuronectes americanus	10	453–843	15.5	34	0.5	In vivo – Resting	
	10	453–843	39.2	52.4	0.74	In vivo – Swimming	
Thunnus alalunga	16.5	9,100	28[b]	87	0.32	Anesthetized	Lai et al. (1987)
	21.9	9,300	44[b]	115	0.38	Anesthetized	White et al. (1988)
Thunnus albacares	25	1,400	126[b]	96.7	1,3	Spinally blocked	Bushnell and Brill (1992)
	25	1,400	75[b]	112	0.67	Anesthetized	Jones et al. (1993)
	5	~6,700	19	23	0.86	In situ perfused heart	
	10	~6,700	21	36	0.57	In situ perfused heart	
Thunnus orientalis	15	~6,700	20	55	0.37	In situ perfused heart	Blank et al. (2004)
	20	~6,700	17	78	0.23	In situ perfused heart	
	25	~6,700	18	100	0.18	In situ perfused heart	

[a] Data as a % of initial values at 10°C; [b] Data calculated based on f_H and S_v values

Table 2 Q_{10} values for resting heart rate (f_H–*bpm*) during temperature changes for different tropical, temperate and polar teleost species

Species	°C	Q_{10}	Reference
Bathygobius soporator	25–5	1.6	Rantin et al. (1998)
Chaenocephalus aceratus	2 to −1.8	6.3	Bastos-Ramos et al. (1998)
Gadus morhua	5–10	2.9	Webber et al. (1998)
Hoplias malabaricus	25–35	3.0	Olle 2003
	5–15	1.4	
Leiopotherapon unicolor	15–25	1.2	Gehrke and Fielder (1988)
	25–35	1.1	
Notothenia neglecta	2 to −1.8	4.8	Bastos-Ramos et al. (1998)
Oreochromis niloticus	25–35	2.4	Costa et al. (2000)
	−1 to 3	3.3	
Pagothenia borchgrevinki	3–6	2.5	Lowe et al. (2005)
	6–10	1.2	
Piaractus mesopotamicus	25–35	3.1	Anelli-Jr et al. 2004
	4–10	2.2	Joaquim et al. (2004)
Pleuronectes americanus	5–10	1.4	Cech et al. (1976)
Prochilodus lineatus	25–35	2.3	Kalinin, unpublished data
Synbranchus marmoratus	25–35	1.6	Rocha et al. (2007a)
	25–15	2.3	
Thunnus albacares	10–20	3.6	Blank et al. (2002)
	15–25	2.6	
Thunnus orientalis	5–15	2.4	Blank et al. (2004)
	10–20	2.1	
	15–25	1.8	

malabaricus, which is a sedentary erythrinid species that may increase f_H from 35 to 105 bpm (Olle 2003) if exposed to acute temperature transitions from 25 to 35°C. Surprisingly, this also applied to the Antarctic notothenioid *Pagothenia borchgrevinki,* which increased f_H from 16 to 33.7 bpm during temperature transitions from −1 to 6°C (Lowe et al. 2005). This response seems large in relation to the extremely low temperature of the environment. Table 2 presents selected Q_{10} values for f_H, underlying the considerable variability among species.

Taken together, these results suggest that increases in f_H can be the main adjustment triggered to maintain adequate cardiac performance during increases of temperature. Changes of f_H are considered most important for modulations of cardiac output, which has also been described for the tuna heart by Farrell (1996) and Blank et al. (2002). As to low temperatures, Webber et al. (1998) recorded Q_{10} values for resting values of Q and f_H in cod (*Gadus morhua*). As calculated from 5 to 10°C, the value was 1.65 for Q and 2.93 for f_H. These authors concluded that the reduction in Q with lower temperature can be attributed to considerable reductions of f_H (Armstrong 1986; Gehrke and Fielder 1988; Kolok et al. 1993; Keen and Farrell 1994; Claireaux et al. 1995).

Elevated temperatures increase the depolarization frequency of the pacemaker cells and, consequently, of the heart rate (for a review, see Tibbits et al. 1992a). In addition, higher temperatures shorten the duration of the ventricular action

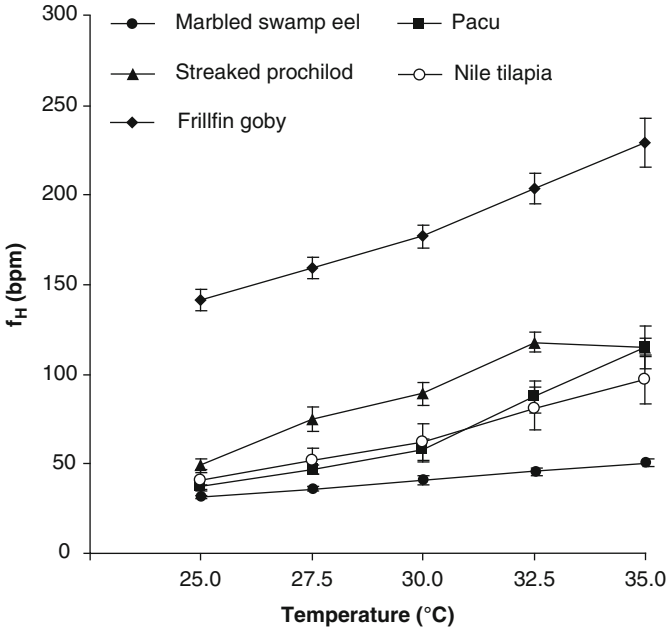

Fig. 1 Effects of temperature on heart rate (f_H) for Nile tilapia, *Oreochromis niloticus* (Costa et al. 2000), frillfin goby, *Bathygobius soporator* (Rantin et al. 1998), pacu, *Piaractus mesopotamicus* (Anelli-Jr et al. 2004), marbled swamp eel, *Synbranchus marmoratus* (Rocha et al. 2007a), and streaked prochlilod, *Prochilodus lineatus* (Kalinin, unpublished data), acclimated to 25°C

potential (VAP_D) (Lennard and Huddart 1991; Aguiar et al. 2002). This effect can be attributed to an increased permeability of the sarcolemma (SL), leading to an earlier final repolarization. Consistent with these temperature effects, several tropical species increase f_H when exposed to high temperatures (Fig. 1).

In 1988, Gehrke and Fielder combined increases in temperature and different oxygen levels to analyze the cardiorespiratory responses of the spangled perch, *Leiopotherapon unicolor.* Widespread throughout inland waters of Australia, this species survives temperatures from 5 to 44°C and dissolved oxygen concentrations down to $1\,mg\,L^{-1}$. The purpose was to simulate steep thermal gradients without any acclimation. The f_H of *L. unicolor* increased in response to rising temperature, and decreased as the temperature became reduced (Fig. 2). At 100% O_2 saturation of the water, f_H reached a maximum mean rate of 104.1 bpm at 35°C, compared to the minimum mean rate of 12.2 bpm at 5°C, which gives an overall Q_{10} value of 2.85. Q_{10} values for f_H at other oxygen tensions ranged from 2.50 to 3.05. Progressive hypoxia caused bradycardia and heart rate became dependent on available oxygen before oxygen consumption declined, with the exception of individuals at 35°C.

Little information is available on the in vivo effects of temperature on cardiorespiratory systems, whereas substantial information has been provided by in vitro

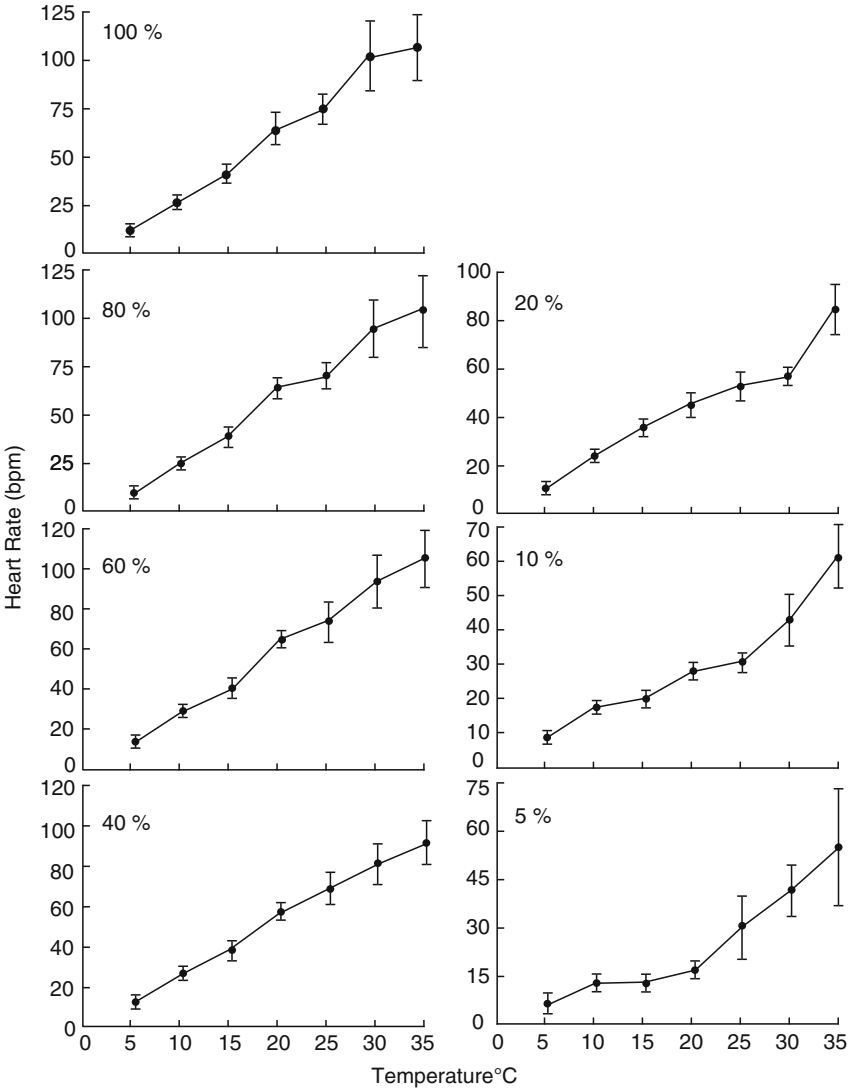

Fig. 2 Heart rate (f_H) of splangled perch, *Leiopotherapon unicolor*, in response to temperature at levels of oxygen saturation from 100% to 5% (from Gehrke and Fielder 1988)

studies on perfused hearts (Farrell 2002). Furthermore, the majority of the studies were conducted with temperate fish species.

Several in vitro studies have demonstrated an inverse relationship between f_H and V_S in fish hearts in response to acute temperature changes (Graham and Farrell 1985; Webber et al. 1998; Yamamitsu and Itazawa 1990; Farrell 2002).

Studying the lincod, *Ophiodon elongates*, Stevens et al. (1972) pointed out that changes of f_H are most important for modulation of Q, in vivo, while changes in

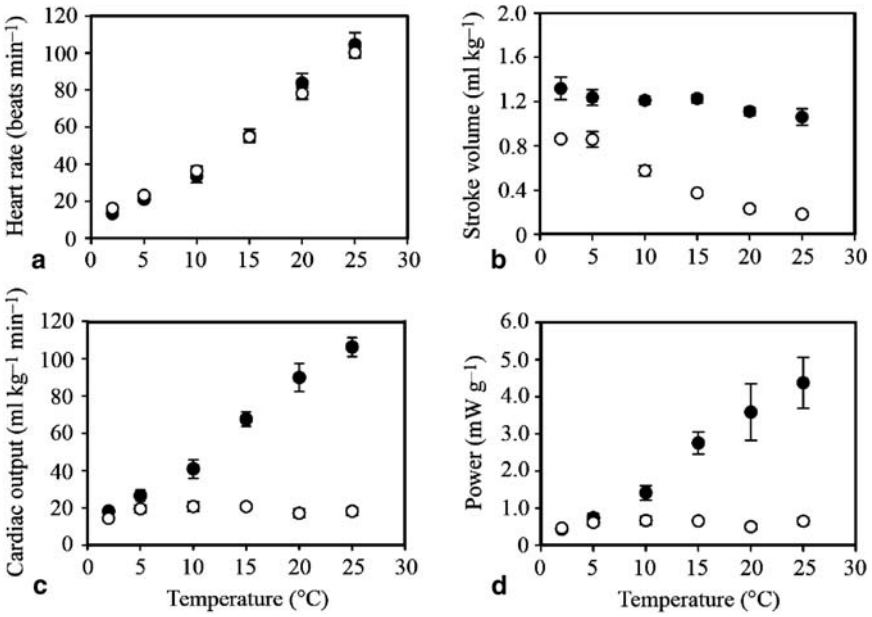

Fig. 3 Values of cardiac parameters recorded from spontaneously beating yellowfin tuna hearts *in situ* at temperatures of 10–25°C: heart rate (**a**), stroke volume (**b**), cardiac output (**c**), myocardial power output (**d**). Standard conditions are shown as *open symbols*, maximal conditions as *filled symbols*. (from Blank et al. 2002)

V_S are of minor importance. Blank et al. (2002) evaluated the effects of acute temperature changes on cardiac function of the in situ-perfused heart preparation of yellowfin tuna (*Thunnus albacares*), and they found that f_H became reduced in a linear manner, falling from ~106 bpm at 25°C to ~20 bpm at 10°C. Meanwhile, S_V increased as temperature fell, resulting in a very limited effect on cardiac output under standard conditions (Fig. 3). Based on these results, and earlier in vitro experiments (Freund 1999), it was proved that changes in S_V are, very probably, important for maintained cardiac output in tuna confronted with changing temperatures (cf. Farrell et al. 1996). Consistently, an increase in V_S (Webber et al. 1998) partially compensated for the large reduction in f_H from 10 to 5°C recorded in cod.

Joaquim et al. (2004) conducted the first direct measurements of resting and maximum cardiac function in winter flounder, *Pleuronectes americanus,* determining the critical swimming capacity at 4 and 10°C in relation to temperature. In resting fish, they found a temperature-induced increase in Q (from 9.8 to 15.5 ml kg^{-1} min^{-1}), exclusively achieved by an elevated f_H (from 20.5 to 34.3 bpm – Q_{10} of 2.35), while resting S_V was independent in relation to acclimation temperature (0.50 at 4°C and 0.47 ml kg^{-1} at 10°C)P. Previously, Cech et al. (1976) studied the same species, and reported a significantly lower Q_{10} (1.36) for f_H between 5 and 10°C. This could be due to a higher resting f_H at the lower temperature (30 bpm) combined with an increase in S_V from 0.55 ml kg^{-1} at 5°C to 0.90 ml kg^{-1} at 15°C. According to

Joaquim et al. (2004), the Sv values obtained by Cech et al. (1976) were calculated from Q values based on the Fick equation. This calculation does not include temperature effects on cutaneous O_2 uptake, and it is possible that the reported S_V increases resulted from temperature effects on cutaneous oxygen uptake. The authors emphasized that their results on winter flounder differed from those of Kolok et al. (1993), who studied the largescale sucker, *Catostomus macrocheilus*, in which Q failed to increase, when acclimated to temperatures of 5–10°C. This occurred because a 25% increase in f_H (Q_{10} of 1.65) was counteracted by 30% decrease in S_V. These intraspecific differences suggest that Q of some species may be rather insensitive to some segments of temperature (Joaquim et al. 2004).

As exposed above, it is clear that alterations in teleosts' heart function caused by temperature changes are highly variable, as shown in Tables 1 and 2. In addition, the methodological differences combined with interspecific characteristics and thermal acclimation exerts a pronounced effect on cardiac parameters.

Matikainen and Vornanen (1992) recorded f_H in response to acute increases of temperature in the crucian carp (*Carassius carassius*) acclimated to 5°C and 15°C (Fig. 4). The responses were identical at the lowest temperatures (2 or 4°C), but above this range f_H was higher in warm-acclimated summer fish, when compared to cold-acclimated winter fish. According to the authors, this type of temperature acclimatization, where the cold-acclimated animal has the lower Q_{10} value, is designated as noncompensatory or inverse acclimation by Prosser (1973).

Likewise, specimens of Nile tilapia, *Oreochromis niloticus*, acclimated to 35°C and then submitted to acute temperature reductions down to 15°C, had a higher f_H at 15°C and 20°C than the cold-acclimated (15°C) specimens submitted to

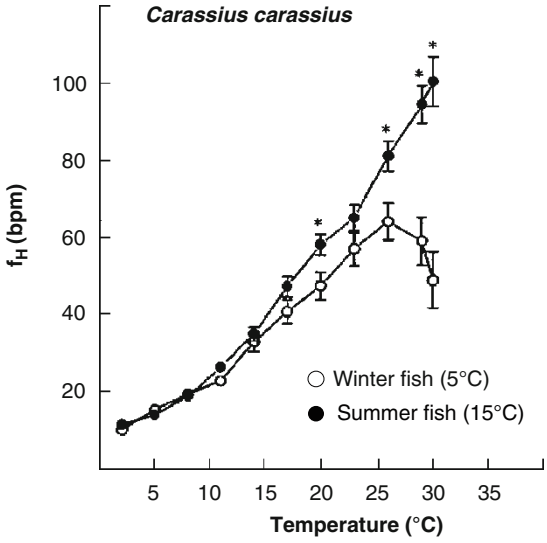

Fig. 4 Temperature dependence of in vivo heart rate (f_H) in crucian carp, *Carassius carassius*, during summer and winter (from Matikainen and Vornanen 1992)

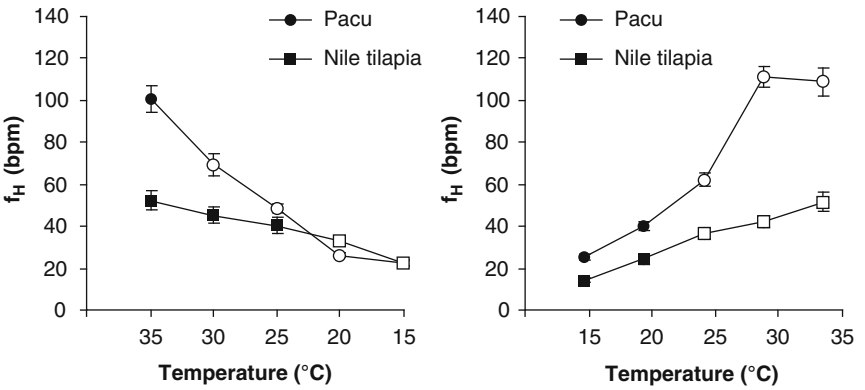

Fig. 5 The effects of temperature on heart rate (f_H) for Nile tilapia, *Oreochromis niloticus* (Maricondi-Massari et al. 1998), and pacu, *Piaractus mesopotamicus* (Aguiar et al. 2002) acclimated to 35°C (*left panel*) and 15°C (*right panel*). *Open symbols* indicate a significant difference ($p < 0.05$) in relation to the initial values

temperatures up to 35°C (Fig. 5) (Maricondi-Massari et al. 1998). On the other hand, Aguiar et al. (2002) reported significantly higher values of f_H in cold-acclimated (15°C) pacu, *P. mesopotamicus*, when compared to the warm-acclimated (35°C) group (Fig. 5). Similarly, goldfish, *Carassius auratus*, acclimated to 10°C displayed a higher f_H than the specimens acclimated to 25°C (Morita and Tsukuda 1995). Likewise, Seibert (1979) recorded a lower f_H for warm-acclimated European eel, *Anguilla anguilla*, when compared to the cold-adapted eels at all temperatures, and this difference could be abolished by vagal blockade. Similar results were also obtained for medaka, *Oryzaias latipes*, guppy, *Lebistes reticulatus* (Tsukuda 1961) and the sole, *Solea vulgaris* (Sureau et al. 1989).

Taken together, these results indicate that the cardiac alterations following temperature changes are not uniform among teleosts, and the effects of temperature acclimation on f_H are rather species-specific, reflecting seasonally induced changes in the temperature sensitivity of the pacemaker cells.

As in other vertebrates, the f_H of teleost fish is determined both by the intrinsic rate of pacemaker cells in the sinus venosus and the extrinsic control by the autonomic nervous system along with humoral factors (Laurent et al. 1983), and temperature affects both intrinsic and extrinsic components. According to Haverinen and Vornanen (2006), low temperatures slow down f_H, which tends to decrease cardiac output as well as the level of activity of ectothermic animals, in particular within habitats of large seasonal temperature changes. Several fish species of the north temperate latitudes such as rainbow trout (*Oncorhynchus mykiss*), perch (*Perca fluviatilis*), European eel (*A. Anguilla*), and sole (*S. vulgaris*), are able to oppose or avoid the effects of cold temperatures on f_H, which occurs by long-term mechanisms that are triggered upon exposure to low temperature. These authors suggest that such compensatory mechanisms involve a cold-induced decrease in the

inhibitory cholinergic control of the pacemaker rate and are likely to involve the pacemaker mechanism itself.

Teleost hearts usually operate under some degree of vagal tone, which varies due to physiological and environmental conditions. The extent of sympathetic cardio-acceloratory innervation in teleosts remains unclear, but some species possess adrenergic nerve fibers, and an adrenergic tonus has been verified in vivo, but the relative importance of neuronal versus humoral influences has not been evaluated. Increased ventricular force contraction and f_H (positive inotropy and chronotropy) are mediated by β-adrenoreceptors on pacemaker cells and myocytes, whereas α-adrenoreceptors mediate negative chronotropy (Egginton et al. 2006).

According to Seibert (1979), vagal tone increases with temperature in the eel (*A. anguilla*), and this also seems to be the case in pacu (*P. mesopotamicus*) described by Aguiar et al. (2002), as shown in Fig. 5. However, in trout and carp parasympathetic control dominates at low temperatures, with the sympathetic control of f_H assuming a greater importance at higher temperatures (Farrell 1984). In the notothenioid *P. borchgrevinki*, an increased f_H during temperature transitions from −1 to 6°C was due to a 45% increase in excitatory adrenergic tone, masking a 37% increase in inhibitory cholinergic tone (Lowe et al. 2005). Additionally, Egginton et al. (2006) emphasized that, in this species, acclimation to a high temperature resets resting f_H to a lower level, which indicates an increased vagal inhibition (Seebacher et al. 2005). These findings reinforce the absence of uniform chronotropic responses to temperature acclimation in teleosts.

2.1 Heart Pacemaker

Unfortunately, the size and exact location of the primary pacemaker area of the fish heart are incompletely defined and, according to Satchell (1991), the pacemaker location within the fish heart is not uniform. In carp, *Cyprinus carpio* (Saito 1973), and Japanese loach, *Misgurnus anguillicaudatus* (Yamauchi et al. 1973), the primary pacemaker area has been located to the sinoatrial junction. In trout, Haverinen and Vornanen (2007) exclusively recorded primary pacemaker potentials from the base of the sinoatrial valve, where a morphologically distinct ring of tissue comprising myocytes and neural elements was detected by histological methods (Fig. 6).

According to Haverinen and Vornanen (2007), the diastolic depolarization of the pacemaker cells, which elicits the regular firing of pacemaker action potentials (APs), results from a combined action of many sarcolemmal ion currents, including T- and L-type Ca^{2+} current, slow and fast components of the delayed rectifier K^+ current, transient outward K^+ current, inward Na^+ current, and the pacemaker current carried by both K^+ and Na^+ ions. They suggest that the pacemaker mechanism could involve a close interplay between Ca^{2+}-induced Ca^{2+} release (CICR) of the sarcoplasmic reticulum (SR) and sarcolemmal Na^+/Ca^{2+} exchange (NCX), since subsarcolemmal SR Ca^{2+} release can accelerate diastolic depolarization via inward

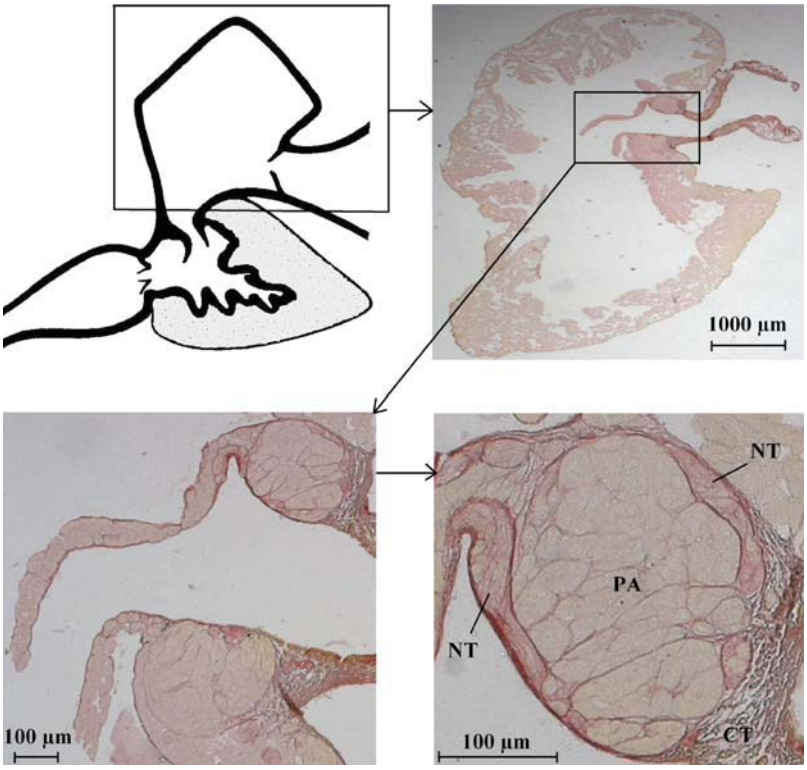

Fig. 6 Light microscopic histology showing structure and location of the sinoatrial nodal tissue of the rainbow trout heart. *Top left panel*: a schematic presentation of the heart structure and the area for histological inspection (within the *box*). *Top right panel*: a photomicrograph of the trout atrium and the adjacent part of the sinus venosus. The junctional area between sinus venosus and atrium, including the sinoatrial valve, is shown framed in the *box*. *Bottom left panel*: a closer look at the nodal tissue at the base of the sinoatrial valve. *Bottom right* panel: an enlarged image of the sinoatrial nodal tissue. *CT*, connective tissue; *NT*, nervous tissue; *PA*, pacemaker area (from Haverinen and Vornanen 2007)

NCX current. The mechanisms involved in cardiac Ca^{2+} management and related effects of temperature will be discussed in the in vitro section of this chapter.

Previous studies have shown that the inherent rhythmicity of pacemaker activity of the marine teleost plaice (*Pleuronectes platessa*) measured in vitro has a high temperature coefficient (Harper and Watt 1990; Windram et al. 1993). Furthermore, Harper et al. (1995) reported that temperature acclimation results in compensatory changes, so that pacemaker rhythms in cold-acclimated fish are higher, when compared to their warm-acclimated counterparts. At least in the plaice, the results show that the temperature coefficient of discharge activity of the cardiac pacemaker tissue displays seasonal variations. Accordingly, annual changes would imply that f_H of winter plaice would be maintained at a rate similar to that of the summer season.

Working on trout, Haverinen and Vornanen (2007) reported that the intrinsic beating rate of the sinoatrial pacemaker was higher in cold-acclimated (46 ± 6 APs min^{-1}) than in warm-acclimated fish (38 ± 3 APs min^{-1}), and similar responses were obtained from isolated pacemaker cells (44 ± 6 vs. 38 ± 6 APs min^{-1}), which corroborates the hypothesis that thermal acclimation modifies the intrinsic pacemaker mechanism of fish heart.

Unfortunately, information is very limited and only temperate species have been studied, which makes conclusions difficult concerning the effects of acclimation on the pacemaker activity. Nevertheless, some techniques, e.g., electrocardiography, can provide some information on the electrical activity of heart cells.

2.2 ECG

As discussed above, temperature strongly influences the contractile performance of cardiac muscle and the discharge rate of pacemaker cells, but the mechanisms of wave pattern variability in ectothermic vertebrates are incompletely understood, and data are few concerning the electrical properties of fish heart in vivo and in vitro.

One of the methods employed to characterize the state of the heart is electrocardiography. Cardiac alterations are extensively used for clinical diagnosis in human cardiology, while the mentioned methods have been little used for the analysis of cardiac function in fish, which probably relates to difficulties in obtaining consistent ECG patterns.

A detailed analysis of cardiac electric activity requires several ECG leads, sufficient to detect each component of the ECG waves and to establish the electrical axis of the heart. However, the use of several leads in fish is difficult for several reasons, including the anatomic diversity of these animals and, in particular, a lack of information on the best sites to place the electrodes in relation to the heart. In this respect, the ECG pattern depends on body shape and should be established for each species. In addition, most of the studies on fish electrocardiography have been performed on anesthetized fish and/or under other stressing conditions that influences the ECG. Nevertheless, important information on myocardial function can be obtained using a single lead provided that the procedure is well standardized for the studied species (for a review, see Satchell 1991; Rantin et al. 1995). The electrocardiogram (ECG) of fish presents the basic electrical events known from mammalian ECG, i.e., the P wave represents the atrium depolarization, the QRS complex indicates the activation of the ventricle, and the T wave corresponds to the ventricular repolarization (Rantin et al. 1995).

Studies conducted with two tropical species, the Nile tilapia, *O. niloticus* (Maricondi-Massari et al. 1998), and pacu, *P. mesopotamicus* (Aguiar et al. 2002) have shown that neither thermal acclimation nor increases or decreases in temperature affected the QRS and the T wave amplitudes (Fig. 7). These results are consistent with Morita and Tsukuda (1995), who studied QRS complexes of goldfish acclimated to 10°C and 25°C. Based on standard electrocardiography, the

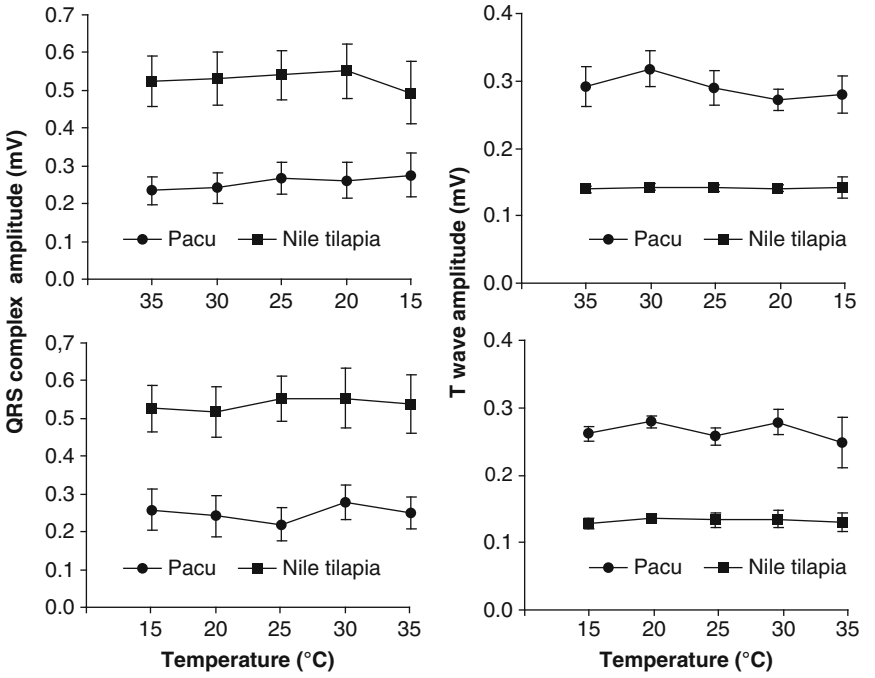

Fig. 7 Effects of temperature on the QRS and T wave amplitudes for Nile tilapia, *Oreochromis niloticus* (Maricondi-Massari et al. 1998), and pacu, *Piaractus mesopotamicus* (Aguiar et al. 2002)

projection of the depolarization and repolarization vectors on the derivation line of the ECG provides the amplitudes of the QRS and T waves respectively. Thus, the ECGs indicated that, in these species (goldfish, Nile tilapia and pacu) the directions of the ventricular depolarization and, moreover, repolarization remained constant, in spite of large variations of temperature. Further, both QRS complexes and T waves recorded for *O. niloticus* and *P. mesopotamicus* were consistently positive, indicating that the ventricular repolarization and depolarization followed the same direction.

According to Cotter and Rodnick (2006), the QRS complex duration indirectly expresses the conduction velocity of electrical signals through ventricular tissue, which is inversely related to f_H during and after exercise in healthy mammals. The precise control of QRS duration is not known, but is probably modulated by autonomic tone and possibly other factors (Trautwein 1963; Goldberger and Bhargava 1983; Nakagawa et al. 2000; Cotter and Rodnick 2006).

In warm-acclimated *O. niloticus*, its QRS duration increased progressively during stepwise increments of temperature, while the QRS duration only increased at 15°C in *P. mesopotamicus*. In the cold-acclimated groups, QRS duration was significantly lower at 20, 25, 30 and 35°C for O. niloticus, and at 30 and 35°C for *P. mesopotamicus* (Fig. 8). These results indicate that the depolarization processes were less sensitive to temperature changes in *P. mesopotamicus* than in

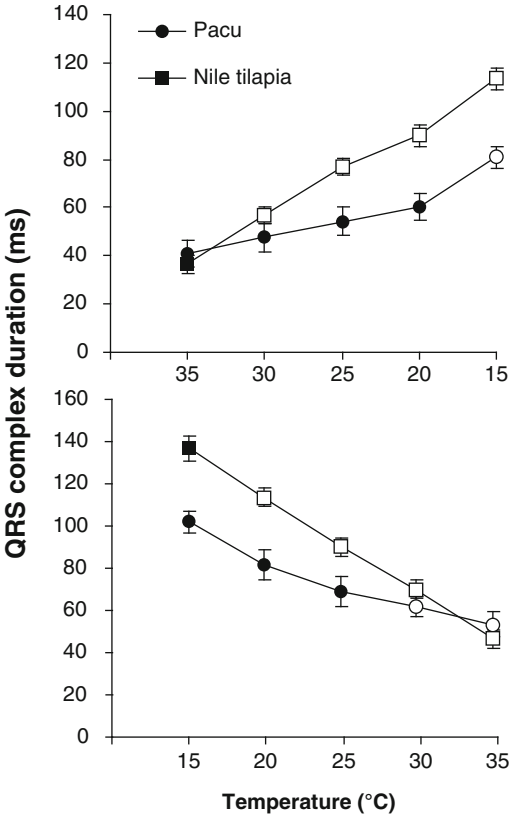

Fig. 8 Effects of temperature on the QRS complex duration for Nile tilapia, *Oreochromis niloticus* (Maricondi-Massari et al. 1998), and pacu, *Piaractus mesopotamicus* (Aguiar et al. 2002) acclimated to 35°C (*upper panel*) and 15°C (*lower panel*). *Open symbols* indicate a significant difference ($p < 0.05$) in relation to the initial values

O. nilotucus. Additionally, in spite of the significantly higher values presented by the warm-acclimated fish, *P. mesopotamicus* increased or decreased the T wave duration with the same magnitude (Fig. 9).

A segment in an electrocardiogram is the region between two waves: the ST segment is the isoelectric line followed by the QRS, which terminates at the onset of the T wave, and represents the time at which the entire ventricle is depolarized and roughly corresponds to the *plateau* phase of the ventricular action potential. The ST segment is important in the diagnosis of ventricular ischemia or hypoxia because, under those conditions, the ST segment can either become depressed or elevated (Klabunde 2004). Temperature changes also have a considerable effect on ST segment, as shown in Fig. 10. The magnitude of this effect seems to be species-specific since the Q_{10} for ST segment in *P. mesopotamicus* is about 3 while *O. niloticus* does not exceed 2, irrespective of temperature acclimation. Consistently, Liu and Li (2005) pointed out that increases in f_H can be caused by the shortening

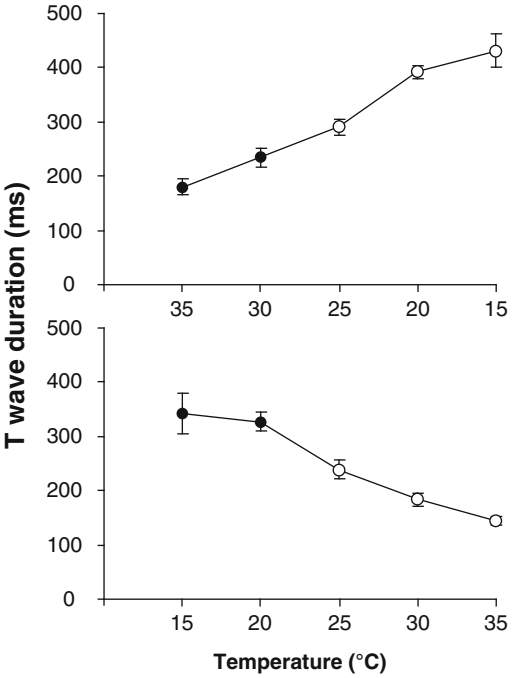

Fig. 9 Effects of temperature on the T wave duration for pacu, *Piaractus mesopotamicus* acclimated to 35°C (*upper panel*) and 15°C (*lower panel*). *Open symbols* indicate a significant difference ($p < 0.05$) in relation to the initial values. (from Aguiar et al. 2002)

of rest period and acceleration of atria-ventricular conduction, with a shortened rest period as the most important factor.

The Q–T interval represents the time for both ventricular depolarization and repolarization, and, therefore, roughly estimates the duration of an average ventricular action potential (VAP_D). This interval depends on heart rate. At high f_H, the duration of ventricular action potentials shorten, which decreases the Q–T interval, while the opposite occurs at low f_H (Klabunde 2004). In the vertebrate heart, VAP_D depends on body temperature, in a way that reminds of the QRS duration, i.e., it is longer at low body temperatures and shorter as temperature is increased (Shattock and Bers 1987). The conduction velocity determines the excitation and relaxation of the ventricle and, as it slows down, the ventricular activity lasts longer.

Shortened VAP_D due to increases in temperature were earlier reported for flounder ventricle by Lennard and Huddart (1991) and by Chapovetsky and Katz (2003) working on toads. The same effect was reported for the tropical species *O. niloticus* (Maricondi-Massari et al. 1998) and *P. mesopotamicus* (Aguiar et al. 2002) acclimated to 15°C and submitted to increases of temperature up to 35°C. The opposite occurred when fish acclimated to 35° were submitted to stepwise decreases in temperature down to 15°C (Fig. 11). However, the VAP_D of *P. mesopotamicus* is shorter

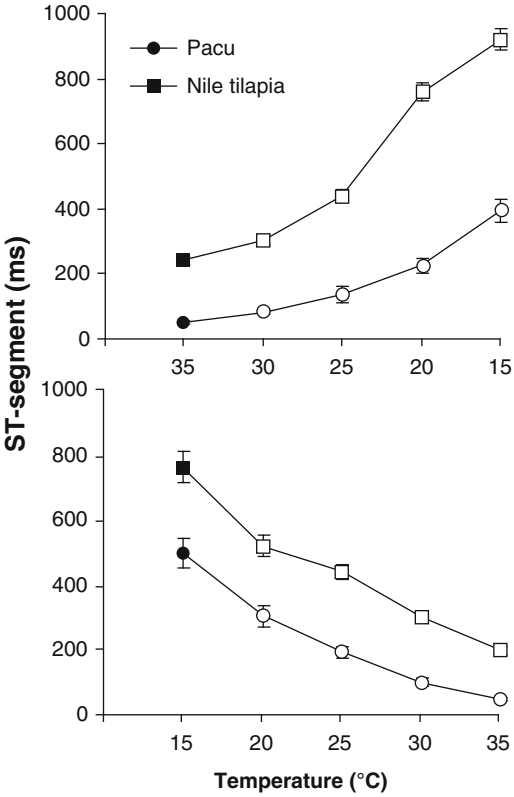

Fig. 10 Effects of temperature on the ST-segment (ms) for Nile tilapia, *Oreochromis niloticus* (Rantin and Kalinin, unpublished data), and pacu, *Piaractus mesopotamicus* (Aguiar 1996) acclimated to 35°C (*upper panel*) and 15°C (*lower panel*). *Open symbols* indicate a significant difference ($p < 0.05$) in relation to the initial values

than that of *O. niloticus* (Fig. 11), which increases f_H of *P. mesopotamicus* to values exceeding those of *O. niloticus* (Fig. 5).

Cotter and Rodnick (2006) analyzed the effects of different anesthetics on electrical properties of the rainbow trout heart, and discovered that the QRS duration and QT interval (VAP_D) were independent of f_H. According to these authors, the QT interval varies inversely with heart rate in mammals, in which cardiac output predominately increases by elevated f_H and sympathetic activation. Most fish, including rainbow trout, increase cardiac output predominately via a larger stroke volume, which depends less on electrical activity (i.e., decreasing the time from depolarization to repolarization of the ventricles would not have significant effects in stroke volume versus heart rate regulators). These authors also define how QT interval and f_H are related in unanesthetized fish, which would increase the understanding of the relationship between the electrical and mechanical activities of the ventricle.

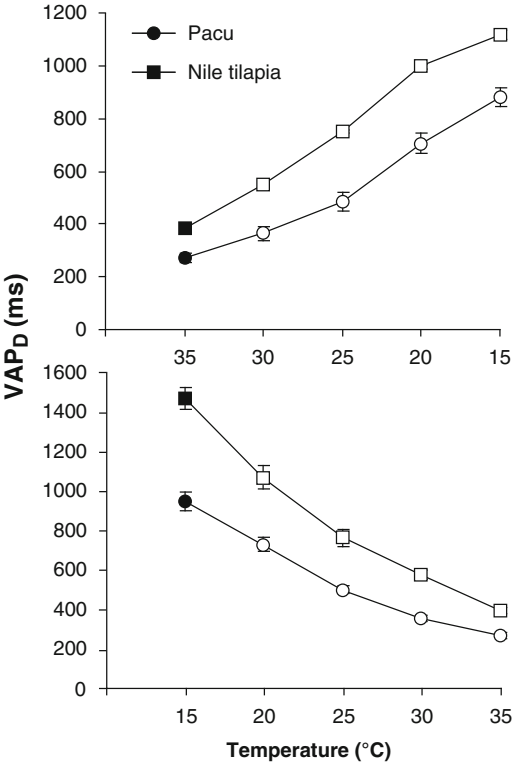

Fig. 11 Effects of temperature on the ventricular action potential duration (VAP_D–ms) for Nile tilapia, *Oreochromis niloticus* (Maricondi-Massari et al. 1998), and pacu, *Piaractus mesopotamicus* (Aguiar et al. 2002) acclimated to 35°C (*upper panel*) and 15°C (*lower panel*). *Open symbols* indicate a significant difference ($p < 0.05$) in relation to the initial values

3 In vitro Cardiac Performance

Most of the studies on cardiac contractility were developed with endothermic animals, in which cardiac muscle works in vivo on essentially constant temperatures (Hove-Madsen 1992). Furthermore, the literature dealing with the effect of temperature on cardiac Ca^{2+} management of tropical fish has been rather scarce, since most studies performed with these animals only analyzed the effect of acute changes in temperature, lacking data on the seasonal adaptive effects. In contrast, much attention has been paid to few species of temperate teleosts, specially the rainbow trout, *Oncorhynchus mykiss*, and the crucian carp, *Carassius carassius*.

Temperature modifies the rate of enzymatic reactions, including those of cardiac tissue (Marengo et al. 1997). As a consequence, cardiac contractility in fish can directly be influenced by temperature effects on the duration of active state (i.e., contraction and relaxation dynamics) (Bailey and Driedzic 1990). Altered

contraction dynamic allows the total amount of Ca^{2+} activator to bind to troponin C in a single contraction, affecting peak force. Conversely, these temperature-induced alterations in the active state can also modify contraction frequency, changing cardiac contractility with temperature (Shiels et al. 2002a).

The ability to tolerate acute temperature changes (daily and/or water column dislocation-related) is an inherent genetic property, which triggers an adequate cardiac performance to match fast thermal variations. Therefore, an adequate cardiac contractility in response to acute changes in temperatures is only present in species that possess a sufficient myocardial plasticity to avoid disturbances in electrical excitation and metabolic imbalance (Paajanen and Vornanen 2004).

As a consequence, twitch cardiac force can increase, decrease, or even remain unaltered after an acute change in temperature. For instance, ventricle strips from Nile tilapia (Costa et al. 2000), *O. niloticus*, and the frillfin goby (Rantin et al. 1998), *B. soporator*, showed a marked reduction in twitch force during acute increases in temperature from 25 to 35°C, in spite of such thermal transitions in the habitat (Fig. 11). Nonetheless, both Nile tilapia and frillfin goby presented an increase in f_H as temperature was acutely elevated (Fig. 1), which may increase cardiac output through a higher contractility, rather than by elevations in stroke volume. Additionally, chronotropy induced by high temperature has a negative inotropic effect, due to the typical negative force staircase in teleost fish (Driedzic and Gesser 1985; Vornanen 1989).

On the contrary, pacu presented an accentuated increase in chronotropism (Fig. 1) while the inotropic response remained the same (Fig. 11) during the acute elevation in temperature from 25 to 35°C (Anelli-Jr et al. 2004). A marked temperature-induced increase in cardiac output of pacu is well correlated with its high activity pattern, since the cardiac demand increases in proportion to an acute elevation in temperature.

Conversely, an acute reduction in temperature from 17 to 2°C will not significantly influence the force of contraction in the atrial tissue of salmon or trout (Fløysand and Helle 1994; Ask et al. 1981), whereas a reduction of temperature from 20 to 1°C increased the force of contraction ventricle of the crucian carp by 60% (Vornanen 1989). In these fish, the reduction in bath temperature was associated with a marked increase in the time to peak force, which would let more Ca^{2+} to enter the cell during a single contraction.

This response is related to the fact that the duration of the action potential (AP) in fish hearts (around 500 ms) is already prolonged, when compared to rat hearts (less than 100 ms) (Vornanen 1989; Møller-Nielsen and Gesser 1992; Shiels et al. 2006). (Fig. 12) On the contrary, an acute increase in temperature decreases the time available for the development of contractile force, since less time is available for Ca^{2+} to reach the contractile apparatus during contraction. According to Paajanen and Vornanen (2004), the mechanism that underlies the temperature-dependent shortening of AP duration observed in cold-acclimated crucian carp ventricular myocytes during acute increases in temperature is correlated with temperature-induced changes in the voltage-dependent rectification of the background inward rectifier K^+ current.

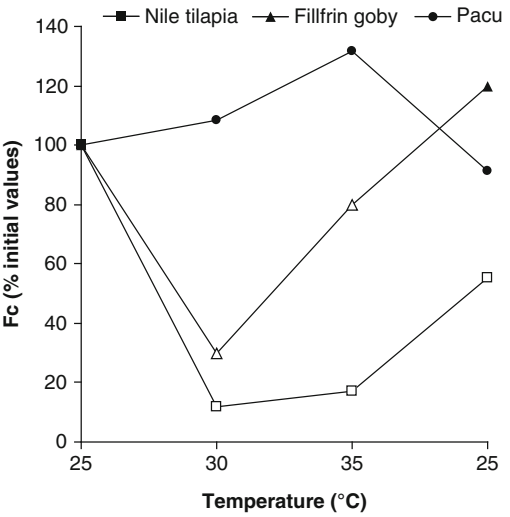

Fig. 12 Twitch force (*Fc* = % initial values) developed by ventricle strips from three species of tropical teleosts ($N = 10$ for all) acclimated to 25°C and subjected to an acute increase in temperature until 35°C and then to the subsequent return to acclimation temperature. *Open symbols* denote a significant ($p < 0.05$) difference in relation to the initial values. Results are presented as means, but error bars have been omitted for clarity. Nile tilapia from Costa et al. 2000; fillfrin goby from Rantin et al. 1998; pacu from Anelli-Jr et al. 2004

Temperature tolerance can be increased by temperature acclimation, because chronic (seasonal) temperature changes can allow the expression of myosin isoforms or Ca^{2+}-transporting proteins more adapted to the new thermal environment (Paajanen and Vornanen 2004). In addition, there is a direct correlation between these responses and the thermal history of the animal, as well as with the activity pattern of the animal at a given temperature. Therefore, fish need efficient mechanisms to achieve thermal adaptation of the cardiovascular system.

Activity levels are matched to variable temperatures, and it can be generalized that changes in cardiac function in response to acclimation is most pronounced in teleosts that can remain active at low temperatures, which contrasts with sedentary animals that become torpid or lethargic in the cold (Bailey and Driedzic 1990). On the other hand, animals that become dormant or little active in the cold may develop a non-compensatory or inverse acclimation of metabolic and enzymatic activities (Prosser 1973; Matikainen and Vornanen 1992).

Some teleosts, including rainbow trout, increase swimming performance after acclimation. An active life style at low temperatures requires compensatory changes in the function of swimming muscles and the cardiovascular system, including increased cardiac contractility and increases of myofibrillar ATPase (Aho and Vornanen 1999).

Conversely, the crucian carp becomes cold-dormant, which results in a concomitant reduction of the cardiac contraction kinetics, which results from a temperature-induced reduction in myofibrillar ATPase activity (Tiitu and Vornanen 2001). The

temperature-dependent expression of different myosin isoforms in fish hearts might be important from a ecological and physiological point of view, since slow myosin types are believed to produce force more economically than fast myosins, which would precondition cardiac muscle to a low energy supply (Tiitu and Vornanen 2001).

Vornanen (1994) described the expression of a myosin heavy chain isoform in crucian carp heart during the cold season. This isoform presents a lower myosin-ATPase activity than its summer counterpart, when an additional myosin heavy chain is expressed. This suggests that both cardiac activity and energy consumption are high in warm, normoxic summer waters but significantly reduced during cold and anoxic winter. Notably, a positive thermal compensation was absent in the ventricular myofibrils of the rainbow trout, which remains active at low temperature (Aho and Vornanen 1999).

According to Pelouch and Vornanen (1996), longer intervals between beats in cold-living fish are due to a prolonged AP duration, which can induce cardiac hypertrophy, which is a direct response to wall stretch, which expands diastolic filling. The cold acclimation involves an increase of the heart, which partly compensates adverse effects of low temperatures on cardiac contractility. These authors found that crucian carp, *C. carassius*, which had been cold-acclimated (2°C) for 3 months developed an 86% increase of ventricular mass. Activity levels are matched to variable temperatures, and it can be generalized that changes in cardiac function in response to acclimation are most pronounced in teleosts that can remain active at low temperatures, which contrasts with sedentary animals that become torpid or lethargic in the cold (Bailey and Driedzic 1990). On the other hand, animals that become dormant or little active in the cold may develop a non-compensatory or inverse acclimation of metabolic and enzymatic activities when compared to warm-acclimated animals at 22°C (Prosser 1973; Matikainen and Vornanen 1992) This cold-induced enlargement occurs mainly by an increase in collagen rather than by myofibrillar or sarcoplasmic proteins. This shifts active force production to more passive elastic elements of the extracellular matrix. Consistently, winter-acclimatized crucian carps had smaller hearts (10%) when compared to fish in the summer (Matikainen and Vornanen 1992). This is related to the inverse (or non-compensatory) temperature acclimation of the crucian carp due to its low activity during the winter. In addition, Matikainen and Vornanen (1992) described how an atypical positive force–frequency relationship in both electrically paced ventricles (Fig. 13a) or spontaneously beating hearts (Fig. 13b) from warm-acclimated crucian carp becomes more evident at higher testing temperatures.

In contrast, Shiels et al. (2002a) showed that the force–frequency response of rainbow trout heart is shifted downwards with acute increases in temperature, and upwards with acute decreases in temperature (Fig. 14). The authors state that these changes in cardiac contractility favor brief exploitation of colder niches after warm-acclimation, but not vice-versa. This response was also described in other cold-active teleosts, including the yellowfin tuna (Shiels et al. 1999) and the mackerel (Shiels and Farrell 2000).

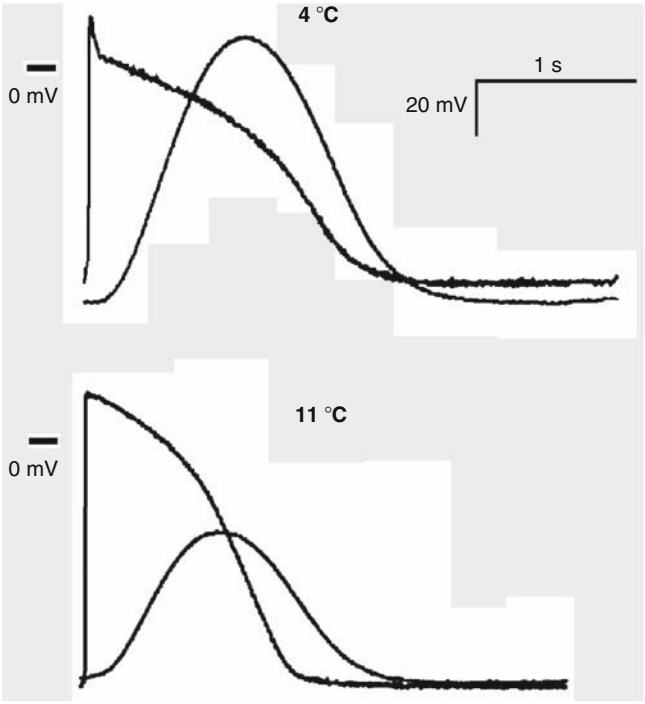

Fig. 13 Representative recordings of ventricular action potentials and associated contractions of the burbot (*Lota lota*) ventricle at 4°C and 11°C. *Scale bars* are for recordings at both temperatures. Force of contraction is in arbitrary units (from Shiels et al. 2006)

Animals subjected to similar temperature conditions and different adaptation strategies can be incorporated into behavioral patterns. Unfortunately, information on in vitro temperature acclimation is lacking with regard to cardiac function in tropical teleosts.

The species-specific differences of the inotropic responses described above are linked to the components of Ca^{2+} management in myocytes (Fig. 15). Myofilaments are activated during the E–C coupling in response to an increased cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$. The increase of $[Ca^{2+}]_i$. can occur in response to mobilization of these ions from the sarcoplasmic reticulum (SR) as well as by influx through the sarcolemma (SL), which can occur via L-type Ca^{2+} channels and/or Na^+/Ca^{2+} exchanger (NCX) acting in the reverse mode (Hove-Madsen et al. 2000). Inversely, cardiac muscles relax when $[Ca^{2+}]_i$ is reduced back to its diastolic resting levels by Ca^{2+} transportation out of the cell via the sarcolemmal Ca^{2+}–ATPase and, in addition, mainly by the NCX and/or its accumulation inside the SR, which occurs by pumping activity via a sarcoplasmic-endoplasmic Ca^{2+}–ATPase (SERCA).

Fish myocytes have several anatomic peculiarities (Fig. 16), including a smaller myocyte diameter than in mammals, and they have peripherally arranged myofibrils and an absence of T-tubules (Farrell and Jones 1992). This results in differences

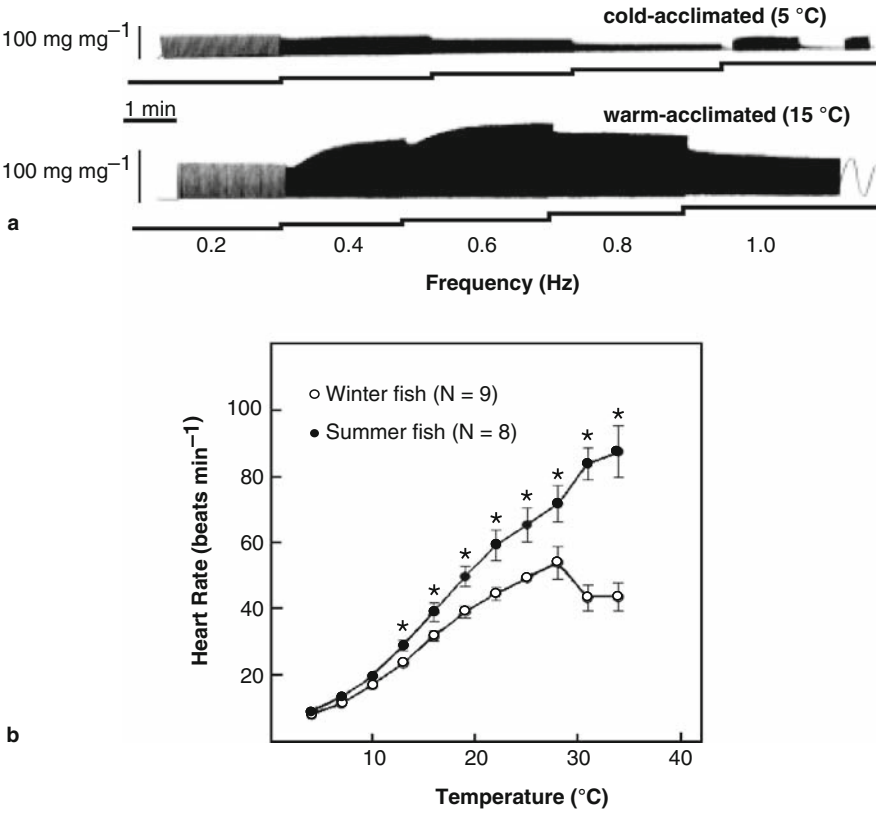

Fig. 14 **a** Original polygraph recordings of the force-frequency relationship for ventricles of warm- and cold-acclimated crucian carp during summer. Pacing rate was raised from 0.2 to 1.0 Hz at 0.2 Hz intervals. The *vertical bar* indicates 100 mg force per mg tissue wet mass. The temperature of the tissue bath was 15°C. **b** Temperature dependence of heart rate in isolated spontaneous beating crucian carp during summer and winter. Results are means ± SE. *Asterisks* indicate a value significantly different from that for winter fish (modified from Matikainen and Vornanen 1992)

in the E–C coupling, which makes the myocytes of most fish more dependent on extracellular Ca^{2+} rather than on SR Ca^{2+} stores. These conditions present a proportionally reduced contribution to Ca^{2+} management, if compared to the sophisticated regulatory mechanisms of mammals. The time and the amount of Ca^{2+} cycled on a beat-to-beat base depend directly on the activity of the Ca^{2+}-transporting mechanisms, which varies between species and depends on temperature (Gwathmey and Morgan 1991; Vornanen 1998). In fact, several physiological differences exist, and among these Ca^{2+}-transporting mechanisms and their thermal dependence will ultimately determine the pattern of the inotropic temperature responses of each species.

In fish myocytes, the temperature dependency of the NCX is lower than the value in amphibians, which itself is inferior to mammalian values (Fig. 17). This relative

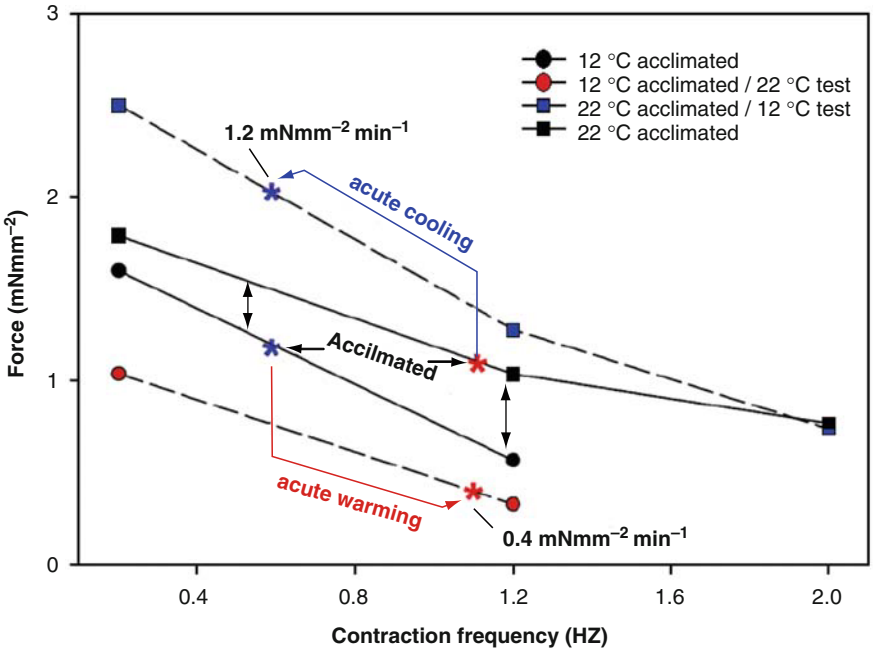

Fig. 15 The effect of acute temperature change and temperature acclimation on isometric force development from ventricular muscle from rainbow trout at high and low pacing frequencies for 12°C-acclimated fish, or high, medium and low pacing frequencies for 22°C-acclimated fish. *Asterisks* indicate the approximate in vivo heart rate under each condition. *Double-headed arrows* indicate the changes in the force–frequency relationship and in the in vivo heart rate associated with temperature acclimation. *Single-headed arrows* indicate the change in force and heart rate associated with acute increases (*red arrows*) and decreases (*blue arrows*) in temperature. Numeric values indicate the pumping capacity of the preparation (product of force and frequency) after heart rate and contractile force adjustment following an acute temperature change. (From Shiels et al. 2002)

temperature-insensitivity of NCX reflects differences in the primary structure of its proteins, which constitutes an adaptation to allow activity of the fish during exposure to low temperatures (Elias et al. 2001).

Nevertheless, it is not clear which is the advantage of temperature insensitivity to species that normally face acute and/or chronic transitions to high temperatures, considering that an increase in NCX activity in response to high temperature would allow a faster contraction–relaxation cycle. In this context, Bailey and Driedzic (1990) emphasize that ATP-dependent Ca^{2+} efflux from the cell is temperature-dependent, increasing directly with temperature.

Ca^{2+} currents through L-channels of mammals present a high temperature dependency ($Q_{10}\sim3$). For temperate fish, however, from evolutionary selection in this direction, one would expect a reduced temperature dependency of these channels (Kim et al. 2000; Shiels et al. 2000). Otherwise their cardiac contractility would be compromised after an acute decrease in temperature (Thomas et al. 1996). In

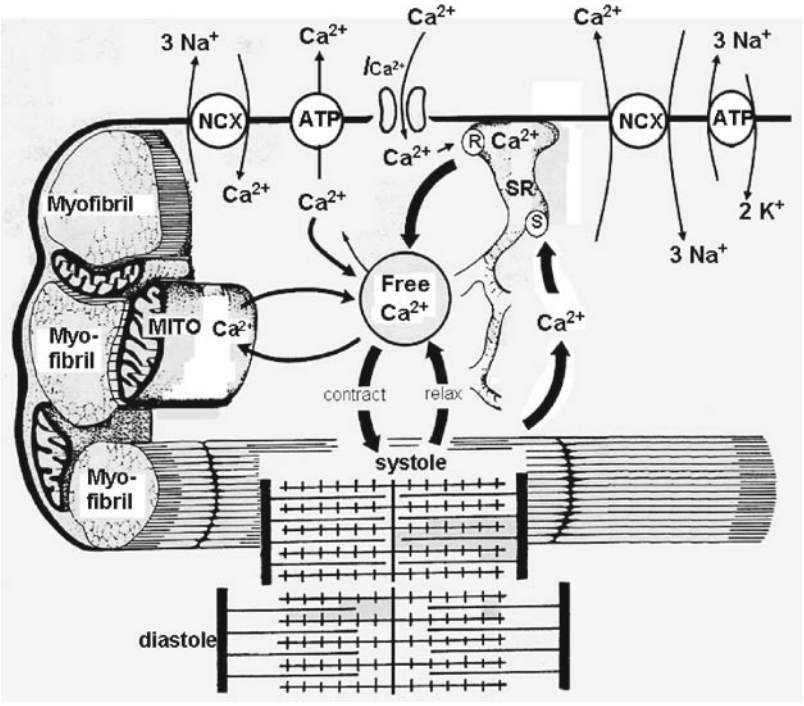

Fig. 16 NCX–Na^+/Ca^{2+} exchanger; *SR* sarcoplasmic reticulum; *R* ryanodine channel; *S* SERCA (sarco-endoplasmic reticulum calcium ATPase); *MITO* mitochondria. ICa^{2+} — (adapted from Opie 1998, for fish myocyte model)

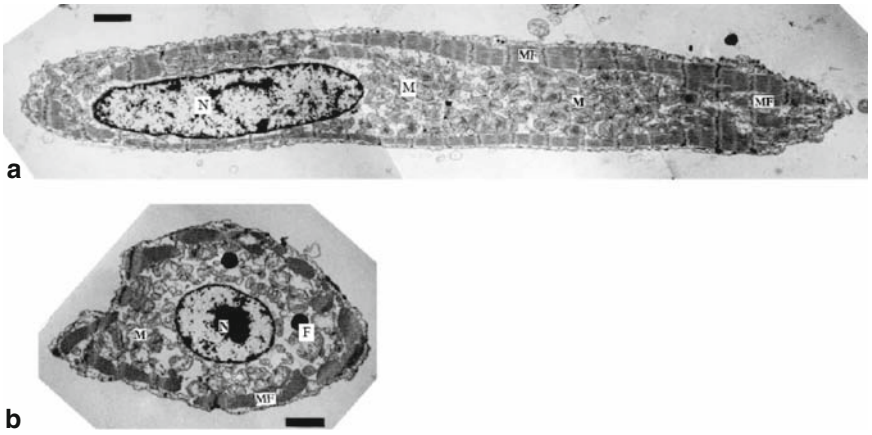

Fig. 17 Electron miographs of enzymatically isolated ventricular myocytes of rainbow trout heart. **a** A longitudinal section through a small part of a ventricular yocyte. **b** A cross-section of a ventricular myocyte. *M* mitochondria; *MF* myofibrils; *N* nucleus; *F* fat. Scale bars: 2μ M. (from Vornanen 1998)

the same direction, the high temperature sensitivity of L-channels would assure a temperature-dependent increase on the Ca^{2+} activator, which would increasse stroke volume in response to higher temperatures.

In this context, studies on teleost hearts established that Ca^{2+} influx through sarcolemma remains almost constant during changes in temperature, despite the temperature sensitivity ($Q_{10} \sim 2$) of L-channels (Kim et al. 2000; Shiels et al. 2000).

Thermal acclimation does not change Ca^{2+} current density (I_{Ca}) through L channels in the ventricular myocytes of rainbow trout and crucian carp (Vornanen 1998). The inactivation kinetics of I_{Ca} is, however, accelerated after acclimation to cold in trout, which would result in a smaller Ca^{2+} influx to the heart of cold-acclimated than of warm-acclimated trout. According to Vornanen (1998), the reduced peak amplitude of I_{Ca} could be compensated by the slower inactivation of I_{Ca}, due to the concomitant prolongation of action potential duration by low body temperature.

Furthermore, there are species-specific differences in relation to the participation of SR Ca^{2+} stores on Ca^{2+} management in fish hearts, and also between the atrium and the ventricle of the same species (atrium > ventricle) (Aho and Vornanen 1999). In spite of this, the following generalization can be made about the effects of temperature on the SR participation in the contraction–relaxation cycle: its role increases after acute elevations in temperature as well as after acclimation to cold (Shiels et al. 2002b).

A cold-induced opening of the SR Ca^{2+}-release channel renders the SR ineffective in sequestering Ca^{2+} (Tibbits et al. 1991, 1992a; Hove-Madsen 1992; Møller-Nielsen and Gesser 1992). Additionally, in the trout heart, the SERCA is temperature-dependent ($Q_{10} \sim 1.6$), and makes Ca^{2+} accumulation inside this organelle less efficient with acute reduction of temperature (Aho and Vornanen 1998; Hove-Madsen et al. 1998; Keen et al. 1994). In spite of this, the fish cardiac SR may accumulate significant amounts of Ca^{2+} during the long-lasting depolarization (Aho and Vornanen 1998).

The combination between temperature sensitivity of the SR Ca^{2+}-release channels and the temperature dependency of SERCA can minimize the role of the SR as a source of Ca^{2+} activator in hearts of fish exposed to acute fluctuations to low temperatures, as rainbow trout (Shiels et al. 2002b).

The high temperature dependency of SR Ca^{2+}-release channels in the fish heart was established by Keen et al. (1994) for rainbow trout, in which ryanodine (an alkaloid that blocks SR function) induced a twitch force decrease, which only appeared at high and unphysiological temperatures (above 15°C). More recent studies (Hove-Madsen et al. 1998, 2001) have verified that L-current magnitude itself can not fully activate myofilaments also at physiological temperatures, which implies that the SR of trout myocytes also plays some role under in vivo conditions. Additionally, Tiitu and Vornanen (2002a) observed that the SR in the atrium and the ventricle of the stenothermic and cold-active burbot, *Lota lota*, is extremely sensitive to ryanodine, even at temperatures as low as 1°C.

Different from the examples cited above, in most temperate fish ryanodine is unable to reduce the cardiac peak force at physiological temperatures. According to Thomas et al. (1996), this lack of effect of ryanodine is due to lower testing

temperatures. Atrial and ventricle strips from skipjack tuna, *K. pelamis* (Keen et al. 1992; Tibbits 1996), and from the mackerel, *Scomber japonicus* (Shiels and Farrell 2000) significantly reduced twitch force after blockade of SR Ca^{2+}-release channels by ryanodine only at temperatures above 20°C, but not at lower temperatures.

In this way, an acute transition to a high temperature can indirectly reveal an increase of SR participation in Ca^{2+} cycling in animals in which this organelle is potentially functional. Shiels and Farrell (1997) state that an acute transition to a high temperature amplifies the response to ryanodine in a more consistent manner than thermal acclimation. This implies that an increase in SR participation on Ca^{2+} management at these conditions represents an adaptive response to an acute and abrupt increase in temperature. Conversely, this implies that ryanodine channels did not suffer any evolutionary selection that could work more adequately after acute transitions to low temperatures.

Nevertheless, thermal acclimation may alter the E–C coupling process of the fish heart, given that cold-acclimation alters the relative importance of the SR Ca^{2+} cycling according to the activity pattern of the fish. The cardiac E–C coupling in the cold-dormant crucian carp is exclusively based on extracellular Ca^{2+} sources (Tiitu and Vornanen 2001), and several studies confirmed that SR Ca^{2+}-release channels from active teleosts remain functional in the cold (Shiels et al. 2002b). Indeed, morphometric analysis of the heart of perch, *Perca fluviatilis*, suggest that the SR is better developed in cold-acclimated than in warm-acclimated fish (Bowler and Tirri 1990). Consistently, Keen et al. (1994) observed a small, but significantly increased anatomical development of the cardiac SR of rainbow trout after thermal acclimation to cold. As a specific feature, this organelle increased force development only when cold-acclimated fish were subjected to acute transitions to high temperature, in particular at subphysiological stimulation frequencies. In cold-acclimated trout, this transition resulted in a more accentuated increase in twitch force, when compared to the warm-acclimated fish. Nevertheless, a high structural development of the SR was not sufficient to ameliorate force development when animals were tested at lower temperatures (Fig. 18).

Functional studies in thermally acclimated trout prove that contraction is more sensitive to ryanodine inhibition in cold-acclimated than in warm-acclimated animals, even at physiological body temperatures (∼4°C) and at physiological pacing rates (∼0.6 Hz). For instance, Aho and Vornanen (1999) recorded that the SR accelerates the recovery of contractility from inactivation and reported that the atrium and the ventricle of cold-acclimated trout allow relatively high cardiac frequencies and permit adequate cardiac outputs at low environmental temperatures. Additionally, they proved that the atrial SR contributes directly to the cytosolic Ca^{2+} under the same conditions.

There are marked species-specific differences among teleosts in relation to the SR Ca^{2+} uptake by SERCA. In this context, Aho and Vornanen (1998) reported that the cold-acclimated heart (4°C) increased the Ca^{2+} uptake rate by the SR of trout heart (complete thermal compensation).The SR Ca^{2+} uptake rate, however, indicated that rainbow trout and perch, which are adapted to low temperature,

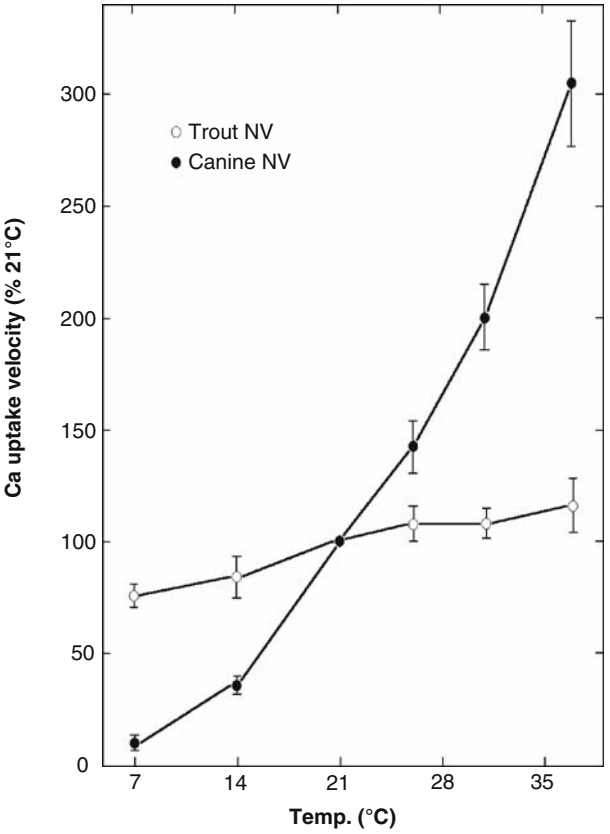

Fig. 18 Initial rates of Na^+-dependent Ca^{2+} uptake in native sarcolemmal vesicles (NV) as a function of temperature, normalized for the rate at 21°C. Extracellular Ca^{2+} concentration ($[Ca^{2+}]_0$) was 20 μM, $n = 4$ different preparations for each group, pH 7.0, and the reaction time was 2 s. NV were maintained at 4°C intil the reaction was initiated. 100% equals 3.0 and 3.5 nmlol mg, protein^{-1} s^{-1} in trot and canine NV respectively. (From Tibbits et al. 1992b)

have an increased volume and Ca^{2+}-handling efficiency of the SR (Bowler and Tirri 1990; Keen et al. 1994). These mechanisms partially compensate for the detrimental effects of cold on cardiac contractility (Fig. 19). Against this background Landeira-Fernandez et al. (2004) described that tuna species in cold waters such as albacore tunas, *Thunnus alalunga*, have an increased SR Ca^{2+} uptake capacity when compared to tropical tunas, such as yellowfin tuna, *Thunnus albacares*. Conversely, species that become inactive or dormant in the cold (e.g., crucian carp) have a limited Ca^{2+}-handling capacity, which was depressed after acclimation to low ambient temperature.

A physiological strategy to modulate the effects of changes in temperature on cardiac contractility is β-adrenergic modulation, which enhances both Ca^{2+} delivery and its subsequent removal during the contraction–relaxation cycle (Shiels et al. 2002a). Ask et al. (1981) and Keen et al. (1993) established that an increase in

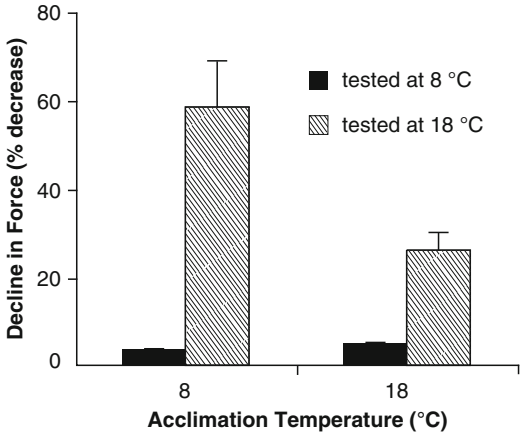

Fig. 19 Effect of ryanodine application (10 μM) on tension development in ventricular strips from rainbow trout (*O. mykiss*) acclimated to either 8 or 18°C and tested at 8 C (*solid*) or 18°C (*diagonal lines*) under the stimulation frequency of 0.2 Hz. Histograms are mean ± SEM (modified from Keen et al. 1994)

the density of β-adrenoceptors in the sarcolemma of cold-acclimated trout myocytes increases the sensitivity of trout heart to adrenaline. An upregulation of the sympathetic system increases not only the heart rate, but also contractile force (Graham and Farrell 1989). These results contrast with data for the stenothermal African catfish *Claris gariepinus*, in which myocardial β-adrenoceptor density did not change in response to temperature acclimation (15, 22, and 32°C). This may indicate that a β-adrenoceptor plasticity similar to that exhibited by eurithermal temperate teleosts is superfluous in tropical fish (Hanson et al. 2005).

In several temperate teleosts, adrenergic sensitivity is also enhanced in response to an acute reduction of temperature (Ask et al. 1981; Ask 1983; Shiels and Farrell 1997, 2000; Aho and Vornanen 2001). In the tropical teleosts *O. niloticus* (Costa et al. 2000) and *P. mesopotamicus* (Anelli-Jr et al. 2004), the effects of an acute change in temperature are opposite to those described for temperate fish, since adrenergic responsiveness of multicellular ventricle preparations increased as temperature was acutely elevated, whereas this failed to occur in *B. soporator* (Rantin et al. 1998). These species-specific differences in adrenergic responsiveness of hearts from tropical teleosts to acute elevations in temperature must be related to the fact that *B. soporator* presented an extremely pronounced temperature-induced increase in chronotropism that may assure adequate increases in cardiac output. Adrenergic modulation seems to represent an important strategy utilized by fish to increase cardiac performance when subjected to acute alterations to environmentally relevant temperatures.

To sum up the considerations, it can be stated that, in less active temperate teleosts, the SR plays a minimal role (if any) as a calcium source to the activation of myofibrils (Tiitu and Vornanen 2001), since ryanodine channels remain in an "open state" over a longer period of time, provided that the test temperatures are

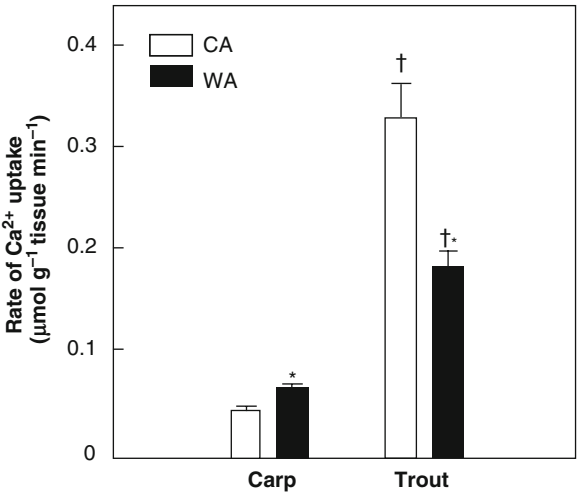

Fig. 20 Sarcoplasmic reticulum Ca^{2+} uptake rate of crude cardiac homogenates from crucian carp and rainbow trout. The results are means + SEM of 6–10 preparations. *Asterisks* indicate a statistically significant difference ($p < 0.05$) between acclimation groups. *Daggers* indicate a statistically significant difference ($p < 0.05$) between trout and carp. *CA* cold-acclimated (4°C); *WA* warm-acclimated (carp: 24°C; trout: 17°C). Modified from Aho and Vornanen 1998

low (Hove-Madsen et al. 2001). The myocytes of cold-adapted fish develop adaptive mechanisms that lead to a relative temperature insensitivity of sarcolemmal Ca^{2+}-transporting systems (particularly L-channels and NCX), which maximizes the proportional contribution of transarcolemmal Ca^{2+} fluxes to the relaxation–contraction cycle. These conditions, combined with an increase in AP duration at cold temperatures, allow a prolonged Ca^{2+} influx through the same number of Ca^{2+} channels and/or NCX molecules, which also makes sarcolemmal Ca^{2+} influx relatively independent of ambient temperature at more realistic (whole animal) situations. As a consequence, Ca^{2+} can be delivered to myosin at a rate and magnitude compatible with the low heart rates observed in most of the temperate fish (Farrell and Jones 1992; Driedzic and Gesser 1994; Lillywhite et al. 1999). This assures their survival at temperatures considered cardioplegic to an endotherm, even in species that lack a functional SR. Indeed, to active species cold acclimation is associated with an increased Ca^{2+}-handling capacity of the SR. This may allow partial compensation for the detrimental effects of cold on cardiac contractility, while an inversal thermal compensation has been described in fish that are inactive during winter (Aho and Vornanen 1998) (Fig. 20).

Conversely, two very active neotropical teleosts, the curimbata, *Prochilodus lineatus* (Kalinin, unpublished data), and the pacu, *Piaractus mesopotamicus* (Anelli-Jr et al. 2004), and two sedentary neotropical species trahira, *Hoplias malabaricus* (Olle 2003), and marbled swamp eel *S. marmoratus* (Rocha et al. 2007a,b), had a direct contribution from the SR to ventricular inotropism at physiological temperatures. This was established in fish acclimated to 25°C, using the inhibitory

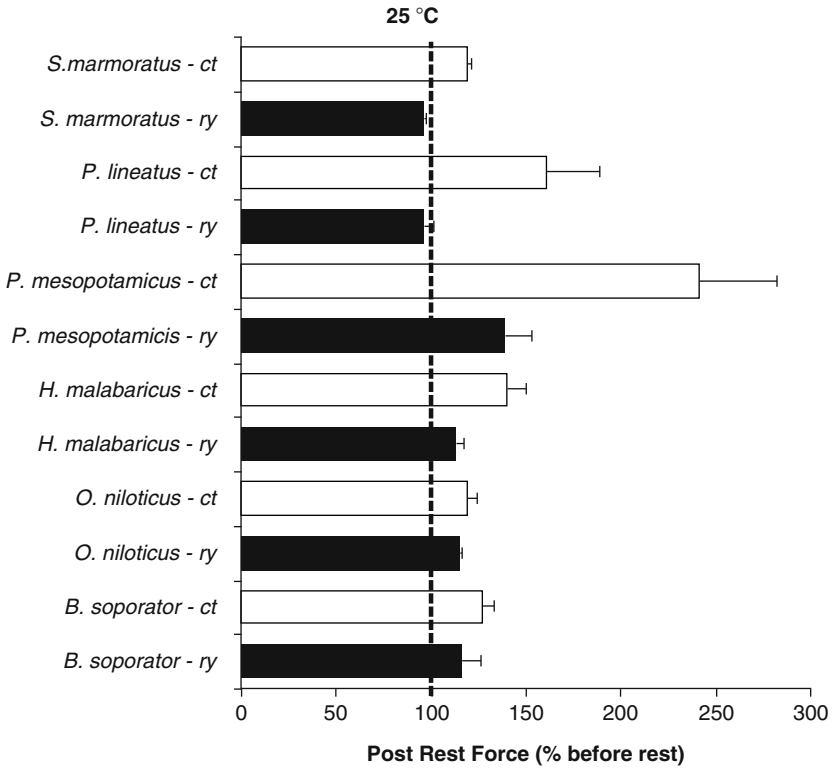

Fig. 21 Twitch force developed after a prolonged diastolic pause (5 min) in ventricle strips of fish before (*ct* = control) and after addition of 10 μM of ryanodine (*ry*)

effect of ryanodine after a prolonged diastolic pause (5 min) (Fig. 21). These data would allow us to state that tropical fish present an increased metabolic rate associated with a faster contraction–relaxation cycle, due to a shorter AP duration and a more direct participation of the SR in the E–C coupling to reduce the diffusion distances and/or faster Ca^{2+}-transporting proteins in a predictable manner. In particular, Fig. 21 shows that ryanodine had no effect on post-rest force of ventricle strips from other tropical teleosts (*O. niloticus* and *B. soporator*), irrespective of their activity pattern. This implies that in tropical teleosts, the SR participation on cardiac Ca^{2+} management is not necessarily dependent upon the activity pattern of the species. In active tropical teleosts lacking a functional SR, the transarcolemmal Ca^{2+} fluxes (mainly via L-channels and NCX) may suffice to provide an adequate myofilament activation, given that temperature-induced increases in heart rate may be enough to increase cardiac performance in response to a temperature-induced increased cardiac demand.

Interestingly, Rivaroli et al. (2006) defend the hypothesis that the species-specific differences in the dependence of the SR among tropical teleosts could be related to their phylogenetical position. While most of the tropical fish presenting a potentially

functional SR (curimbata, trahira, and pacu) belong to the superorder Ostariophysi, most tropical ryanodine-insensitive teleosts (e.g., Nile tilapia, and frillfin goby) belong to the superorder Acanthopterygii. Among the species presented in Fig. 21, however, an exception to this rule is the acantopterygian marbled swamp eel (*S. marmoratus*), in which the SR plays a functional role for the ventricular Ca^{2+} even at physiological f_H and temperatures (25 and 35°C), despite also being a highly sedentary animal (Rocha et al. 2007b). The effects of thermal acclimation to seasonally relevant temperatures are necessary to evaluate and compare the adaptive strategies developed by these animals involving a large number of subtropical and tropical teleost fish.

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Physiological Evidence Indicates Lungfish as a Sister Group to the Land Vertebrates

M.L. Glass

Abstract Current research tends to support that lungfish (Dipnoi) and land vertebrates (Tetrapoda) form a sister group, which has stimulated an interest in these animals. The extant lungfish include: *Protopterus,* the African lungfish (four species) and the South American lungfish (*Lepidosiren paradoxa*) (one species). The African and South American lungfish have well-developed lung and reduced gills, while the Australian lungfish (*Neoceratodus forsteri*) is highly dependent on the gill ventilation, and its lung is one of the simplest among vertebrates. Lungfish and land vertebrates share many features of respiratory control. *Lepidosiren* (and probably *Protopterus* possess central cerebral CO_2 and H^+ receptors, which regulate acid–base by increases or decreases in pulmonary ventilation. This regulatory pattern is also valid for land vertebrates, including human beings. By contrast, teleost fish lack central CO_2/H^+-receptors, which suggests that the lung and the central chemoreceptors evolved together. In this context, any very specific features are common to lungfish and land vertebrates, and these include the Hering–Breuer reflex and the presence of very specific stretch receptors.

1 Introduction

Styloichthys, a 417-million-year-old fossil was found, with the characteristics to be expected for a last common ancestor of tetrapods and lungfish (Zhu and Yu 2002). The descendants of the Sarcopterygians (lobe-finned fish) include the coelacanths (Actinistii) with two species *Latimeria chalumnae*, which was discovered in 1938 at the east coast of South Africa, while *L. menadoensis* was seen for the first time

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in 1999. *Latimeria* possess lungs, but these are filled with fat (Carroll 1988), which makes sense since coelacanths belong to the deep waters. Other descendants of the sarcopterygians include the lungfish (Dipnoi) and the land vertebrates (Tetrapoda), and current research favors the lungfish as the sister group to the tetrapods (Meyer and Dolven 1992; Yokobori et al. 1994; Zardoya et al. 1998; Toyama et al. 2000; Brinkmann et al. 2004). Tetrapod albumin has been studied to a great extent in tetrapods; Metcalf et al. (2007) recently reported its presence in the Australian lungfish (*Neoceratodus forsteri*), and it turned out to have a high degree of similarity to the sequence of tetrapod albumins.

Lepidosireniformes (subclass: Dipnoi; class: Sarcopterygii) include the South American lungfish *Lepidosiren paradoxa*, which the Austrian morphologist Fitzinger (1837) found appropriate to an animal, due to the unexpected combination of a lung and a gill system. This slender lungfish is equipped with appendages and can weigh about 1 kg (Fig. 1), and its popular name is 'pirambóia', taken from the language of the Tupi indians. *L. paradoxa* is found within the Amazon and Paraná-Paraguai regions and inhabits shallow vegetation-covered lakes. Mainly, *L. paradoxa* feeds on invertebrates, including mollusks that can easily be crushed by its tooth plates (Sawaya 1946).

The African lungfish *Protopterus* includes four species (*amphibius*, *annectens*, *aethiopicus* and *dollei*). These lungfish are similar to *L. paradoxa,* although more heavily built, and their habitats are much like those described above. *L. paradoxa* and *Protopterus sp.* are crucially dependent on the lung for gas exchange, while the Australian lungfish (*Neoceratodus forsteri*) has a well-developed gill system

Fig. 1 Photo of the South American lungfish (*Lepidosiren paradoxa*) in the laboratory, making a slow but elegant turn

combined with a simple lung, which is ventilated at very long intervals (one h or more) (Kind et al. 2002). It inhabits slowly moving rivers of the Queensland region of Australia, where it may reach 1.5 m and a weight of 40 kg.

In the nineteenth and twentieth centuries, much effort was devoted to comparative morphology and anatomy, which was backed by the concepts of evolutionary biology and paleontology. Recently, techniques based on amino acid sequences and mitochondrial genome DNA sequences have provided criteria to evaluate the most probable cladograms for specific groups of animals (cf Yokibori et al. 1994; Brinkmann et al. 2004). Clearly, comparative physiology can be used to trace back the origins and evolution of physiological mechanisms. In this chapter, we will focus on some mechanisms of ancient origin. One example is the dual locations of CO_2/H^+-receptors: the peripheral chemoreceptors monitor acid–base status of the blood, while the central chemoreceptors are stimulated by changes of acid–base status of the CSF and the interstitial environment. This would have pleased the Greek philosopher Anaximander, who about 2,500 years ago stated that once man was another animal, perhaps a fish.

2 How Advanced is the Lungfish Lung?

Surfactant is a substance that reduces surface tension of the lung, and it contains disaturated and unsaturated phospholipids along with surfactant proteins. A lung cannot function without surfactant and, interestingly, surfactant is also present in swim bladders and gill systems, and is produced by type II cells (Daniels et al. 2003); see also Chap. . Lepidosireniformes (*Protopterus* and *Lepidosiren*) produce surfactants that are very similar to those of amphibians, whereas *Neoceratodus* has a lipid composition, which is more closely related to that of actinopterygian fish (Orgeig and Daniels 1995). In the same context, Power et al. (1999) stated that the surfactant composition of *Neoceratodus* has been preserved over the last 300 million years. Only the right lung develops in *N. forsteri*, and it receives blood from both pulmonary arteries. Further, the lung is in a dorsal position, while lepidosireniformes and land vertebrates possess bilaterally positioned lungs in a ventral position (Perry 2007). Bassi et al. (2005) reported pulmonary diffusing capacity in *L. paradoxa*, using the equation: $D_L O_2 = \dot{V}O_2 \cdot \Delta PO_2^{-1}$ (Bohr 1909), in which the individual components are: $D_L O_2$ = diffusing capacity; $\dot{V}O_2 = O_2$ flux through the tissue membrane, which separates lung gas and pulmonary capillary blood, or in general O_2 uptake; ΔPO_2 = the O_2 pressure gradient between lung gas and pulmonary capillary blood. $D_L O_2$ was 0.044 mLSTPD $kg^{-1}\,min^{-1}$ at 35°C (Bassi et al. 2005), which is close to the value for a bullfrog (*Rana catesbeiana*), which has a $D_L O_2$ of 0.054 mLSTPD $kg^{-1}\,min^{-1}$ (Glass et al. 1981a). Further, Crawford et al. (1976) report the rather high $D_L CO$ of 0.068 mLSTPD $\cdot\, kg^{-1}\,min^{-1}$ for the Greek turtle (*Testudo greca*).

The lung of *L. paradoxa* was studied by morphometric measurements, which provided the amazing information that 99% of its gas exchange surface belongs to

the lung, while the skin and the rudimentary gills account for the remaining 1% (Moraes et al. 2005). On the other hand, Sanchez et al. (2005) reported that aquatic O_2 uptake accounted for 9% of total uptake. This seeming contradiction can be solved, because Moraes et al. (2005) found a high density of capillaries in all parts of the gill system, and a respiratory role of the gills cannot be excluded.

The D_LO_2-values for *L. paradoxa* and a bullfrog are very similar, whereas the diffusing capacity of the tegu (*Tupinambis sp.*) and the monitor lizards (*Varanus exanthematicus*) is twofold greater than *L. paradoxa* (Glass and Johansen 1982; Glass et al. 1981a, b). The transition from ectothermic to endothermic metabolism greatly increased metabolism which is not surprising, considering that an alveolar mammalian lung has a 16-fold higher D_LO_2 than *L. paradoxa* (Takezawa et al. 1980).

3 Regulation of Acid–Base Status and Oxygen Levels

As explained in Chap. 3, true lungs are found in land vertebrates, lungfish and bichirs (Subclass: Actinopterygii — ray-finned fish. Order: Polypteriformes). Currently, information on respiratory control in land vertebrates is increasing rapidly, and the interest in lungfish is growing. By contrast, the information on the respiratory physiology of bichirs is scarce (see Chap. 3). Holeost and teleost fish regulate acid–base status by ion exchange (Heisler 1984), because O_2 homeostasis has a priority due to the ever-changing O_2 levels of the aquatic environment (Dejours 1981). Oxygen receptors are located within the gill system, where receptor groups screen the blood or the inspired water (Soncini and Glass 2000; Burleson and Milsom 1995a,b). Acid–base regulation of teleost fish depends on cells located in the gill epithelia, and accounts for no less than 90% of the acid–base relevant ion transfers, while the kidney contributes the remaining 10% (Heisler 1984; Claiborne and Heisler 1986).

In land vertebrates and lungfish, the ability to regulate acid–base status and O_2 homeostasis depends on adjustments of the ratio

$$V_{EF}/V\mathrm{CO}_2 = \mathrm{RT}/P_{EF}\mathrm{CO}_2,$$

where V_{EF} = effective ventilation of the lung, $V\mathrm{CO}_2$ = pulmonary CO_2 output, R = the gas constant, and T = absolute temperature (°K), and the equation is derived from the general gas law. In mammalian respiratory physiology, the equation is usually referred to as "the alveolar ventilation equation". Mammals are the only land vertebrates equipped with alveolar lungs, but 'alveolar' ventilation can be substituted by 'effective ventilation' of a lung.

Land vertebrates (Tetrapoda) control acid–base status by means of central and peripheral CO_2/H^+-receptors, and by far most information is available for mammals. The passage of H^+ and HCO_3^- through the blood–brain barrier is very limited, whereas CO_2 traverses. Therefore, in a classical study Loeschcke et al. (1958)

assumed that CO_2 would react with water according to the equation: $CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^-$, which would leave H^+ as the stimulus. As a further progress, H^+ receptors were detected in a bilateral position within the ventral part of the medulla oblongata (Schläfke et al. 1975). We now know that more sites are involved, including the retrotrapezoid nucleus (Guyenet et al. 2005) and the midline raphe (Bernard et al. 1996). The peripheral chemoreceptors are of very ancient origin, and the chemoreceptors of the aortic arch of reptiles and birds and the carotid bifurcation of mammals and amphibians are homologous to the O_2-receptors on the first gill arch of fish (Milsom 2002). It is, therefore, not surprising that the peripheral receptors include the O_2-sensitive glomus cells (For further information see Nattie (1999; 2006)). Studying one specimen of *Protopterus*, Lahiri et al. (1970) injected the afferent gill arteries with hypoxic blood and cyanide, which increased ventilatory movements. As further evidence, bilateral section of the first three gill arches reduced responses to the stimuli, but the procedure would not define the exact locations of the O_2-sensitive sites.

4 Respiratory Control in Lungfish Compared to Amphibians and Other Land Vertebrates

Striedter (2005) stated: "Lungfish brains exhibit very little histological differentiation, and are among the simplest vertebrate brains". This is true; but in some regards, they possess characteristics which strikingly resemble those of the land vertebrates. The Lepidosireniformes (*Protopterus* and *Lepidosiren*) have amazingly high $PaCO_2$, high bicarbonate and a low pHa. As an example, at 35°C PaO_2 was 76 mmHg, $PaCO_2$ 30 mmHg, pHa 7.39 and plasma $[HCO_3^-]$ 25.9 mM (Bassi et al. 2005). The corresponding values at 25°C were PaO_2 81 mmHg, $PaCO_2$ 21 mmHg, pHa 7.53, and plasma $[HCO_3^-]$ 20.0 mM. The values for *Protopterus dolloi* have the same tendency: PaO_2 66 mmHg, $PaCO_2$ 18 mmHg, pHa 7.37 (Perry et al. 2007). This is very distinct from the values for anuran amphibians such as the cane toad (*Chaunus schneideri*), with PaO_2 61 mmHg, $PaCO_2$ 7.7 mmHg, pHa 7.75, and plasma $[HCO_3^-]$ 13.7 mM (Wang et al. 1998). *Neoceratodus* (Ceratodontidae) is certainly different from the lepidosireniformes. Its values are PaO_2 39 mmHg, $PaCO_2$ 4 mmHg, pHa 7.64 (Lenfant et al. 1966, 1967), which reflects its predominant gill respiration and Dejours (1981) has pointed out that the more an animal depends on aquatic respiration, the lower its $PaCO_2$. It should be noted that some early studies report very low PaO_2 values, which are unrealistic due to invasive techniques and/or incorrect handling of the blood samples.

5 Focus on the South American Lungfish *L. paradoxa*

Figure 1 shows the eel-like body of *L. paradoxa*, with which Johansen and Lenfant (1967) obtained pioneering data, in particular concerning gill function. They measured an O_2 extraction from the gills (EO_2) of 30%, which is low compared to

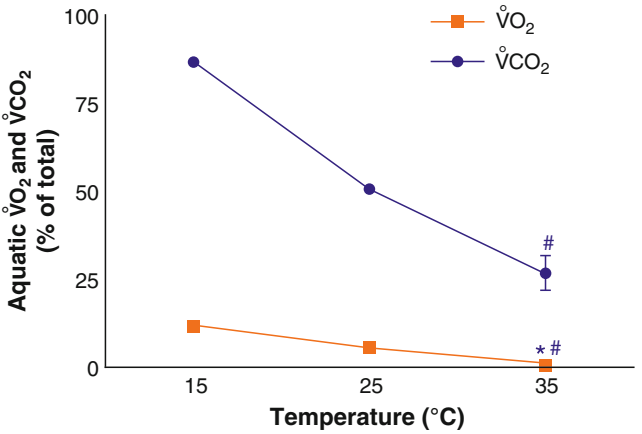

Fig. 2 *Upper curve*: Carbon dioxide elimination to the water as a function of total CO_2 output. *Lower curve*: Total O_2 uptake from the water. The temperature range is 15–35°C. Increasing temperatures reduced the percentage CO_2 output to the water, because a larger percentage of total CO_2 became redirected and eliminated by pulmonary ventilation. A small amount of O_2 was taken up from the water at 15°C, whereas the uptake was practically nil at 35°C, which confirms a high dependence of *L. paradoxa* on lung ventilation (ANOVA, log-nat. transform., Bonferroni, Friedman, Dunn. Mean ± SEM, $n = 5$)

teleost fish, which reach no less than 70–85% (Rantin et al. 2007). Further, they discovered that the gill arches 1 and 2 are practically devoid of gill filaments, and the remaining arches had considerably reduced surface areas. A high density of capillaries might, however, account for some O_2 uptake by the gills, since the proper respiratory exchange surface is negligible (Moraes et al. 2005).

The relative roles of aquatic and aerial gas exchanges were assessed by Amin-Naves et al. (2004), who focused on a temperature range from 15 to 35°C. Aquatic O_2 uptake by the animal was minute, and constant with temperature (about 0.01 mlSTPD kg^{-1} min^{-1}), while pulmonary O_2 uptake increased from 0.06 (15°C) to 0.73 (35°C) mlSTPD kg^{-1} min^{-1}, while pulmonary ventilation increased 17-fold over the same temperature range. Figure 2 shows the pulmonary and aquatic gas exchanges, presented as percentage values for $\dot{V}O_2$ and $\dot{V}CO_2$ at the three tested temperatures. It is clear that the aquatic $\dot{V}O_2$ is practically nil at 35°C, while the lung has taken over. Concurrently, aquatic CO_2 elimination strongly dominates at 15°C, but becomes reduced as temperature increases, and at 35°C only 1/3 of the total CO_2 output is eliminated to the water, while the lung eliminates 2/3 of the remaining output. This occurs because the higher the temperature, the more dominant becomes the gas exchange by the lung.

L. paradoxa possess central chemoreceptors, which were first detected using superfusion of the 4th cerebral ventricle with mock CSF solutions at pH levels ranging from 7.4 to 8.0, while pulmonary ventilation was measured using a method for freely diving animals. A reduction of pH from 8.0 to 7.4 increased ventilation threefold, while respiratory frequency increased from 5 to 12 breaths h^{-1} (Sanchez

et al. 2001a). The next step was to verify the hypothesis that the *L. paradoxa* possess peripheral CO_2/H^+-receptors. To test this, the lungfish was initially kept in aerated water, after which combined aquatic/gas-phase hypercarbia ($PCO_2 = 49$ mmHg) was maintained for 5 h, during which pulmonary ventilation gradually increased 8-fold relative to the initial control value. In a second run, this procedure was repeated with the modification that superfusion of normocarbic mock CSF was applied during the last 2 h of the experiment. This reduced ventilation which, however, remained 3-fold higher than the initial control value. In addition, this hyperventilation was statistically different both from the control value and from the maximum response. With this information, it could be calculated that peripheral CO_2/H^+-receptors accounted for 20% of the ventilatory drive, whereas the bulk part of the drive was central (Amin-Naves et al. 2007a,b).

This was consistent with data on the central chemoreceptor drive in the cane toad *Chaunus schneideri*, and in the alligator (Branco and Wood 1993). The value for mammals is from Smith et al. (2006), and the measurement for birds (duck) is from Milsom et al. (1981). See Fig. 3 for a cladogram that informs on peripheral and central components to the CO_2/H^+ receptor drive in various groups of vertebrates.

Shams (1985) exposed the medulla of anaesthetized cats to an increased PCO_2 while pH was kept constant by superfusion, which stimulated pulmonary ventilation. Harada et al. (1985) studied the brainstem of the newborn rat, and found that hypercarbia increased the respiratory output of the phrenic nerve, while pH was kept constant. Toads (*Chaunus schneideri*. Previously *Bufo paracnemis*) were also evaluated in this context, and it turned out that an increase of mock CSF CO_2 increased ventilation, while pH was kept constant.

These two stimuli (CO_2 and pH) were also tested in *L. paradoxa* and the acid–base environment of the central chemoreceptors was controlled by superfusion, while pulmonary ventilation was recorded. Initially, superfusion was applied to keep $PaCO_2$ at 21 mmHg and pH at 7.45, which corresponds to normal values for animals in the water at 25°C. As a second step, pH continued at 7.45, while PCO_2 was increased to 42 mmHg, and this increased pulmonary ventilation twofold. Conversely, ventilation increased 3-fold, when mock CSF pH was reduced from 7.45 to 7.20, while PCO_2 was kept constant at 21 mmHg.

Peripheral chemoreceptors in mammals also respond to both CO_2 and H^+(cf. Hlastala and Berger 1996), and the advantage of this dual mode of stimulation might be that ventilation can respond to both respiratory and metabolic acidosis.

Evidently, lungfish and land vertebrates share characteristics of respiratory regulation, and the origins of key elements are clearly very ancient. A large number of non-mammalian land vertebrates are equipped with intrapulmonary stretch receptors, in which the firing rate becomes reduced by increases of CO_2 (cf. Milsom et al. 2004). Slowly adapting stretch receptors were discovered in *Protopterus* and *L. paradoxa* (DeLaney et al. 1983), and it turned that increased intrapulmonary CO_2 levels inhibited the firing rate of the receptors. Rapidly adapting receptors were also found, but the slowly adapting type was more common. The firing rate of the slowly adapting receptors was dependent both on rate of inflation and on CO_2 levels. Curiously, such types of receptors have been found in the air-breathing organ of the

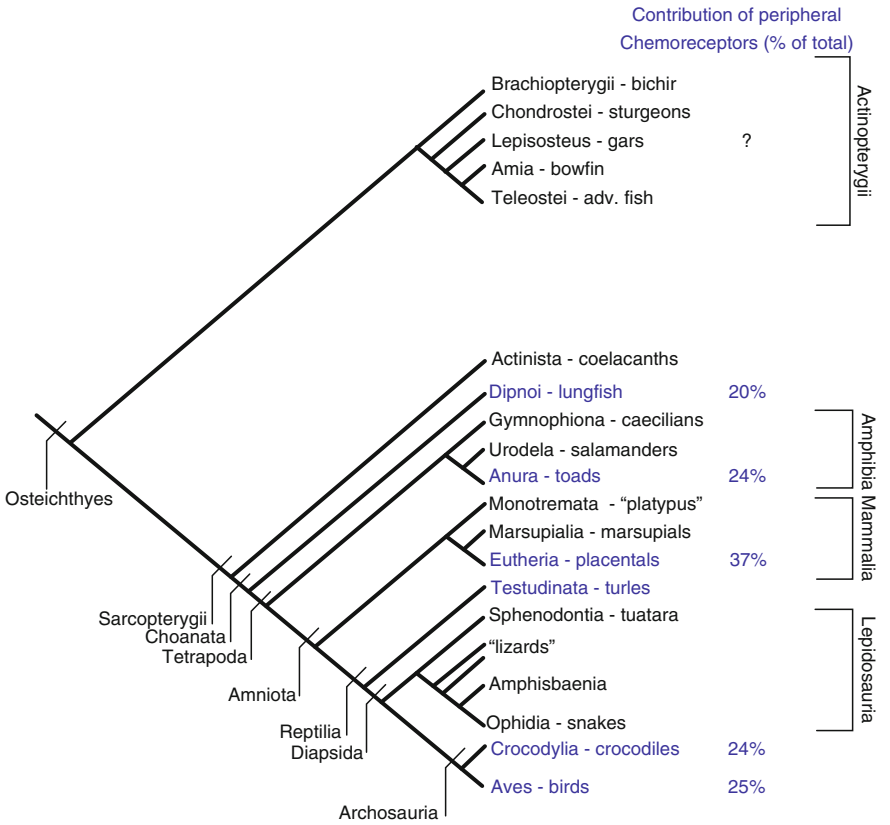

Fig. 3 Cladogram representing probable relationships between the vertebrate groups. *Blue* indicates that the peripheral contribution to the peripheral chemoreceptor drive is known for at least one species of the group. The references are: (1) dipnoi: lungfish *L. paradoxa* 20% (Bassi et al. 2005), (2) anura: toad *Chaunus schneideri* (earlier *Bufo paracnemis*) (Branco et al. 1993), (3) eutheria (placentals): dog 37% (Smith et al. 2006), (4) crocodylia — crocodiles 24%, Branco and Wood (1993), (5) aves — pekin duck 25% (Milsom et al. 1981; Shams and Scheid 1989)

gar *L. oculatus* (Smatresk and Azizi 1987). Two types of receptors were identified. Like in the lungfish, a rapidly adapting receptor was present, and a slowly adapting type was CO_2-sensitive. Further, hypercarbia reduced the firing rate of the slowly adapting receptors, which might suggest an ancient origin rather than a coincidence.

It is easy to detect CO_2-sensitive stretch receptors. Initially the animal breathes air, after which hypercarbia is applied, which increases pulmonary ventilation at a fixed CO_2 level. After some time, the animal suddenly returns to air-breathing. At this point, one would expect a decrease of ventilation. Instead, ventilation increases steeply, because the intrapulmonary CO_2 levels become reduced, which removes the inhibitory action of the CO_2-sensitive stretch receptors. This effect is often referred to as a 'post-hypercapnic hyperpnea' (Milsom et al. 2004). *L. paradoxa*

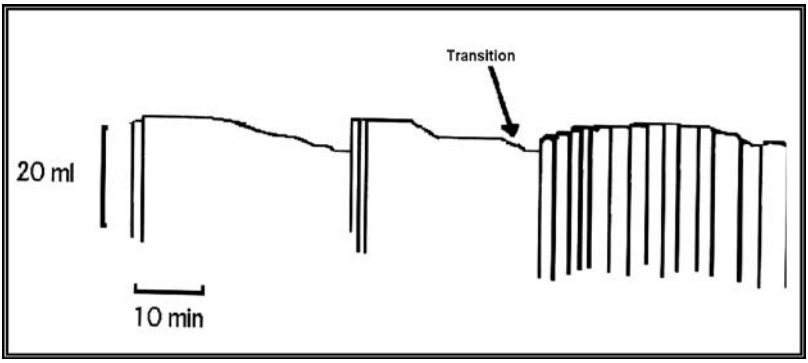

Fig. 4 This figure illustrates the presence of 'post-hypercapnic hyperpnea' in *L. paradoxa.* The recording shows the tidal volume during steady state hypercarbia. Both gas phase and water were kept at $PCO_2 = 55$ mmHg, after the inspired gas was substituted with air. This reduced intrapulmonary CO_2 levels, which stimulated ventilation and caused a transient burst of intensive respiration. This response is a hallmark for the present of intrapulmonary CO_2-sensitive stretch receptors

was tested in that regard, and the recording shows the expected response (Sanchez and Glass 2001); see Fig. 4.

O_2-receptor function has also been studied in *L. paradoxa*, including an evaluation of ventilatory responses to aerial and/or aquatic hypoxia. It turned out that aquatic hypoxia (range 145–153 mmHg; $t = 25°C$) had no effect on pulmonary ventilation, whereas gas phase hypoxia caused a fourfold increase of ventilation. In addition, the O_2 stimulus (O_2 content or, alternatively, O_2 partial pressure) was identified, since a reduction of O_2 content by 50% had no effect on ventilation. This proves that the specific O_2 stimulus is O_2 partial pressure and not O_2 content (Sanchez et al. 2001b). Amphibians such as *Chaunus schneideri* also possess O_2 receptors that monitor O_2 partial pressure, and this modality seems to apply to most land vertebrates, including human beings (Wang et al. 1994; Branco and Glass 1995).

6 Focus on the African Lungfish *Protopterus sp*

Smith 1935) initiated a line of studies on kidney physiology, including *P. aethiopicus* (Smith 1930). In an early pioneering work, Johansen and Lenfant 1968) studied gill function and the relative importance of gas exchange surfaces at 20°C. In this species, the O_2 extraction by the gills ranged from 11 to 36%, which is very low considering that the normal value for teleost fish is about 85% (Rantin et al. 2007), but this is consistent with data for *L. paradoxa*. In addition, Johansen and Lenfant (1968) reported that the O_2 uptale from the water accounted for as little as 11% of total uptake. By contrast, the CO_2 output to the water accounted for

no less than 73% of total CO_2 output. The corresponding values for *L. paradoxa* at 25°C were an aquatic O_2-uptake of 10% of total uptake, combined with 65% of CO_2 produced eliminated to the water (Amin-Naves et al. 2004). The values of *Protopterus sp.* and *L. paradoxa* are strikingly similar, which is surprising since the split of South America from Africa took place some 100 million years ago (Vidal et al. 2007).

Recently, Perry et al. (2007) studied acid–base regulation in the *P. annectens* assuming that ventilatory responses to hypercarbia would occur which, in turn, would indicate the presence of chemoreceptors. A 1-h acid infusion was applied, which temporarily increased respiratory frequency of the gills and lung twofold, after which these values dropped to reach the previous baseline within 5 h. The procedure can not distinguish between central and peripheral chemoreceptors. Nevertheless, the respiratory responses of *P. annectans* were large and immediate, and clearly distinct from the weak responses of teleost fish to acid–base disturbances (Soncini and Glass 2000). The authors also found that extra-renal routes were a key factor in metabolic compensation. On the other hand, during aestivation in *P. aethiopicus*, plasma [HCO_3^-] gradually increased over 7 months, but the mechanism of the increase was not clear, and a possible explanation could be a gradual loss of body water, concentrating plasma [HCO_3^-] (DeLaney et al. 1977).

Compensation of pHa was not evident in *L. paradoxa*, in spite of an exposure to 7% CO_2 (49 mmHg) during 48 h (Sanchez et al. 2005). It should be pointed out that its gills are considerably more reduced than those of *Protopterus sp.*. As a possibility, the pH regulation could be similar to that of salamanders studied by Heisler et al. (1982). Urodeles inhabit a strenuous hypercarbic environment, which makes the regulation of the extracellular environment difficult, whereas the intracellular compartments are regulated in response to hypercarbia.

Carbonic anhydrase (CA) has one of the fastest turnover numbers of all enzymes. Nevertheless, recent studies report that addition of CA will increase CO_2 excretion (Gilmour et al. 2007). Thus, bovine CA slightly decreased $PaCO_2$ of *P. dolloi*, while pH increased from 7.48 to 7.53. The authors conclude that the bulk parts of O_2 and CO_2-excretion occurs by the lung, which is consistent with data on *L. paradoxa* exposed to temperatures from 15 to 35°C (Amin-Naves et al. 2004).

Both amphibians and lungfish inflate the lung by positive pressure, using the buccal cavity as a force pump that inflates the lung with the inspired gas (McMahon 1969). Lungfish have ribs, but these are not activated during respiration (Foxon and Bishop 1968). Reaching the surface, *Protopterus* closes the mouth and compresses the buccal cavity to eject water through the operculum. As the second step, the opercular and buccal spaces expand, while air enters by the mouth. Subsequently, a glottal sphincter opens and the gas is expelled from the lung. After that, several stepwise movements force the gas into the lung (McMahon 1969; Lomholt 1993). These rather complicated movements invite the question asked by Pack et al. (1992) about a possible action of a Hering–Breuer reflex in response to the lung inflation. To this end, the authors placed a tube into the lung of the animal, which allowed to more volume to be added at the onset of the buccal force pump, which would shorten the time for inspiratory buccal force movements. In addition,

it turned out that vagotomy would virtually abolish the relationship between inflation of the lung and expansion of the lung. The presence of this mechanism in a lungfish indicates that a control of lung expansion is a basal feature. See also Pack et al. (1990).

7 Focus on the Australian Lungfish *Neoceratodus Forsteri*

The Australian lungfish, *Neoceratodus forsteri* Krefft, is heavily built and reminds us of ancestors such as the Devonian *Dipterus* (Carroll 1988). As a new and surprising development, this lungfish turns out to be an obligate neotene animal, or in other words, it can be considered as a larval form with capacity to produce, which is backed up by deficiencies in its thyroid function. Concurrently, possible neotenic features in *Lepidosiren* and *Protopterus* are under discussion (Joss and Johanson 2007). *N. forsteri* inhabits river systems in the South-East Queensland region, and aestivation has never been reported for this lungfish. Surprisingly, *N. forsteri* possess ampullary organs that may be used to locate the prey. Based on application of various stimuli, the authors confirmed that *N. forsteri* can detect weak electric fields surrounding living animals, and they also propose that the fish uses this information to locate hidden prey (Watt et al. 1999). To my knowledge, there is no similar information for *Lepidosiren* and *Protopteru*s.

Aestivation is absent in *N. forsteri*, which makes sense, since this animal is not exposed to the strenuous environmental conditions of Lepidosireniformes. Johansen et al. (1967) studied respiratory function in *N. forsteri*, and found that the interval of air-breaths often lasted more than 1 h or more (temp. 18°C). This is not surprising, because its gill system is highly developed, whereas the lung is very simple, when compared to those of other extant lungfish. As could be expected, hypoxia provoked large increases of branchial and pulmonary ventilation. Later, Kind et al. (2002) reported a nearly 8-fold increase of air-breaths, with reduction of O_2 from 120 mmHg to 40 mmHg. Air-breathing was always accompanied by a burst of branchial movements and a large increase of pulmonary perfusion (Fritsche et al. 1993). Based on the principle of Dejours 1981), a high dependence on gill respiration leads to a low $PaCO_2$, and the blood gas values ($PCO_2 = 3.6$ mmHg and pHa = 7.64; $t = 18°C$) are close to those for teleost fish. By contrast, *L. paradoxa* and *Protopterus sp*. are highly dependent on the lung, which is reflected in their high $PaCO_2$ values and low pHa.

8 Aestivation

Aestivation is a seasonal dormancy, which is usually related to adverse environmental conditions such as a dry season and/or limited availability of food items. Different from hibernation, aestivation can occur without any temperature changes.

Aestivation is well-studied in amphibians, and information on reptiles is growing (Abe 1995; Andrade et al. 2004). In amphibians, the reductions in O_2 uptake ranged from 18 to 54% relative to previous baseline value for awake, undisturbed animals (Glass et al. 1997).

DeLaney et al. (1974) decided to address aestivation in *P. aethiopicus.* Initially the lungfish caved out a burrow, when the mud gradually dried out, and this was followed by mucous secretion from the skin of the animal. Soon, the mucous hardened to cover the animal, except for an opening at the mouth, which allowed respiration via a breathing channel. The transition from water to cocooned conditions, correlated with reduction of O_2 uptake to half of the previous value for the animal in water (DeLaney et al. 1974). Over 2 weeks the mean blood pressure fell from 24 mmHg to 15 mmHg, and f_H decreased from 35 to 11–16 beats min^{-1}. As an extreme case, Lomholt (1993) reported that *P. amphibius* could remain in the cocooned state for 6–7 years, and in one specimen the O_2 uptake had decreased to 15% of the initial value for the animal in water. Using X-rays, Lomholt (1993) claimed that one of the animals (0.4 kg) occupied the entire space of the burrow, which would require a lung volume of 250 ml. The survival time for cocooned *Protopterus* is amazing, but one *P. amphibius* achieved a possible record, with 7 years of survival (Lomholt 1993). If the animal really fills out the space of the burrow, then it should be possible to apply pneutachography, since inspiration and expiration would be the only major movements. Later, DeLaney et al. (1977) studied the effects of aestivation on blood gases of *P. aethiopicus* at 25°C. With the animal in water the values were: $PaCO_2 = 26$ mmHg; pHa = 7.60. During the second weak of aestivation, $PaCO_2$ increased at a lower rate to reach a $PaCO_2$ of 49.8 mmHg, with a pHa of 7.37. These changes can be explained based on several mechanisms. When in water, a large fraction of the CO_2 output becomes eliminated to the water. Assuming that the observation of a close fit to the burrow is correct (Lomholt 1993), then the whole CO_2 output would be eliminated by the lung. Once again the principle of Dejours (1981) can be applied to predict a large increase of $PaCO_2$ which, consequently, lowers pHa. The authors were, however, uncertain about the nature of a slow increase of plasma [HCO_3^-]. DeLaney et al. (1974) reported a downregulation of mean blood pressure, and a heart rate that was reduced to 50% of the value for the animal in water. The breathing frequency decreased from 20 to 8 breath h^{-1} (values before and after aestivation). Unfortunately, respiratory signals provide no informationregarding the possible reduction of V_T after transition to aestivation. Returning to long-term data, one *P. amphibius* that had been in the cocoon for 6 years had an end-tidal PCO_2 of 40 mmHg and an end-tidal PO_2 of 120 mmHg, which would practically fit into the data for some weeks of aestivation (Lomholt 1993).

Recently, Perry et al. (2008) studied aestivation in *P. dolloi*, and under favorable conditions in the laboratory would induce secretion of a cocoon, which became hard after 4–5 days. Before aestivation, *P. dolloi* consumed 0.35 $mlSTPD\,kg^{-1}\,min^{-1}$ (5°C) but in the cocoon the O_2 uptake increased to 0.45 $mlSTPD\,kg^{-1}\,min^{-1}$, which reduced $PaCO_2$ from 18 to 14 mmHg. Greenwood 1986) states, however, that this lungfish does not aestivate in its normal habitat. The authors realized that the animals were not in a state of aestivation and, therefore, coined the word ‘terrestrialization’,

Table 1 Blood gases in *Lepidosiren* in water and after 40 days of aestivation

	In water	*Aestivation 40 days*
PaO_2 (mmHg)	87.7 ± 2	$77.1 \pm 3^*$
$PaCO_2$(mmHg)	21.8 ± 0.4	$34.4 \pm 3.2^*$
pHa	7.51 ± 0.05	7.53 ± 0.05

Mean values $\pm$ SEM; $n = 5$ ($p < 0.05$ paired t-test)

and this condition was maintained for more than 1 month, which is important, because true aestivation could have appeared after a longer period. The solution to this enigma would be highly interesting, and interactions between various disciplines would be required.

The South American lungfish also aestivates, but for much shorter periods, typically 1 or 2 months. When a lake dries out, the aestivation is initiated by a position in which it assumes a U-shape in which the tail approaches the head. For breathing, the animal slides the head upward, and breaks the surface to respire. There is no cocoon formation, but the characteristics of aestivation are very similar as seen from Table 1.

In conclusion, it is clear that lungfish and land vertebrates share very fundamental mechanisms of physiological regulation. These concern the control of pulmonary ventilation. These involve the relative drives of central and peripheral CO_2/H^+ receptors, where their relative roles are very similar. Moreover, in mammals, toads and lungfish the common central stimuli are both CO_2 and H^+, and intrapulmonary stretch receptors that lower firing rate if intrapulmonary PCO_2 increases are also found in lungfish as well as in tetrapods. A Hering–Breuer reflex is even present in lungfish, which once again shows common traits which most likely evolved before the now rather likely ramification between lungfish and tetrapods.

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Aestivation in Amphibians, Reptiles, and Lungfish

M.L. Glass, J. Amin-Naves, and G.S.F. da Silva

Abstract Some regions of the world have very limited variations in temperature during the year. Adverse conditions such as lack of appropriate food items and/or the drying out of shallow lakes may induce a state of torpor. This is different from hibernation, which involves a reduction of temperature. Torpor is characterized by a cessation of feeding, and eventually lack of movement. In addition, tissue metabolism and cardiac activity become downregulated, mainly by reduced cardiac frequency. The state of torpor occurs in some amphibians, reptiles and lungfish, and the transition also involves increases in CO_2, accompanied by reduced O_2 levels. African lungfish have records of surviving in a cocoon with up to 7 years without food intake, but amphibians and reptiles survive in torpor for several months.

1 Introduction to Aestivation

Aestivation is a dormant state, which usually takes place on a seasonal basis. This word is derived from "aestes," which in the Latin language signifies "summer." Alternative words are "dormancy" or "torpor," which are both used in the literature. Fishman et al. (1989) stated that: "aestivation is a loose term that signifies little more than summer torpor as a mechanism for surviving torrid ambient conditions." This torpor is, however, quite distinct from hibernation, which takes place at considerably reduced ambient temperatures. Some subtropical regions may be at virtually the same temperature during the whole year. In this case, cold is not the factor which forces the animal to aestivate. Rather, specific factors such as lack of food or the dry conditions may force the animal to lower metabolism. As stated by Abe (1995), in amphibians exposed to a dry environment this may involve substantial losses of water.

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Such conditions may force animals to downregulate ventilation, perfusion and gas exchange. In land vertebrates, this downregulation may lower metabolism down to 20–50% of the metabolic level before aestivating, which raises questions about the underlying mechanisms.

1.1 Metabolic Downregulation and Aestivation in Anuran Amphibians

Flanigan et al. (1993) reported a considerable metabolic downregulation in the aestivating Australian goldfields frog (*Neobatrachus wilsmorei*). They also confirmed a metabolic reduction in isolated tissues, but there was no damage to the metabolism of the brain and kidney. Muscle and skin preparations indicated a 50% reduction of tissue metabolism relative to the awake animal. In spite of that, the N^+ and K^+ gradients were maintained across the membrane of muscle cells. Withers and Guppy (1996) found that desert frogs accumulated large amounts of urea (100–200 mM) when in torpor. They also suggested that the aestivating frogs were insensitive to the effects of urea or, alternatively, that the accumulation of urea was involved in a mechanism for metabolic depression. Bayomy et al (2002) addressed the effects of body water and mass, along with the acid mucopolysaccaride (AMPS) and its degrading enzyme components in tissues of *Cyclorana maina, C. platycephala, C. australis* and *Neobatrachus sutor*. The authors suggested that, during aestivation, the desert frogs were protected by a cocoon formed by AMPS and other material, and this substance limited the water loss to the environment through the cocoon. In addition, the kidney reabsorbed water, preventing dehydration. Moreover, the Australian desert frog (*Neobatrachus centralis*) was studied by Furey et al. (1998), who addressed metabolic depression and a 67% reduction of protein synthesis.

Hudson et al (2005) focused on the effects of long-term aestivation on structure and function of the green-striped burrowing frog (*Cyclorana alboguttata*), which stayed in torpor for no less than 6 months. At the end of this period, awake animals were compared to animals at the end of the dormant period. It turned out that the resting end plate potentials were the same for the two groups, about −067 mV, while the evoked end plate potentials were 3.2-fold larger in the awake frogs. Further studies reported that, as an important feature, *C. alboguttata* aestivates within a cocoon formed by shed skin and mucous, which immobilizes its hind limbs, but in spite of this, metabolic depression during dormancy may provide a protection against muscle atrophy (Hudson and Franklin 2002). According to Lavidis and Hudson (2008), this frog can survive in the cocoon for months or years, and then recover in the rainy season. In their experiments, the frogs remained in aestivation for 3 months, while a control group was provided with food and water. It turned out that atrophy was absent and the in vitro performance of the gastrocnemius muscle was intact. The protection of aestivating amphibians acts in a complex and integrated manner. Withers and Guppy (1996) suggested that high levels of urea accumulated during aestivation could be part of the mechanism to protect tissues. In the end, aestivation

in amphibians has many spectacular surprising aspects, but the physiological key event for dormancy has not yet been identified.

Few studies deal with blood gases and cardiovascular function in amphibians exposed to aestivation. The toad *Chaunus schneideri* (earlier *Bufo paracnemis*) is rather common in regions of São Paulo State, where the seasonal temperature changes are quite limited. During the dry season (June–July), the toads dig out burrows and aestivate. Glass et al. (1997) reported a downregulation of cardiac function, which resulted from a reduction of cardiac frequency (f_H) (Fig. 1), while the arterial pressure was the same. Moreover, the changes of f_H closely reflected total O_2 uptake (Fig. 2). At 25°C, the PaO_2 of aestivating animals was 53 mmHg, whereas the value for spring animals was a PaO_2 of 60 mmHg.

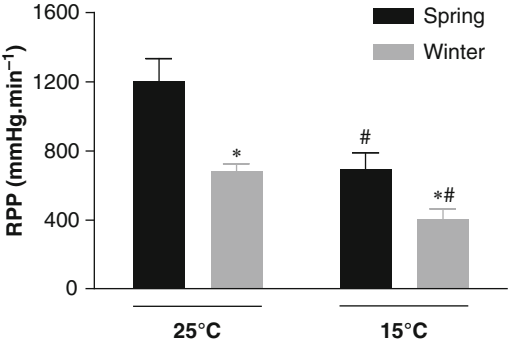

Fig. 1 Seasonal variations in O_2 uptake (VO_2) in *Chaunus schneideri* at 25°C and 15°C. Data are expressed as mean values ± SE. Results were considered significantly different when $P < 0.05$. *Asterisk* means difference between winter and spring toads at the same temperature. *Hash* means difference between the two temperatures of the same season (winter or spring). Modified from Glass et al. (1997)

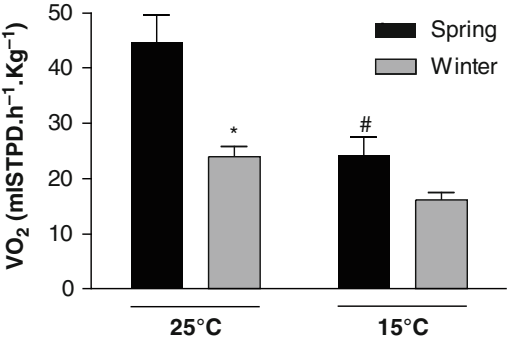

Fig. 2 Seasonal variations of the rate–pressure product (RPP) in *C. schneideri* at 25°C and 15°C. Data are expressed as mean values ± SE. Results were considered significantly different when $P < 0.05$. *Asterisk* means difference between winter and spring toads at the same temperature. *Hash* means difference between the two temperatures of the same season (winter or spring). Modified from Glass et al. (1997)

An earlier study by Boutilier et al. (1979) addressed the related toad *Bufo marinus,* which escapes from adverse conditions and digs out a hole in the mud, while it maintains access to air. The transition was quite quick, lasting 2 days, during which pHa fell from about 8.00 to 7.40 and, simultaneously, $PaCO_2$ rose from 8 to 14 mmHg. In addition, plasma [HCO_3^-] rose from about 12 to 24 mM, which nearly restored pHa after 4 days. A Davenport diagram showed that the toad closely followed the buffer line for the first 36 h, after which $PaCO_2$ increased within the isopleths of 14–16 mmHg, which increased [HCO_3^-] to 28 mM. This compensated pHa from 7.64 to 7.80. Consistently, PaO_2 fell from 90 to 65 mmHg and f_R became downregulated during aestivation. A steady-state condition had already developed during the first week.

1.2 Dormancy in Reptiles

The data on aestivating reptiles are rather few. In addition, Abe (1995) points out that aestivation is not well-defined and adaptations are variable. As an advantage for the studies, the temperature changes over the year in Brazil are minimal, which invites the study of dormancy. Seasonal changes in O_2 transport and acid–base status have been studied in the torpid tegu lizard *Tupinambis merianae*. It turned out in one study that its metabolism was about one third, relative to awake lizards, and the dormant state resulted in long periods of apnea (de Andrade and Abe 1999). Andrade et al. (2004) reported that dormancy increased plasma [HCO_3^-], $PaCO_2$ and plasma [O_2]. Recently, Sanchez et al. (unpublished data) measured the ventilatory responses and blood gases of *T. merianae* in both the awake and dormant states ($t = 25°C$). Normocarbia and two levels of hypercarbia were applied. It turned out that dormant lizards had much reduced ventilatory responses, compared to those of awake animals. In addition, these responses occurred at much higher $PaCO_2$ values. Also working on *T. merianae*, de Souza et al. (2004) found resting rates in various seasonal states such as the late autumn.

Also, Winne et al. (2006) reported for the first time that aestivation behavior occurred in an aquatic snake (*Seminatrix pygaea*) which inhabits isolated wetlands, where the habitats are subject to periodic droughts. This snake has a small body size, and feeds on small aquatic prey. The authors concluded that the snake aestivated below the dried surface of its habitat as a survival strategy for severe drought and lack of food items. In addition, *S. pygaea* exhibited a well-adapted reproductive policy. Many ectotherms accumulate energy to reproduce (capital breeding) and become anorexic while pregnant. *S. pygaea* continued feeding throughout pregnancy, which allowed the population to increase immediately after the dry period.

A recent paper from Ligon and Peterson (2002) supported the work by Rose (1980), which considered the mud turtle (*Kinosternon flavescens*) as an aestivating species, with a capacity to survive for no less than 2 years without water. These yellow mud turtles are also known to exhibit low levels of activity and minor increases of plasma osmolality during torpor.

1.3 Dormancy in Lungfish

The lungfish was widespread in the middle Devonian, but was reduced by 70% in the Permian, which contrasts with the abounding and diversified evolution of land vertebrates (Tetrapoda). The highest degree of morphological diversity occurred in the late Devonian, whereas the Permian period was characterized by a morphological stasis (Lee et al. (2006). The *Latimeria*, with two species (*menadoensis* and *chalumnae*), possess lungs, but these are filled with fat (Carroll 1988), and lungfish is the most probable sister group to the tetrapods (cf. Tohyama et al. 2000; Brinkmann et al. 2004). This is backed up by the discovery of a 417 million-year-old fish fossil (*Styloichthys*), which has the characteristics expected of a common ancestor to tetrapods and lungfish (Zhu and Yu 2002). These two groups possess central and peripheral acid–base CO_2/H^+-receptors to control the ventilatory drive (Sanchez et al. 2001; Amin-Naves et al. 2007a, b). Further, the O_2-related drive in lungfish and tetrapods is PO_2, while reduced O_2 content, and Hb–O_2 saturation failed to increase V_E. The lepidosirenid lungfish *Protopterus sp.* and *Lepidosiren paradoxa* aestivate, whereas the river-dwelling Australian lungfish (*Neoceratodus forsteri*) has no period of torpor (Lenfant 1967; Kind et al. 2002).

Lepidosireniformes include the African lungfish (*Protopterus*) with four species (*P. aethiopicus*, *P. amphibius*, *P. annectens*, and *P. dolloi*), and the South American lungfish *Lepidosiren paradoxa.* Dry conditions induce *Protopterus* to dig out a burrow into mud and clay, and then a mucous secretion from the skin covers the animal, forming a cocoon, except for the mouth, from where the animal can respire via an opening at the surface (DeLaney et al. 1974, 1977). *L. paradoxa* is different, since it does not form a cocoon, which permits the animal to move the body position within a site. Data on burrowing, and a downregulation of f_H, were obtained by Harder et al. 1999, while Mesquita-Saad et al. (2002) found that skeletal and heart muscles had a high anaerobic capacity during aestivation. This torpor suppressed enzyme activity, which downregulated metabolism.

DeLaney et al. (1974, 1977) studied aestivation in *P. aethiopicus* for no less than 9 months, the first of which was dominated by metabolic reduction: f_H became decreased from 25 to 14–16 beats $\cdot$ min^{-1}, while the mean blood pressure (f_{mean}) fell from 25 mmHg to 15 mmHg. In particular, VO_2 became reduced from 25 to 5 ml kg^{-1} h^{-1}, which corresponds to 17% of the value for the aroused fish. In addition, the gill system was shut down, leading the blood flow to the lung (Johansen and Reite 1967), which would probably direct the entire CO_2 output to that organ, and accounted for a large increase of $PaCO_2$ during aestivation. Also, transition to dormancy was accompanied by large decreases in O_2 uptake, which was 0.2–0.5 ml kg^{-1} min^{-1} when in water, but fell to 0.084 ml kg^{-1} min^{-1} during aestivation (DeLaney et al. 1974).

Later, Delaney at el. (1977) addressed the impressive effects of conversion from aquatic life to the cocooned condition. With the animals in water at 25°C, their pHa was 7.60 with a $PaCO_2$ of 26 mmHg and a plasma [barHCO_3^-] of 31 mM. Later, $PaCO_2$ followed a buffer line to 45 mmHg at pHa 7.41. After that, $PaCO_2$ rose to no less than 50 mmHg, while plasma [HCO_3^-] reached 40 mM (DeLaney

at el. 1977). Aestivation caused dehydration and a shut down of kidney function, accompanied by a progressive increase of osmolality. In this situation, it is a surprise that two *P. annectens* survived in a laboratory for 6–7 years in the cocooned state (Lomholt 1993).

Recently, the lungfish *P. dolloi* was studied by Perry et al. (2008), although Greenwood (1986) had warned that this particular species might not aestivate within its region. Initially, the lungfish produced a cocoon under appropriate laboratory conditions. The expected next step was a downregulation of VO_2, but just the opposite happened, since metabolism increased by 17% relative to the values for the animal in water. Consistently, $PaCO_2$ fell from 18 to 14 mmHg due to hyperventilation. To explain this rather unique behavior of the animal, Perry et al. (2008) coined the word "terrestrialization." The rather unusual circumstance was that the cocoon acted as a trap and not as a protection, retaining CO_2 and forcing the animal to hyperventilate (Glass 2008). Chew et al. (2004), however, reported that *P. dolloi* can be induced to aestivate within a layer of dried mucous in a plastic bag. Nevertheless, this will not identify the specific signals to induce aestivation in this lungfish.

Recently, Ip et al. (2005) induced *P. aethiopicus* to aestivate for 46 days. Rates of urea and ammonia accumulation were used as indicators of metabolism, and samples were taken after zero, 12, 34 and 46 days. Fasting animals in water were taken as a control group and presented a large endogenous ammonia production, while the aestivating animals had much lower ammonia levels. The authors proposed two possibilities, which were an increase of urea synthesis, or a decrease in the rate of ammonia production. As to the first assumption, they found that the overall urea production decreased during the whole test period. However, they also noticed that the first 12 days were rather different from the rest of the experiment. During this period, the urea synthesis was 2.8-fold greater than on day zero, which was attributed to an upregulation of some OUC (ornithine–urea cycle) hepatic enzyme activity, which detoxified ammonia. It was concluded that long-term aestivation in *P. aethiopicus* suppressed ammonia production, since the animal depended on an increased urea synthesis to detoxify ammonia produced in a fasting condition. A similar phenomenon of urea production under fasting conditions had been observed in *P. dolloi* (Chew et al. 2004), but instead of excreting the produced excess of urea as *P. aethiopicus* (Ip et al. 2005), *P. dolloi* retained the substance in its body (Chew et al. 2004).

Few studies have addressed the question of hypoxic and/or hypercarbic environment conditions in aestivating lungfish. Loong et al. (2008) focused on ammonia toxicity, and proposed that hypoxia would result in a more severe metabolic depression. Therefore, they exposed *P. aethiopicus* to an inspired PO_2 of 15 mmHg ($\sim 2\%O_2$), which is severely hypoxic. It turned out that application of 2% O_2 during 12 days significantly reduced concentrations of urea in liver, muscles, and plasma. Ambient hypoxia reduced ATP and creatine phosphate levels, leading to a reduced buildup of urea. Working on *P. annectens,* Lomholt (1993) evaluated the transition to aestivation and, in particular, the question of possible hypercarbic and/or hypoxic conditions. A specimen of *P. annectans* was studied after 6–7 years of aestivation, as mentioned above. The end-expired PCO_2 was 40 mmHg, and the corresponding PO_2

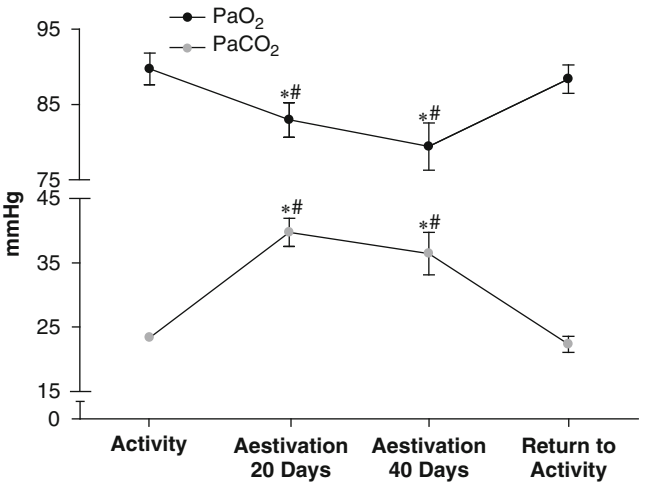

Fig. 3 Effects of aestivation on $PaCO_2$, $[HCO_3^-]$ and pH in *Lepidosiren paradoxa*. Data are expressed as mean values ± SE. Results were considered significantly different when $P < 0.05$. *Asterisk* means difference from the activity period. *Hash* means difference from the return to activity, after dormancy period. Modified from da Silva et al. (2008)

was 120 mmHg. At this point, data are too few to describe the precise conditions within the cocoon and the tube that connects to the air.

Recently, aestivating specimens of *L. paradoxa* were followed for 40 days. When in water, $PaCO_2$ was 22 mmHg, but torpor for 20 days increased $PaCO_2$ to 38 mmHg (Δ73%), while 40 days of torpor resulted in 34 mmHg (da Silva et al. 2008) (Fig. 4). Using the same sequence, animals in water had a plasma $[HCO_3^-]$ value of 23 mM, while 20 days of aestivation increased it to no less than 40 mM, but after 40 days the plasma $[HCO_3^-]$ became slightly reduced to 36 mM (Fig. 3). As it turned out, $PaCO_2$ and $[HCO_3^-]$ rose in parallel, and the consequence was that pHa became confined to a narrow range of 7.49–7.53. This fact suggests that pHa is the regulated variable (Fig. 3). On the other hand, PaO_2 was subject to change in response to the ambient impact. Initially, in water the PaO_2 was 88 mmHg, and torpor reduced PaO_2 to 77 mmHg (Fig. 4). Transition to torpor had no effect on blood pressures, but f_H decreased from 31 to 22 beats min^{-1} after 40 days of aestivation (Fig. 5). Hb–O_2 dissociation curves (ODCs) were also done in *L. paradoxa* and indicated a low affinity, which predicted HbO_2 saturations in the range of 20–40% (Bassi et al. 2005; Kind et al. 2002).

Unlike *P. aethiopicus* (DeLaney et al., 1974, 1977), cited above, *L. paradoxa* maintained the same mean blood pressure (27–32 mmHg), whether in the water or within the burrow (da Silva et al. 2008). This is consistent with data from the toad *Chaunus Schneideri*, in which blood pressures remained the same during dormancy and activity, while VO_2, f_H, and RPP decreased by 50% or less, relative to aroused toads (Glass et al. 1997).

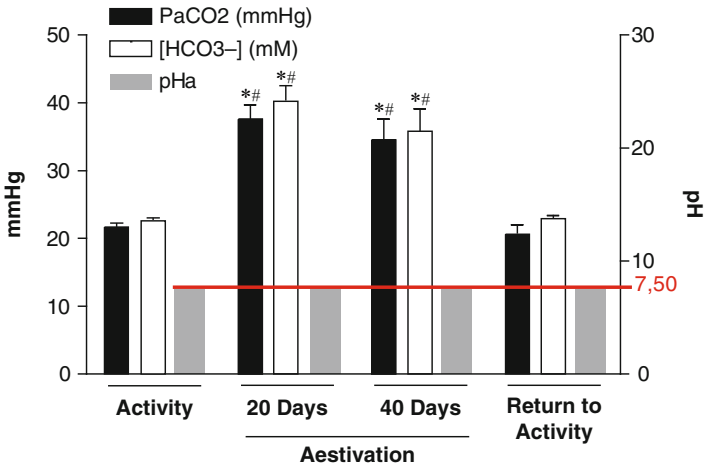

Fig. 4 Effects of aestivation on $PaCO_2$ and PaO_2 in *Lepidosiren paradoxa*. Data are expressed as mean values ± SE. Results were considered significantly different when $P < 0.05$. *Asterisk* means difference from the activity period. *Hash* means difference from the return to activity, after dormancy period. Modified from da Silva et al. (2008)

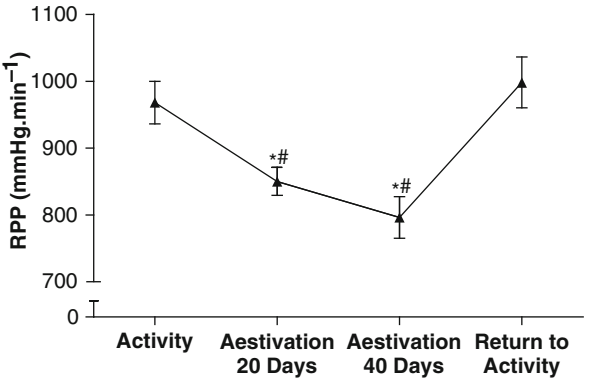

Fig. 5 Effects of aestivation on heart frequency and arterial pressure, represented by the rate–pressure product in *Lepidosiren paradoxa*. Data are expressed as mean values ± SE. Results were considered significantly different when $P < 0.05$. *Asterisk* means difference from the activity period. *Hash* means difference from the return to activity, after dormancy period. Modified from da Silva et al. (2008)

Da Silva et al. (2008) also measured the osmolality and the leptin-induced respiratory responses in *L. paradoxa.* Osmolality showed a significant increase from $\sim 232 \pm 2.6$ Osmol (before aestivation) to a range of 261 ± 4.5 (20 days) to 267 ± 2.2 Osmol (40 days) (see Fig. 6), which is in agreement with the data for *P. aethiopicus* (DeLaney et al. 1974). As for leptin, there are studies that show its interaction with the central chemoreceptor systems in mammals. In particular, the genetically modified mice ob/ob, which are unable to produce leptin, showed highly

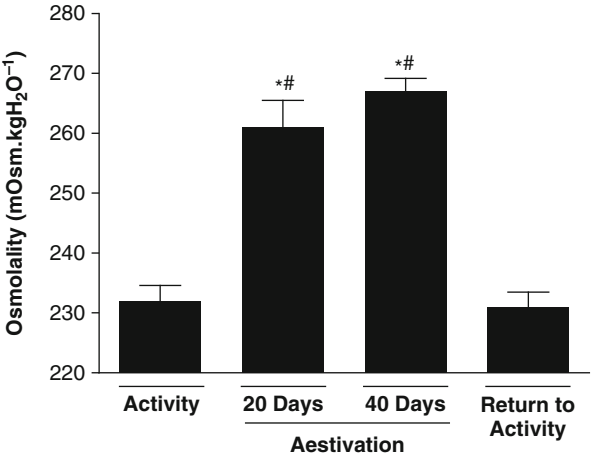

Fig. 6 Effects of aestivation on plasmatic osmolality in *Lepidosiren paradoxa*. Data are expressed as mean values ± SE. Results were considered significantly different when $P < 0.05$. *Asterisk* means difference from the activity period. *Hash* means difference from the return to activity, after dormancy period. Modified from da Silva et al. (2008)

reduced ventilatory responses to hypercarbia (O'Donnell et al. 1999, 2000; Polotsky et al. 2004). However, in *L. paradoxa* the respiratory responses for Leptin could not be recognized, even though it turns out that leptin is present in *Lepidosiren* (da Silva et al. (2008).

In conclusion, the crisis strategies of *Protopterus sp.* and *L. paradoxa* are different only with regard to the formation of the coccon. The former aestivates in a cocooned state, which restricts its movements, and the latter aestivates in a simple burrow of mud, which allows it to escape from an unfavorable site. In respect of the cocoon, it may protect the animal for years, but as reported by Lomholt (1993), may lead it to death.

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Part II
Evolution of Pulmonary Mechanics and Respiratory Control

Trade-offs in the Evolution of the Respiratory Apparatus of Chordates

S.F. Perry, W. Klein, and J.R. Codd

Abstract In the evolution of the respiratory apparatus repeated trade-offs between it and other organ systems are evident. Gills of non-vertebrate chordates have both a respiratory and alimentary function, whereby in amphioxus the gills filter incoming water, extract and transport suspended particles, and ventilate the atrial epithelium, which is the main respiratory surface. In craniates a muscular pump replaces the ciliary ventilator. The branchial pump of gnathostomes encloses the heart, resulting in the biomechanical expedience of coupling branchial and cardiac beat frequency. For aquatic vertebrates an air-filled air breathing organ means a trade-off between buoyancy and respiratory functions. In terrestrial tetrapods, multi-functionality of lungs and of respiratory musculature results in complex trade-offs and synergic combinations. The shift to aspiration breathing has resulted in a mechanical constraint in some lizards, due to the dual locomotor and ventilatory role of the hypaxial musculature. The most highly derived amniotes, mammals and birds, however, evolved along different pathways to high-performance aerobes. Whereas the kinetic bronchoalveolar mammalian lung results in a trade-off between large surface area and work of inflation, the avian system combines a large surface area and thin air–blood barrier of the constant volume lung with ease of inflation of the air sacs, resulting in energy-efficient gas exchange.

1 Introduction

The vertebrate respiratory apparatus consists of: (1) a gas exchanger, (2) a circulatory component that connects it with the heart, (3) a ventilatory pump, and (4) a central nervous control element. The evolution of respiratory control and the double circulatory system are covered in other chapters. This review focuses on

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the trade-offs involved in the coupled evolution of the gas exchanger and the ventilatory pump. These terms target structure–function complexes (faculty; Bock and Wahlert 1998) rather than anatomical or physiological units. The systematics of Janvier (1998) will be employed for basal craniates, whereby the hagfish (Hyperotreti) are considered to be the sister group of vertebrates (Vertebrata), which include the lampreys (Hyperoartia) and jawed vertebrates (Gnathostomata).

In a multidimensional complex such as the vertebrate body rarely does a given structure have a single function or is a given function carried out by a single structure. As expressed by Cuvier in the law of correlation of parts: "Everything is organized as a unit, a closed system in which all parts mutually correspond . . . . None of these parts can change without also changing the others. Thus each of them taken separately contains elements of all the others" (Cuvier 1812).

Some interdependent relationships are mutually beneficial, and are referred to as synergistic relationships. Well-known examples are (1) Starling's law of the heart (increased stretching of the cardiac muscle increases the power of contraction), and (2) the increase in breathing frequency with a fall in blood pH, resulting in better pulmonary ventilation/perfusion matching during exercise. Sometimes, however, improving one faculty will decrease the function of another. One example of such a trade-off in the respiratory gas exchanger is that an increase in alveolar surface area in mammalian lungs results in an increase in work of breathing, due to the decreased radius of alveolar curvature, increased surface tension, and thus decreased lung compliance. We define a trade-off as a change that improves one faculty to the detriment of another: in the present example increasing the diffusing capacity of the gas exchanger makes more work for the ventilatory pump. In the evolution of the respiratory faculty, trade-offs frequently occur and have resulted in the evolution of compensatory mechanisms, which may in turn open the door to new evolutionary pathways.

2 Aquatic Water Breathers: Interaction of Respiratory, Cardiovascular, Locomotor and Nutritional Faculties

A branchial basket as part of the head gut is a synapomorphy of the phylum Chordata. In tunicates and acraniates (e.g. amphioxus) the gills are enclosed in a peribranchial atrium, which led to the earlier classification of these two groups as 'atriozoa'. Although the terminology (branchial, gill) implies respiratory function, it remains to be demonstrated to what extent these structures are involved in respiration and/or in feeding. From the very beginning the ancestral organ was involved in a respiration/nutrition trade-off.

The branchial skeleton is covered with ciliated epithelium, which is an integral part of the filter feeding apparatus. An iodine-rich secretion is produced in the ventral hypobranchial groove (endostyle) and carried up the inside of the branchial basket by a system of cilia. At the same time cilia on the anterior and posterior sides of the branchial bars create a current that propels water from the lumen of the

branchial cavity through the lace-like secretion product into the atrium. The water then exits the animal through an outflow opening, or atriopore. The secretory product and trapped particulates are collected in the dorsal epibranchial groove (epistyle) and transported by yet another ciliary band to the intestine.

Although the basic circulatory pattern in acraniates and most tunicates is similar to that of vertebrates, with dorsal and ventral aortic vessels connected by branchial arteries, the respiratory function of the 'gills' is compromised by the thick ciliated cells that cover the vessels. In the amphioxus, *Branchiostoma lanceolatum,* most of the morphometric diffusing capacity resides in the atrial epithelium, with only 1–2% in the gills (Schmitz et al. 2000). Thus, in the trade-off between gas exchange and filter feeding, the latter is clearly the major function of the branchial basket. In some pelagic tunicates (salps) the gills furthermore take on a locomotor function. The atrium lies posterior rather than lateral to the ventilatory ciliated cells, and the water current created by the gills moves the animal by jet propulsion (Goldschmid 1996).

In craniates, the trade-off between breathing and feeding has been resolved by the partitioning of the cranial gut into both a mouth that functions in (often predatory) prey acquisition and more caudally located gills with a primarily respiratory role. The mouth or the most anterior gill arches act as jaws whereas the floor of the mouth and gill rakers serve in prey capture, while the distal regions of the gills — in gnathostomes the epibranchial and ceratobranchial elements of arches 1 through 4, sometimes also 5 and 6 — are specialized for gas exchange and ionic regulation. The branchial pump is muscular rather than ciliary, and the mouth is usually provided with dental plates (hagfish), toothlike structures (adult lampreys) or true teeth (most jawed vertebrates).

The gross structure of the exchanger in hagfish is completely different from that of vertebrates. Instead of the familiar filaments and secondary lamellae we find a system of respiratory folds that are oriented to the unidirectional water flow, such that countercurrent exchange takes place as in true vertebrates. This unique gross anatomy is in contrast to the microscopic structure, which exhibits pillar cells and blood channels similar to those of vertebrates (Mallat and Paulsen 1986).

The muscular pump in hagfish (and also in larval lampreys) is the velum, which is essentially a plunger that is slanted backwards. It traps pharyngeal water and pushes it caudally into the gill pouches. This mechanism is very different from the constriction and expansion of the branchial space which occurs in vertebrates.

The transition from ciliary to muscular branchial ventilation allowed efficient gas exchange for the first time. The presence of anatomically distinct countercurrent systems in hagfish (velum, respiratory folds) and vertebrates (gill pouch constriction and expansion) suggests that this physiological attribute may have predated the origin of the muscular pump. The apparent homology of pillar cell systems in craniates also indicates that gills dedicated to water breathing may have arisen together with the loss of filter feeding and the beginning of a more pelagic life style, including prey detection and tracking. Hence in hagfish the telencephalon is highly developed as a centre of integration of chemoreception and its translation of this information into behavioural patterns, whereas the diencephalon contains diverse and complex neuroendocrine systems including light-sensitive lateral and median

eyes. The branchiomeric cranial nerves (V, VII, IX, X) are also present in the most basal craniates, and the central nervous control of breathing is similar to that of true fish (Rovainen 1996).

The origin of muscle-powered branchial ventilation is a prerequisite for metabolism-matched respiratory activity. Whereas the velar respiratory pump is suitable both for filter feeding and for branchial breathing with a stiff gill skeleton, the constriction and dilatation of gill pouches as seen in lampreys and elasmobranchs requires a flexible branchial region. The five-part gill arch skeleton of gnathostomes is consistent with this functional attribute and is considered a synapomorphy of the group (Janvier 1998) Therefore, the muscular gill pump may have arisen at least three times (among the ancestors of hagfish, lampreys and gnathostomes, and possibly also in conodonts), emphasizing the importance of respiratory gills among craniates.

Muscular pumps and jaws constitute a solution for the trade-off between feeding and breathing, the constriction–dilation pump, however, also results in a new trade-off. Since the heart is surrounded by the gill pouches, coupling of heartbeat and gill movement results in a biomechanical exacerbation of both these functions. The coordination of gill and heart activity is well-documented neurobiologically, suggesting a functionally meaningful rather than coincidental mutual biomechanical influence (Taylor et al. 2006). At the same time this anatomical arrangement represents a constraint, since any divergence from event coupling will result in a loss of efficiency in both of these systems. Such neurological coupling also exists in bony fish, although the relatively inflexible encasement of the gills between the skull and operculum restricts the mutual biomechanical influence of cardiac and respiratory dynamics. Cardiac-modulated respiratory neurons have also been observed in post-metamorphic anuran amphibians (Naoki Kogo, personal communication, 1996).

3 Aquatic Air Breathers

Numerous fish groups contain multimodal breathers, which can exchange gas with water using gills, and with air by one or more accessory respiratory organs. These include specialized pharyngeal surfaces, all regions of the gut, the skin and even the secondary lamellae of gills themselves (Graham 1997; Roy and Munshi 1996; Hughes and Munshi 1973; Maina and Maloiy 1986). These parallel systems usually do not necessarily represent trade-offs, if the development of one does not influence the function of the other. On the contrary, oxygen-detecting structures (e.g. neuroepithelial bodies, aortic and carotid bodies) can result in local vasoconstriction or vasodilation and thus in the regulation of perfusion of a region that is exposed to low oxygen tension (e.g. shunting blood past the gas exchange surfaces in *Protopterus* (Nilsson and Axelsson 1987).

Pulmonary ventilation in anuran and caudate lissamphibians differs from group to group (Brainerd 1999; Brainerd and Owerkowicz 2006; Brainerd and Simons

2000; Brainerd et al. 1993), but always involves forcing air from the buccal cavity into the lungs. This inspiratory mechanism may be ancient: it involves branchial and hypobranchial muscles and appears to be derived from modified gill ventilatory mechanisms as seen in the lungfish, *Protopterus* (McMahon 1969). During aestivation, however, *Protopterus* is reported to use costal breathing (Lumholt et al. 1975; Lumholt et al. 1993). Due to gaps in the fossil record it is unknown if recent lissamphibians lost this double inspiratory capacity or if their ancestors lacked costal breathing altogether. Although energy-efficient and adequate for a frog (West and Jones 1975), the buccal pump imposes a constraint on the head shape and therefore on feeding strategies, as it requires a broad and voluminous mouth.

4 Terrestrial Air Breathers

The buccal pump and costal, aspiration breathing do not pose a trade-off, since both mechanisms can exist simultaneously, involve different sets of muscles, and represent different biomechanical approaches to lung ventilation. In the savanna monitor lizard (*Varanus exanthematicus*), for example, costal breathing is employed when the animal is at rest, but while walking the lungs are ventilated, using buccal pumping as an accessory mechanism (Owerkowicz et al. 1999). Buccal pumping has also been reported in other lizard species, when the lungs become over-distended in defensive behaviour (von Saalfeld 1934) or during exposure to hypercapnia (Klein et al. 2002).

Locomotion and aspiration breathing can, however, be in conflict with one another, and therefore present a potential trade-off. Carrier (1987a, b, 1990) demonstrated that the green iguana (*Iguana iguana*) is not capable of running and breathing at the same time, thereby explaining the inability of this species to sustain activity (Mitchell et al. 1981). Many lizards, in particular scincomorphs, display a nearly complete reduction of the bronchi (Klein et al. 2005), consistent with the hypothesis that one lung may directly ventilate the other during undulating body movements, as suggested by Carrier (1991). But obviously the ability of such a mechanism to oxygenate the blood depends on the amount of oxygen stored in the central lumen of these single-chambered lungs, on lung perfusion, and on the metabolic demand. Monitor lizards show a much greater aerobic endurance than other lizards. This is only partly due to the high diffusing capacity of their lungs: the tegu lizard (*Tupinambis merianae*) has the same diffusing capacity as the savanna monitor lizards (Perry 1983) but its endurance during treadmill exercise is much less (Klein et al. 2003a).

Like the savanna monitor, many other ‘reptilian’ groups are characterised by accessory breathing apparatus that effectively preclude the breathing/locomotion trade-off. These can be divided into two categories: (1) structures that increase the efficiency of inspiration, and (2) special (apomorphic) inspiratory muscles.

4.1 Structures that Increase the Efficiency of Inspiration

Probably the oldest efficiency increasers are the gastralia: derivatives of ventral scales, which stiffen the belly wall and restrict paradoxical inward movement during inspiration. In extinct amniote groups such as ichthyosaurs, plesiosaurs, pterosaurs, 'pelycosaurs', basal therapsids, and sauropod dinosaurs, as well as in extant crododilians, turtles and the rhynchocephalian *Sphenodon*, this function persisted, whereas in theropod dinosaurs kinetic gastralia appear to have been part of an active breathing aid (Claessens 2004a; Codd et al. 2008). If gastralia are so instrumental in resolving the problem of paradoxical belly movement and the breathing/locomotion trade-off, how can we explain the disappearance of these structures in the very groups that gave rise to the most highly aerobic amniotes: mammals and birds?

To understand this paradox we must consider the mechanics of breathing, and the relationship between parenchymal distribution and the ease of inflation (compliance) of the lung. Lung compliance is inversely proportional to the work of breathing (Crossfill and Widdicombe 1961; Perry and Duncker 1980; Milsom and Vitalis 1984), and also to the degree of internal partitioning within the lung (Perry and Duncker 1978). If the distribution of internal partitioning is heterogeneous, those regions with the greatest surface-to-volume ratio will tend to collapse and will be most difficult to inflate. The one answer lies in the law of Laplace, which states that surface tension of a fluid/gas interface is inversely proportional to the radius of curvature of the gas bubble (alveoli in mammals, air capillaries in birds, and faveoli and ediculae in 'reptiles' and lissamphibians).

In most groups within the Squamata, the dorsal parts of the lungs, especially in the anterior half, are attached by a broad ligament or cohere to the curvature of the rib cage, thus mechanically preventing their collapse (Perry 1998; Wallach 1998). In snakes the situation is extreme: the left lung is reduced or absent. The right lung is completely attached to the body wall or viscera, the anterior part supporting a fine faveolar parenchyma that can extend far forward onto the trachea, while the posterior part is a parenchyma-free sack that completely lacks pulmonary vasculature (Brongersma 1957; Wallach 1998). The analogy to the avian lung-air-sac system is striking (Duncker 1971, 1978). In both groups, the biomechanical faculty is characterised by a low-work strategy (Perry 1992), whereby the extremely high compliance of the sacs results in low trans-pulmonary pressure differences that are required to inflate them, and the forces required to overcome plastic deformation and cohesion within the body cavity are much greater than the resistance of the sacs. Thus the sacs can be filled and the lungs ventilated without the need for gastralia.

In most lizards the low-work strategy also predominates. Among scincomorph lizards the internal partitioning varies greatly from highly heterogeneous in sit-and-wait predators with low metabolic rate (such as *Mabuya brevicollis*) to homogeneous in the active hunters such as tegu lizards (Klein et al. 2005). In intermediate forms such as lacertid lizards, complex septal relationships within the visceral cavity restrict movement of the viscera, and hold the lungs in place during deflation,

whereas the tegu possesses a nearly complete post-hepatic septum (Klein et al. 2000). The latter structure effectively divides the body cavity transversely into two compartments: one with lungs and liver, and another containing the remaining viscera (Klein et al. 2000). The heart lies mostly anterior to the lungs. During inspiration the post-hepatic septum prevents encroachment of the viscera into the hepatopulmonary space (Klein et al. 2003b). It is probably not coincidental that the tegu has the lowest lung compliance of any reptile measured to date (Klein et al. 2003c). The post-hepatic septum also contains copious smooth muscle, the function of which remains to be determined.

Varanoid lizards, chameleons, crododilians, mammals and many turtles possess – affixed to the ventral surface of the lung and separating it from the pericardium and liver – a postpulmonary septum, which probably originated separately in each group (Duncker 1978; Broman 1937). In all cases this septum stabilizes at least the anterior part of the lungs against the liver and secures a viscera-free compartment for them. The membranous framework of the mammalian diaphragm is derived in large part from extensions of the transverse septum that support the ductus cuvieri, but also has dorsal components originating ontogenetically in the cranial nephric fold (Broman 1937; Duncker 1978; Perry 1985). In emydid and trionicid turtles (Gräper 1931; George and Shah 1954) and in mammals, the postpulmonary septum and its derivatives contain skeletal muscle.

4.2 Special (Apomorphic) Inspiratory Muscles

The mammalian diaphragm can certainly be considered a special respiratory muscle. Rarely, however, does any structure arise anew and despite nearly a century of speculation it is still not known for sure where the diaphragm musculature originated from and what it did before mammals arose. Contrary to earlier suggestions that the sternocostal and crural parts of the diaphragm may be completely different muscles (de Troyer et al. 1981), Greer et al. (1999) demonstrated that both parts have the same origin in the first postotic somites (as also indicated by the cervical origin of the phrenic nerve), but it remains uncertain if this musculature was originally part of the hypobranchial system, the rectus system or neither. Pickering and Jones (2002) and Pickering et al. (2004) suggest that a muscle, which may be homologous to the diaphragmatic muscle, was described by (Beddard 1895a, b; Keith 1905) in the discoglossid anurans. This muscle forms a sling around the esophagus in *Xenopus laevis*, and prevents regurgitation of stomach contents during breathing. In other words, the diaphragm may have originated as part of a solution to a trade-off between aspiration breathing and feeding.

We do know, however, that the structure of the diaphragm in the most basal extant mammals (monotremes) does not differ from that in more highly derived forms (Owen 1868; Perry et al. 2000), and that due to the diaphragm, the muscular efforts of breathing and walking can be de-coupled (Boggs 2002). During galloping gait, these activities can indeed become coupled, but not as a trade-off.

Rather, extension of the spine and spreading the ribs aid diaphragmatic inspiratory effort, and the reverse is the case during expiration and vertebral flexion (Bramble and Jenkins 1993). An analogous synergistic effect of locomotor and respiratory muscles also exists in flying bats (Thomas 1981; Carpenter 1986).

Crocodilians possess a powerful diaphragmaticus muscle that retracts the liver during inspiration, and together with rib movement causes the lungs to expand into the thoracic cavity (Gans and Clark 1976; Claessens 2004b). The homolog of this muscle in other vertebrates is not known. Only its innermost location and its innervation by segmental spinal nerves suggest that it may be derived from the transversus system (Duncker 1978), although the rectus system also remains a possibility. In addition, during inspiration the ischiopubis and ischiotruncus muscles retract the mobile pubis, making room for the viscera as the lungs and liver move caudally (Farmer and Carrier 2000). Expiration involves the transversus musculature as in all tetrapods investigated to date (Brainerd and Owerkowicz 2006), and thus the diaphragmaticus muscle effectively supplements the intercostals in inspiration.

Interestingly, the compliance of the crocodilian lung is high: comparable with that of lizards such as the tokay gecko and the savanna monitor, which both have heterogeneous lungs and little or no accessory breathing musculature. In addition, the body wall compliance is low (Perry 1988). Therefore, it is unlikely that the diaphragmaticus–gastralia complex is necessary to prevent paradoxical belly wall movements and extraneous visceral displacement during breathing in extant species.

Birds are the closest living relatives to crocodilians and have no trace of a diaphragmaticus muscle. On the contrary, transverse, internal and external oblique, and rectus abdominal muscles are present in much the same configuration as seen in lizards or mammals (Zimmer 1935). Thus it remains uncertain if the diaphragmaticus muscle is plesiomorphic for archosaurs, or is an autapomorphy of crocodilians. Bird lungs are constant volume structures that are firmly attached to the vertebrae and the dorsal ribs: ventrally they are separated from the viscera by the horizontal septum and thoracic air sacs.

The main inspiratory muscles are the appendicocostals, which connect the uncinate process of each rib with the following rib or its uncinate process (Zimmer 1935; Codd et al. 2005). The uncinate process acts as a lever arm that projects from the posterior margin of the ribs, and increases the mechanical advantage for movements of the ribs and therefore sternum during respiration (Codd et al. 2005; Tickle et al. 2007). In addition, the appendicocostals can participate in postural control, and the external intercostal muscles, which usually are postural, can support forced inspiration (Fedde et al. 1964; Codd et al. 2005). The uncinates also play a role during expiration, where they act as a mechanical strut for the insertion of the finger-like projections of the external oblique musculature (Codd et al. 2005). Interestingly, the length of these processes differs in extant birds with adaptations to different forms of locomotion, suggesting the mechanics of breathing in birds may be more complex than originally thought (Codd et al. 2005; Tickle et al. 2007).

Rib movement changes the volume of the air sacs, resulting in *en passant* ventilation of the tubular parabronchi of the lungs. Since the blood flow is in general oriented perpendicular to the air movement (bidirectional in the neopulmo,

unidirectional in the paleopulmo), the gas exchange model is called 'cross-current' (Scheid and Piiper 1997). This faculty achieves greater oxygen extraction in resting birds than does the ventilated pool model in mammals (Scheid and Piiper 1970). The combination with the low work breathing strategy is synergistic: extreme heterogeneity of the lung airsac system exacerbates the efficiency both of gas exchange and of breathing mechanics.

In mammals the situation is dramatically different. Although the compliance of the body is similar to that in birds (Perry 1983), the small radius of curvature of the alveoli results in a pulmonary compliance that is several orders of magnitude lower than that of avian air sacs (Perry and Duncker 1980; Perry 1983). The evolution of mammals to high-performance aerobic animals can be explained by the coupled development of the bronchoalveolar lung and the diaphragm in the phylogenetic history of the group (Perry and Duncker 1980).

In small mammals the lungs are much stiffer than the body wall and this situation is more pronounced in newborn animals of the same size as adults of different larger-bodied species (Mortola 1987). Thus inspiratory effort in newborns would tend to collapse the abdominal wall and displace the viscera before it fills the lungs if this trade-off were not counteracted by the muscular diaphragm. It therefore follows that the bronchoalveolar lung could not have evolved in the absence of the diaphragm or its functional equivalent (Perry and Duncker 1980). This functional equivalent could have been the gastralia, which were present in basal synapsids and persisted into the Permian in anomodont therapsids (Romer 1956; Jörg Fröbisch, personal communication, 2006). Among the cynodont therapsids, however, some lines that display a pronounced rib-free lumbar region also lack gastralia. Since mammals are believed to descend from cynodonts, it has been postulated that the muscular diaphragm existed as early as the Permian (Brink 1956) and functioned as a solution to the trade-off imposed by the bronchoalveolar lung, opening the door to mammalian radiation.

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Environmental Selection Pressures Shaping the Pulmonary Surfactant System of Adult and Developing Lungs

S. Orgeig and C.B. Daniels

Abstract Pulmonary surfactant is comprised of lipids and proteins. Environmental variables — temperature, pressure and hypoxia — represent powerful evolutionary selection pressures that have shaped the evolution of the system in adult and developing lungs. We review how the composition, structure and function of surfactant changes dramatically in response to temperature. Physical forces, e.g. stretching of the alveolar basement membrane, fluid distension of the lung during development, or cyclical compression and relaxation of the lipid film also affect the secretion, maturation and physical function of surfactant. Here we also review how high hydrostatic pressures experienced by diving mammals influence the molecular, biochemical, cellular and physiological characteristics of surfactant. Also, hypoxia predominantly exerts its effect on maturation of the surfactant lipids and proteins via the endocrine system in developing organisms. However, the influence of hypoxia on the adult surfactant system is unknown. We summarise the major discoveries concerning how temperature, pressure and hypoxia have influenced the pulmonary surfactant system.

1 Introduction

The pulmonary surfactant system is a complex, integrated, highly conserved, yet dynamic system that has lent itself well to both evolutionary and mechanistic studies. The surfactant system is comprised of the alveolar epithelial type II cell which synthesises, stores and releases the complex mixture of lipids and proteins that form a dynamic lipid–protein film at the air–liquid interface of the lung, to regulate surface tension (Daniels and Orgeig 2001). This system is highly conserved among

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the vertebrates, and we have shown that it probably predated the evolution of lungs and swimbladders (Daniels et al. 2004). We have investigated the evolution of the pulmonary surfactant system from a morphological, molecular (gene, protein and lipid), cellular, developmental and biophysical perspective (Sullivan et al. 1998, 2003; Daniels and Orgeig 2001; Johnston and Daniels 2001; Daniels et al. 2004; Lang et al. 2005a; Foot et al. 2006; Orgeig et al. 2007). In particular we have concentrated on the roles of three specific environmental variables that represent powerful evolutionary selection pressures — temperature, pressure and hypoxia — in shaping the evolution of the system in adult and developing lungs. Interpreting how an environmental selection force, might select for modifications of a physiological system is made difficult by the fact that this system is composed of multiple components each of which may be affected differently by the selection force and they will also interact with each other. Moreover, the possibility of multiple physical or phenotypic effects resulting from the alteration of a single gene, a process known as pleiotropy, may further complicate the elucidation of the evolution of a particular system (Foot et al. 2006). Nevertheless, by careful selection of species and comprehensive analyses of different components, we have built up a significant body of knowledge concerning how the environment has shaped the evolution of the surfactant system in adult and developing vertebrate lungs. In this review we first provide a brief overview of the general biology of the vertebrate pulmonary surfactant system before summarising the major discoveries concerning how temperature, pressure and hypoxia have influenced the pulmonary surfactant system.

1.1 The Structure and Composition of the Pulmonary Surfactant System

Pulmonary surfactant is a complex mixture of phospholipids (PL), neutral lipids, particularly cholesterol (Chol), and proteins. The phospholipids which make up ~80% by weight of surfactant consist predominantly of phosphatidylcholine (PC). The most abundant individual molecular species in most mammalian surfactants is the disaturated phospholipid (DSP), dipalmitoylphosphatidylcholine (DPPC). The lipids and proteins are assembled in the endoplasmic reticulum and the Golgi apparatus of alveolar type II cells, and are stored in lamellar bodies until exocytosis (Fig. 1). The lamellar bodies consist of a dense proteinaceous core with lipid bilayers arranged in concentric, stacked lamellae surrounded by a limiting membrane. After the lamellar bodies have been released into the alveolar space, they swell and unravel into a characteristic cross-hatched structure, termed tubular myelin (Fig. 1). It is this structure that supplies the lipids for the surface film which regulates the surface tension of the liquid lining of the lung (Goerke 1998).

The protein component of pulmonary surfactant represents about 10% by weight, and four surfactant proteins have been described. These are surfactant protein-A (SP-A), SP-B, SP-C and SP-D, all of which are synthesised in alveolar type II cells and are associated with purified surfactant (Haagsman and Diemel 2001). Both the

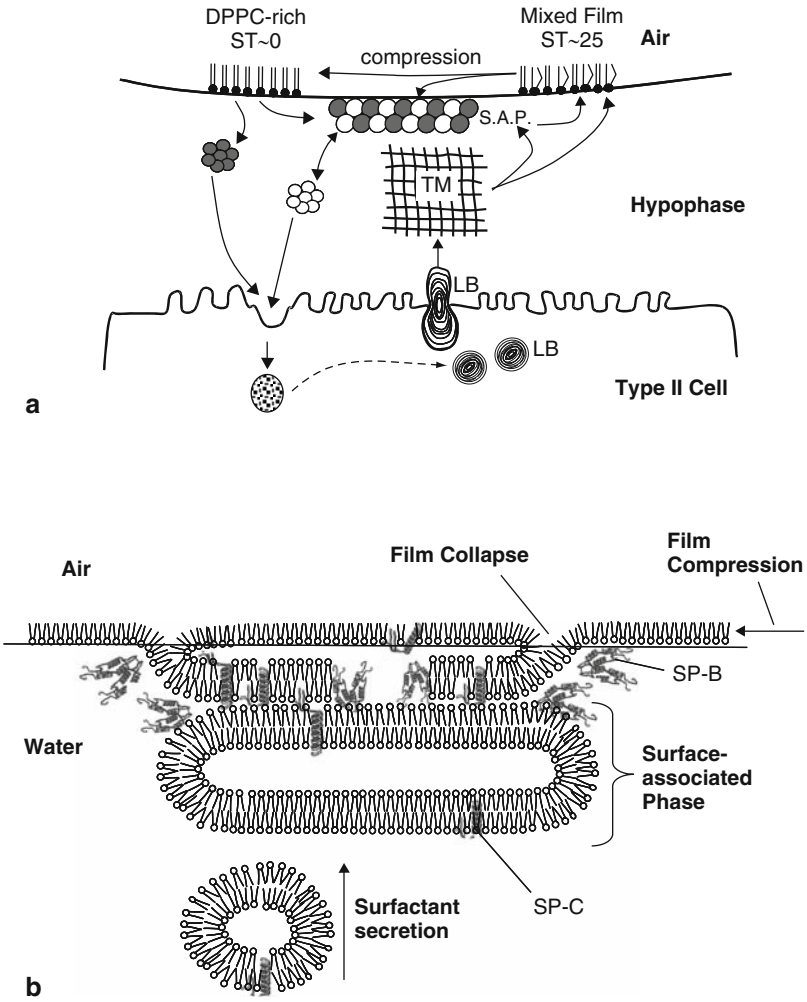

Fig. 1 **a** Schematic diagram of the life cycle of pulmonary surfactant. Pulmonary surfactant is packaged in lamellar bodies (*LB*) that are secreted into the liquid lining the alveoli (*hypophase*) via exocytosis across the type II cell plasma membrane. Here the lamellar bodies swell and unravel, forming a crosshatched structure, termed tubular myelin (*TM*), which consists of lipids and proteins. This structure supplies lipids to the surface film at the air–liquid interface as well as the surface-associated phase (*S.A.P.*), which functions as a reservoir of lipids and proteins. As the mixed molecular film is compressed, lipids are squeezed out of the film into the S.A.P. to produce a DPPC-enriched film, which is capable of reducing surface tension (γ) to near $0\,\mathrm{mN\,m^{-1}}$. Upon reexpansion, some lipids from the S.A.P. re-enter the surface film. Lipids from the surface film and the S.A.P. are eventually recycled and taken back up by the type II cell via endocytosis. **b** Hypothetical model of the surfactant film and surface-associated phase demonstrating film collapse under dynamic compression. The interaction of the two hydrophobic surfactant proteins (*SP-B* and *SP-C*) with the lipid mono- and bilayers is indicated. This interaction aids in the regulation of movement of lipids between the interfacial monolayer and the surface-associated phase. Figure reproduced from Foot et al. 2006 with permission from Elsevier

secretion and the reuptake of surfactant phospholipids into type II cells appear to be regulated by SP-A. Both SP-A and SP-B are essential for the formation and structural integrity of surfactant components. The hydrophobic surfactant proteins, SP-B and SP-C, strongly interact with the lipids and promote the formation and adsorption of the surface film to the air–liquid interface (Haagsman and Diemel 2001). They are integral to the regulation of the movement of the surfactant lipids between the surface-associated phase, the multilayer structure in the hypophase associated with the surfactant film, and the interfacial surfactant film itself (Fig. 1). Hence, they are intricately involved in the surface tension lowering function of the entire system. However, the hydrophilic surfactant proteins, SP-A and SP-D, are predominantly involved in the innate host defense system of the lung (Haagsman and Diemel 2001).

1.2 The Function of the Pulmonary Surfactant System

The main function of the pulmonary surfactant system is to reduce and vary the surface tension at the air–liquid interface. When a film is formed initially, either in vivo or in vitro, as the surface active molecules adsorb to the air–liquid interface, the surface tension is lowered from approximately $70\,\mathrm{mN\,m^{-1}}$ (surface tension, denoted γ, of pure water) to approximately $25\,\mathrm{mN\,m^{-1}}$, defined as the equilibrium surface tension (γ_{eq}). When the film is dynamically compressed, the surface area and hence the surface tension are reduced. The lowest surface tension that can be measured at the lowest surface area is the minimum surface tension (γ_{min}). The extent, expressed as a %, of surface area compression required to reach γ_{min} is known as the % surface area compression (%SAcomp).

The ability to lower and vary surface tension with changing surface area is attributed to the interactions between the disaturated phospholipids (DSP), particularly the most abundant surfactant lipid, dipalmitoylphosphatidylcholine (DPPC) and the other lipids, such as the unsaturated phospholipids (USP) and Chol. Upon expiration, dynamic compression of the mixed surfactant film results in the 'squeezing out' of USP and Chol, resulting in a DPPC-enriched surface film (Fig. 1). The DPPC molecules can be compressed tightly together by virtue of their two fully saturated fatty acid chains. In so doing, they exclude water molecules from the air–liquid interface, thereby virtually eliminating surface tension (Possmayer 2004). Hence, DPPC is regarded as the main surface active component of pulmonary surfactant.

The level to which surface tension reduction occurs in vivo depends very much on the type and function of the particular vertebrate lung (Daniels et al. 1998a; Daniels and Orgeig 2001). For example, in the mammalian lung, with its approximately spherical alveoli, the main function of the surfactant system is to promote alveolar stability by promoting equivalent transmural pressures among interconnected alveoli (Bachofen and Schürch 2001). This alveolar stability function is best served by very low surface tensions (Schürch et al. 2001). Furthermore, these low surface tensions ensure small transmural pressures which reduce the potential for the transudation of interstitial fluid into the alveolus, i.e. the formation of pulmonary edema

(Staub 1983). On the other hand, in animals with much larger and non-spherical respiratory units, e.g. lizards that have faveoli, which are much larger, relative to body size, than alveoli (McGregor et al. 1993; Daniels et al. 1994a), the predominant function of the pulmonary surfactant system is to act as an anti-adhesive (Daniels et al. 1995b,1998a), preventing the delicate epithelial surfaces from adhering to each other during the expiratory phase of the breathing cycle. For such a function it is not essential that surfactant reduces surface tension to extremely low values, as the relative forces are not as great nor the radii of curvature as small, as those in the lung of similar sized mammals (Daniels et al. 1998a). Hence, to fulfil different functions in different animals, the surfactant composition must vary between species.

1.3 The Control of the Pulmonary Surfactant System

The cellular secretion of surfactant is regulated primarily by the autonomic nervous system, via β_2-receptors and by physical forces, particularly stretch of the basement membrane of the alveolar type II cell, associated with ventilation (Mason 1998). The lung distension brought about by the accumulation of fetal lung fluid may also stimulate the production of surfactant lipids during the latter part of gestation (Torday et al. 1998). In addition, there are numerous other biochemical factors that mediate surfactant secretion, including ATP, mediated via purinergic receptors, metabolites of arachidonic acid, calcium ionophores, endothelin-1, vasopressin, lipoproteins and phorbol esters (Orgeig et al. 2004).

There are a number of mechanisms and neurohormonal factors that regulate the maturation of the surfactant system during fetal development. However, the major mechanism is via glucocorticoids which are increasingly produced by the fetal adrenal cortex during late gestation (Winter 2004). Administration in vivo of glucocorticoids to the fetus decreases respiratory distress probably by increasing surfactant phospholipid synthesis via stimulation of the synthetic enzymes and the secretion of phospholipids into the alveolar compartment (Rooney 2004). Administration of exogenous glucocorticoids also stimulates the extent and rate of surfactant protein gene expression (Orgeig et al. 2004). In addition, glucocorticoids enhance or accelerate other indicators of lung maturation, such as alveolarisation (Snyder 2004), the rate of fluid clearance (Wallace et al. 1995) and the density and expression of β-adrenergic receptors (Cheng et al. 1980; Maniscalco and Shapiro 1983). Importantly, it is possible for the expression of glucocorticoids to be altered by environmental stressors, such as hypoxia, which can affect the outcome of lung and surfactant maturation in the fetus, e.g. in growth-restricted fetuses (Orgeig et al. 2004). Other neuro-hormonal and growth factors that stimulate surfactant maturation include adrenergic agonists, thyroid hormones, thyrotropin-releasing hormone, prolactin, estrogen, leptin, parathyroid hormone, insulin-like growth factor, epidermal growth factor and keratinocyte growth factor (Rooney 2004).

1.4 Environmental Influences on the Surfactant System

The function of the pulmonary surfactant system is reliant on the physical interactions of the lipid and protein components. Hence, any environmental force that is capable of altering or influencing these interactions has the potential to exert a long-term selection effect on the pulmonary surfactant system. The three most widely examined environmental forces have been temperature, pressure and hypoxia.

Temperature has a direct and profound effect on the physical state of lipids. Specifically, all lipids can exist in one of two physical states, i.e. either the fluid, disordered (liquid-expanded) state or the ordered, solid (liquid-condensed) state. The transition between these two phases occurs at the phase transition temperature (T_m) of that lipid. In surfactant the major phospholipid, DPPC, has a T_m of 41°C. Hence, at all biologically relevant temperatures, a highly enriched DPPC film at the end of expiration will exist in the ordered gel form. However, in order for the surfactant film to spread over the expanding surface area upon inspiration, the lipids must exist in the disordered, spreadable state. The addition of other lipids, e.g. Chol or USP, into the surface film upon inspiration, lowers the T_m of the lipid mixture, enabling it to exist in the liquid-expanded state at the same body temperature. In this state, the lipids are able to disperse to coat the surface of the expanding fluid layer. Hence, the body temperature of the animal is crucial in determining the physical state of the lipids. Moreover, on an evolutionary scale, it is crucial that the selected lipid composition is appropriate for the thermal biology of the animal, so that surfactant can function optimally. For a general review of phospholipid thermal properties see Chapman (1973, 1975), and for a detailed description of the biophysical properties of pulmonary surfactant see Possmayer (2004).

Physical forces, such as stretching of the alveolar basement membrane (Edwards 2001) or distension of the lung by fetal lung fluid during development (Hooper and Wallace 2004), have profound effects on the secretion and maturation of surfactant, respectively. In addition, the regular compression and relaxation of the lung upon expiration and inspiration exert significant cyclical forces on the lipids of the surfactant film directly (Possmayer 2004). Hence, any environmental force that alters these physical forces, such as changes in the surrounding hydrostatic pressure on an organism, has the potential to alter the function of the pulmonary surfactant system (Foot et al. 2006).

The influence of hypoxia on the function of the pulmonary surfactant system is less intuitively obvious because this factor does not interfere physically with the interaction of the surfactant lipids. Hypoxia exerts its effect on the development and maturation of the surfactant lipids and proteins via the endocrine system (Orgeig et al. 2004). Hence, the effect of hypoxia is most profoundly evidenced in developing organisms, which have been widely used as experimental models.

2 Temperature

Temperature has a profound influence on the structure and function of surfactant lipids (Lang et al. 2005a). Given that the majority of animals have much lower body temperatures than homeothermic mammals, comparative biologists have questioned how animals with low and/or fluctuating body temperatures can regulate the fluidity of their surfactant film. Hence, of all the evolutionary selection pressures, temperature and its effect — short term, long term and on an evolutionary scale — on the pulmonary surfactant system has been the best documented. In the early 1980s, Kevin Keough and his students working with turtles and squirrels investigated the effect of long term (of the order of weeks) thermal changes on the pulmonary surfactant system. Chris Daniels in the late 1980s initiated our long-term research into the comparative thermal biology of pulmonary surfactant by examining the effect of relatively short-term, i.e. of the order of hours, changes in body temperature on the lipid composition of the pulmonary surfactant system in lizards. Hence, temperature has held a long-standing and enduring interest for surfactant researchers for nearly 30 years.

2.1 Temperature: A Selection Force for the Evolution of Surfactant Lipid Composition across the Vertebrates

We have previously discussed and reviewed in detail the pattern of changes in surfactant lipid composition across the vertebrate groups (Daniels et al. 1995b, 1998b; Daniels and Orgeig 2001, 2003; Lang et al. 2005a). Our interpretation of these broad-scale evolutionary changes is that it is the interaction of the body temperature preferenda together with the physicochemical interactions of the major surfactant lipids — the saturated and unsaturated phospholipids and cholesterol — which has placed evolutionary constraints upon the system and hence have driven the evolution of surfactant lipid composition. Briefly, the patterns of surfactant lipid composition across the vertebrate groups are such that, when this composition is expressed as a percentage of total phospholipid (PL), fish contain much greater levels of cholesterol (Chol) and unsaturated phospholipid (USP) than members of the other vertebrate groups, and the percentage of Chol relative to disaturated phospholipids (DSP) decreases 10- to 15-fold in the tetrapods (Daniels et al. 1995a) (Fig. 2).

Animals with lower preferred body temperatures have much higher ratios of Chol/DSP in their surfactant (e.g. fish and amphibians, generally $T_b < 25°C$) than animals with 'warm' body temperatures (some reptiles, birds and mammals, $T_b \sim 37°C$) (Daniels and Orgeig 2001) (Fig. 2). This pattern is consistent across the vertebrates, despite differences in lung structure and phylogeny, and is undoubtedly a result of the thermal and biophysical properties of the surfactant lipids. Cholesterol-rich surfactant can function at low body temperatures because cholesterol lowers the phase-transition temperature of the lipid mixture over a broad

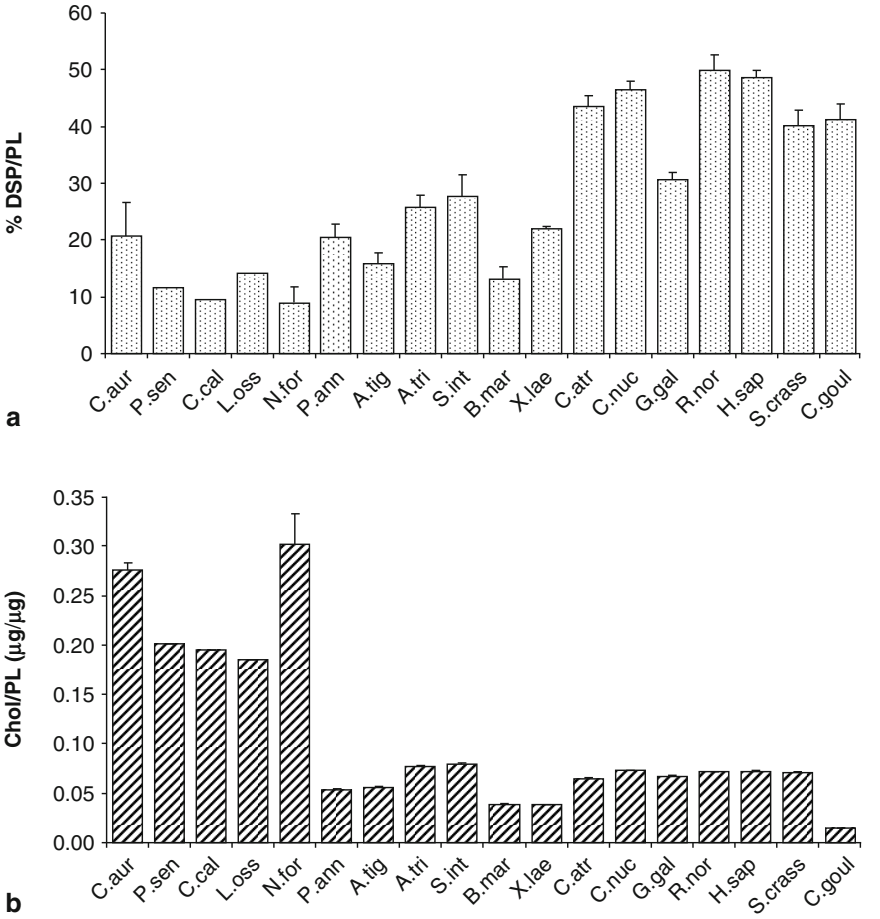

Fig. 2 Relationship of (**a**) disaturated phospholipid as a % of total phospholipid (%DSP/PL) and (**b**) cholesterol as a fraction of total phospholipid (Chol/PL, µg/µg) during the evolution of the vertebrates. Data are presented as mean ± SEM. Animals are grouped into (1) fish: *C. aur* (=*Carassius auratus*, goldfish) (Daniels and Skinner 1994), *P. sen* (=*Polypterus senegalensis*, bichir), *C. cal* (=*Calamoicthys calabaricus*, ropefish), *L. oss* (=*Lepisosteus osseus*, gar) (Smits et al. 1994), (2) lungfish: *N. for (=Neoceratodus forsteri*, Australian lungfish), *P.ann* (=*Protopterus annectens*, African lungfish) (Orgeig et al. 1995), (3) amphibians: *A. tig* (=*Ambystoma tigrinum*, tiger salamander) (Orgeig et al. 1994), *A. tri* (=*Amphiuma tridactylum*, three-toed salamander), *S.int* (=*Siren intermedia*, two-toed salamander), *B. mar* (=*Bufo marinus*, cane toad), *X. lae* (= *Xenopus laevis*, African clawed toad) (Daniels et al. 1994b), (4) reptiles: *C. atr* (=*Crotalus atrox*, rattlesnake) (Daniels et al. 1995c), *C. nuc* (=*Ctenophorus nuchalis*; central netted dragon) (Daniels et al. 1990), (5) birds: *G. gal* (=*Gallus gallus*, chicken) (Johnston et al. 2000), and (6) mammals: *R. nor* (=*Rattus norvegicus*, rat) (Orgeig et al. 1995), *H. sap* (=*Homo sapiens*, human) (Doyle et al. 1994), *S. crass* (=*Sminthopsis crassicaudata*, fat-tailed dunnart) (Langman et al. 1996), *C. goul* (=*Chalinolobus gouldii*, Gould's wattled bat) (Codd et al. 2000b). The lizard, the dunnart and the bat were at their warm-active body temperature (33–37°C)

range of temperatures and acts as a fluidiser at the air–liquid interface (Langman et al. 1996; Lopatko et al. 1998, 1999). Specifically, Chol disrupts the van der Waal's forces between adjacent PL fatty acid chains and forces the mechanical separation of PL head groups (Presti et al. 1982), which is thought to enhance adsorption and promote surfactant respreading upon inspiration (Orgeig and Daniels 2001). USPs also have much lower phase transition temperatures and can increase surfactant fluidity at low temperatures (Daniels et al. 1998a). There is, however, an evolutionary trade-off between temperature and the relative amount of saturated PL, as the addition of USP or Chol at low temperature will also decrease the surface-tension lowering ability of the surfactant mixture (Daniels et al. 1998a, b; Lopatko et al. 1998, 1999). In non-mammalian vertebrates, a decrease in surface activity is a feasible evolutionary trade-off, as pulmonary surfactant in these lungs has primarily an 'anti-adhesive' function which does not require very low surface tensions. However, in heterothermic mammals that are capable of varying their body temperature, as they alternate between periods of torpor and activity, the surfactant must be fluid at cold body temperatures, yet still remain surface-active (Lang et al. 2005a). Hence, the most interesting evolutionary question is: how do heterothermic animals — both ecto- and endotherms — regulate surfactant fluidity and surface activity during periods of activity and torpor?

2.2 Temperature: A Selection Force for Acute Changes in Surfactant Composition, Structure and Function Within Individuals

2.2.1 Surfactant Lipid Composition

Keough and colleagues (Lau and Keough 1981; Melling and Keough 1981) were the first to demonstrate that surfactant lipid composition could alter in response to relatively short-term changes in body temperature. Lau and Keough (1981) observed that surfactant collected from cold-acclimated map turtles, *Malaclemys geographica*, after 2–3 months of hibernation was less lavageable and higher in unsaturated fatty acids than surfactant from warm-acclimated turtles. Since then, our group have demonstrated that the central netted dragon, *Ctenophorus nuchalis*, doubles the Chol/PL ratio in its surfactant after a 4 h decrease in body temperature from 37°C to 14°C (Daniels et al. 1990). Hence, whether individuals increase the relative concentration of Chol or USP, we have hypothesised that these changes reduce the phase-transition temperature, thereby reflecting homeoviscous adaptations in the surfactant of these ectothermic animals. Although the actual effect of these compositional changes on the biophysical properties and behaviour of the surfactant has not been addressed in lizards, we have completed many such studies on heterothermic mammals.

In heterothermic mammals, the surfactant system is very dynamic and responds rapidly to changes in physiological conditions experienced throughout torpor and

activity (Codd et al. 2000a). Most research on the effects of temperature on the surfactant of heterothermic mammals has involved three species: the marsupial fat-tailed dunnart, *Sminthopsis crassicaudata*, the microchiropteran bat, *Chalinolobus gouldii* and the golden-mantled ground squirrel, *Spermophilus lateralis*. Each of these three model animals experiences different patterns of torpor. Dunnarts are small marsupial mammals that live in the semi-arid regions of Australia and enter short-term periods of torpor (up to 8 h) in response to food deprivation and low ambient temperatures (Godfrey 1968). In this species body temperature varies between ~35°C in warm-active dunnarts to a few degrees above ambient temperature (~15–20°C) in torpid dunnarts (Godfrey 1968). Gould's wattled bats are small insectivorous bats that are widespread and common throughout Australia. These species enter torpor spontaneously throughout the year on a daily basis for a period of a few hours even in the face of constant ambient temperatures and food availability. In this bat, body temperature varies from ~37°C during periods of activity to as little as 5°C during torpor (Hosken and Withers 1997, 1999). Ground squirrels on the other hand enter periods of hibernation during the winter, with bouts lasting several weeks and body temperatures decreasing to as low as 2–7°C (Barros et al. 2001).

Amongst these three mammal species, torpor does not appear to be accompanied by any major compositional changes in the composition of the surfactant phospholipids. Using electrospray ionisation mass spectrometry (ESI-MS), we did not observe any differences in phosphatidylcholine (PC) saturation between warm-active and torpid bats, dunnarts or squirrels. Moreover, there were no consistent changes in the molecular species composition of the major phospholipid groups, i.e. PC, phosphatidylglycerol (PG) or phosphatidylinositol (PI) (Lang et al. 2005b). Hence, neither PL saturation nor changes in molecular species composition appear to be a necessary adaptation for torpor in bats, dunnarts or ground squirrels.

However, in all three mammal species, torpor is consistently accompanied by an increase in the amount of cholesterol (Langman et al. 1996; Codd et al. 2002; Lang et al. 2005b). Only in one recent study were we unable to demonstrate the change in cholesterol with torpor in dunnarts (Orgeig et al. 2007). We analyzed the biophysical properties of the large aggregate (LA) fraction of dunnart surfactant, the complex associated with the greatest surface activity and commonly regarded as the 'active' fraction of surfactant, and did not observe changes in the Chol/DSP ratio. It is probable that the increased cholesterol observed in whole lavage is associated predominantly with the small aggregate, less surface-active component of lavage fluid, which is eliminated in the isolation of LAs.

In addition to changes in cholesterol, we have also reported that the amount of lathosterol (LaSL) and the ratio of LaSL/Chol increased significantly in lung lavage fluid isolated from torpid ground squirrels (Lang et al. 2005b). LaSL is a precursor of Chol and is commonly used as a marker of Chol synthesis or metabolism in plasma and other tissues (Larking 1999). Assuming that LaSL is a valid marker for cholesterol synthesis in torpid as well as active animals, the observed increase in LaSL/Chol may indicate that Chol synthesis is significantly higher in the lung during torpor in ground squirrels (Lang et al. 2005b). However, whether this is a common occurrence amongst heterothermic mammals is unknown.

Thus, Chol appears to be very important to the thermal dynamics of surfactant, both in a long-term evolutionary sense and during daily or seasonal changes in body temperature. Given the profound influences of cholesterol on the biophysics of lipids, it is likely that these compositional changes have profound effects on the biophysical behaviour and surface activity of pulmonary surfactant during the different metabolic states of these heterothermic mammals.

2.2.2 Surfactant Protein Composition

The effects of changes in body temperature on the amount, synthesis, behaviour and function of SP-A, SP-B, SP-C and SP-D have not been thoroughly examined. Only one study has investigated the levels of SP-A and SP-B in three species of heterothermic mammal (Lang et al. 2005b). SP-A decreased significantly in surfactants isolated from torpid dunnarts and squirrels. However, the decrease in SP-A was not observed in bats during torpor, suggesting that this response may not be a general phenomenon (Lang et al. 2005b). Due to the diverse roles of SP-A in the lung, the decrease in SP-A could be due to many different mechanisms, including the downregulation of SP-A gene expression. Moreover, low temperatures (Van Breukelen and Martin 2002) can also destabilise the hydrophobic interactions required to maintain proper protein conformation, and thus increase the potential for protein denaturation (Somero 1995). In contrast, SP-B relative to total PL did not change between surfactant isolated from warm-active and torpid mammals (dunnarts, bats and ground squirrels), and this implies that SP-B levels in warm-active animals are sufficient for adequate function at the lower temperatures, during torpor (Lang et al. 2005b). In fast breathing neonatal mammals, SP-B is increased (Rau et al. 2004), but, given that breathing parameters are reduced during torpor, and there is no increase in air–liquid interface dynamics, there would not be a requirement for more SP-B in torpid heterothermic mammals (Lang et al. 2005b). Any changes in SP-C and SP-D (if they occur at all) remain to be described.

2.2.3 Surfactant Protein Structure and Function

In order to determine the possible role of changes in surfactant protein structure on the regulation of surfactant function in animals that experience torpor, we recently tested for the presence of positive (adaptive) selection in the primary sequence of surfactant protein C (SP-C) during evolutionary transitions between heterothermy and homeothermy (Potter et al. 2007). An understanding of the evolutionary pattern of mode of body temperature regulation in mammals was required to determine the direction of selection on SP-C in relation to transitions between homeothermy and heterothermy. Using body mass as a proxy for mode of body temperature regulation, we reconstructed ancestral body masses, to enable an estimation of the pattern and number of transitions in body size during the radiation of the mammals (Potter et al. 2007). The data indicated that ancestral species were generally

$>10\,kg$, suggesting a high frequency of transitions from large homeothermic to small heterothermic species in the mammalian phylogeny. Such transitions enabled us to test for an association between positive selection on SP-C and heterothermy during the transition from large to small body size, which we hypothesised occurs due to selection for a protein that must be able to function under the more physiologically demanding conditions of variable body temperature (Potter et al. 2007). We sequenced SP-C from genomic DNA of 32 mammalian species from groups of closely related heterothermic and homeothermic species (contrasts). We used phylogenetic analysis by maximum likelihood estimates of rates of non-synonymous to synonymous substitutions and fully Bayesian inference of these sequences to determine whether the mode of body temperature regulation exerts a selection pressure driving the molecular adaptation of SP-C (Potter et al. 2007). The results from the maximum likelihood and Bayesian analyses of the *sp-c* gene revealed strong purifying selection in heterothermic and homeothermic species, indicating that constraints are placed on the evolution of SP-C to maintain protein function in surfactant despite variation in mode of body temperature regulation (Potter et al. 2007). The protein sequence of SP-C is highly conserved, with synonymous or highly conservative amino acid substitutions being predominant (Fig. 3).

The evolution of SP-C among mammals is characterised by high rates of transition/transversion and by high codon usage bias. As transitions involve purine–purine or pyrimidine–pyrimidine substitutions (Zhang 2000), they lead either to more synonymous nucleotide substitutions or conservative amino acid substitutions. Similarly, a high codon usage bias suggests that substitutions of amino acids in SP-C are favoured by conservative amino acid replacements. Amongst all the heterothermic/homeothermic contrasts analysed, the only contrast to show evidence of positive selection was that of the bears (*Ursus americanus* and *U. maritimus*). The significance of this result is unclear (Potter et al. 2007). Hence, there is insufficient evidence to suggest that the mode of body temperature regulation has influenced the evolution of SP-C in mammals. Rather, the protein is under strong evolutionary constraints, i.e. purifying selection, to maintain surfactant function despite the variability in mode of mammalian body temperature regulation (Fig. 3). Moreover, in terms of the ability of surfactant from heterotherms to regulate surfactant function under torpid conditions, modulation of SP-C primary sequence does not appear to be the answer. Given the functional redundancy of SP-C and SP-B, it is possible that there are adaptations in the SP-B primary sequence in heterothermic species. Alternatively, it may be possible that there is a differential regulation of the level of SP-C expression in torpid versus warm-active individuals (Potter et al. 2007). This remains to be tested.

2.2.4 Surfactant Biophysical Function During Activity, Torpor and Arousal

As in homeothermic mammals, decreases in body temperature in heterothermic mammals are associated with decreases in tissue elasticity and thus, decreases in lung tissue compliance, at least in the early stages of torpor (Langman et al. 1996).

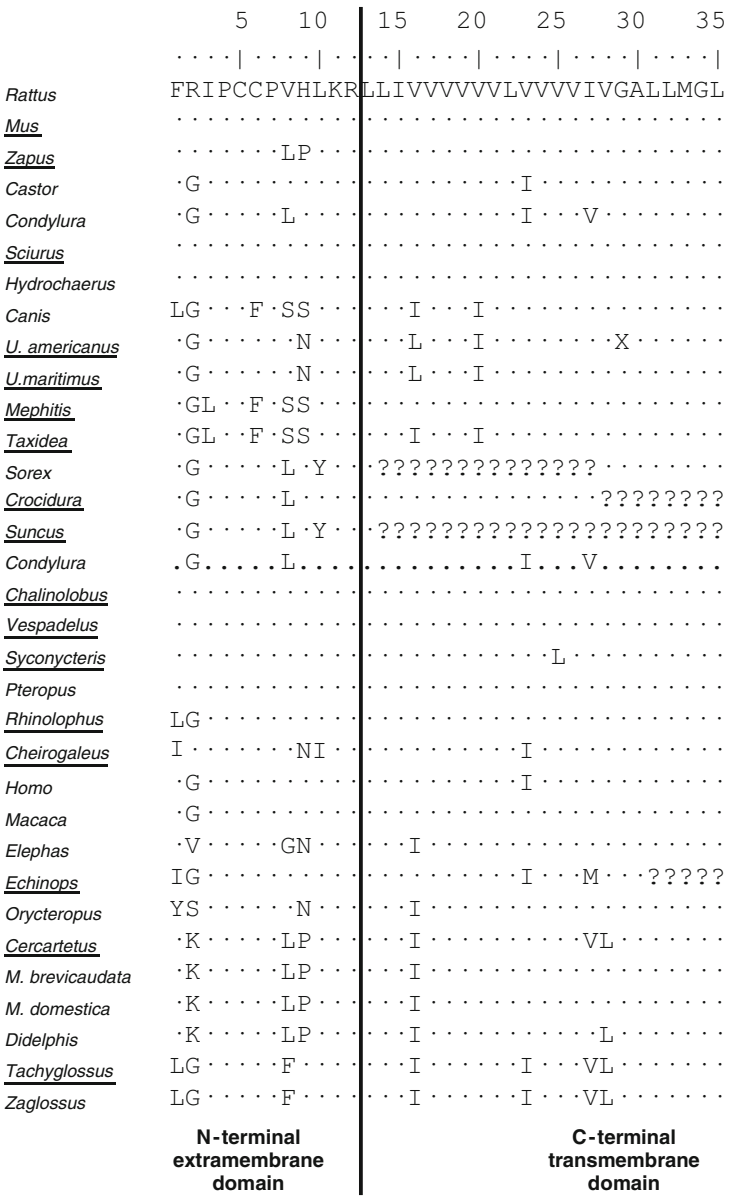

Fig. 3 Aligned amino acid sequences of the mature SP-C protein inferred from the translation of the nucleotide sequences published in Potter et al. (2007). The genus names are listed, except in the case of *U. americanus* and *U. maritimus*, for which the genus is *Ursus* and in the cases of *M. brevicaudata* and *M. domestica*, where the genus name is *Monodelphis*. Heterothermic species are *underlined*. Amino acid position is indicated by the numbers along the *top* of the figure. The *black vertical line* indicates the boundary between the N-terminal extramembrane and the C-terminal transmembrane domains at amino acid position 12. *Dots* indicate conserved amino acids relative to the first sequence, i.e. *Rattus*. *Question marks* indicate residues that were not obtained. '*X*' at site 29 in *Ursus americanus* represents heterozygosity for the amino acids arginine (*R*) or glycine (*G*) (Foot et al. 2007). Figure reproduced from Potter et al. 2007 with permission from Elsevier

However, in order to maintain high lung compliance and hence maintain the work of breathing at a minimum, the surface activity of surfactant must increase to counteract the lower tissue elasticity. Therefore, heterothermic mammals must optimise their surfactant at low temperatures to decrease surface tension in the lung and thus reduce the work of breathing. The fluidity of surfactant must also be maintained at cold body temperatures and this can only be achieved by lowering the phase transition temperature of the lipid mixture, and thus, changing the composition of the surfactant mixture.

We have consistently shown, with a variety of techniques and in both bats and dunnarts, that the in vitro surface activity of surfactant is altered such that it is optimal at that in vitro temperature which matches the body temperature of the animal from which the surfactant was isolated, i.e. whether isolated from either warm-active or torpid animals (Lopatko et al. 1998, 1999; Codd et al. 2002, 2003; Orgeig et al. 2007). These findings are summarised in Table 1. For example, when surfactant from active and torpid Gould's wattled bats were analysed on a captive bubble surfactometer (CBS) at a temperature matching the body temperature of the bat, γ_{eq} of 25 mN m^{-1} and γ_{min} of 1 mN m^{-1} were achieved (Table 1). These values are similar to the literature values for other mammals (Goerke and Clements 1985; Codd et al. 2002). Adsorption was significantly slower when surfactant from active bats was analysed at 24°C compared to 37°C. Conversely, surfactant from torpid bats demonstrated much faster adsorption at 24°C compared with 37°C. Quasi-static and dynamic cycling of surfactant from active bats at 37°C yielded a lower γ_{min} and required a smaller %SAcomp to reach γ_{min} than when measured at 24°C (Table 1). Conversely, surfactant from torpid bats reached a lower γ_{min} and required less %SAcomp to reach low γ_{min} at 24°C than at 37°C (Codd et al. 2002) (Table 1). Hence, in heterothermic mammals, surfactant from active (warm) animals appears to be more suited to function at higher temperatures (37°C) and surfactant from torpid (cold) animals appears to function better at lower temperatures (Lang et al. 2005a).

In addition, we have demonstrated very rapid and precise adjustments in surface activity during arousal from torpor in both bats (Codd et al. 2003) and dunnarts (Lopatko et al. 1999). Arousal from torpor by heterothermic mammals can be very rapid, with rates of 0.7–1°C per minute (Geiser and Baudinette 1990) recorded for dunnarts and up to 0.81°C per minute (Codd et al. 2000a) recorded for bats. Surfactant isolated from bats arousing from torpor (T_b = 28–32°C) adsorbs much faster at 37°C than at 24°C and functions optimally at 37°C (as indicated by a decrease in γ_{min} and %SAcomp) (Codd et al. 2002) (Table 1). Thus, surfactant from heterothermic mammals undergoes rapid changes in surface activity and lipid composition that enable the mixture to function effectively at rapidly increasing body temperatures (Lang et al. 2005a).

2.2.5 Surfactant Film Structure

Recently we have begun an examination of the film structure of surfactants isolated from torpid and warm-active surfactant (Orgeig et al. 2007). We discovered

that differences in film behaviour seen on the Langmuir balance at 23°C were matched with significant differences in film structure as assessed by epifluorescence microscopy of doped phospholipid films and atomic force microscopy (AFM) (Orgeig et al. 2007). Specifically we demonstrated unstable phase partitioning (i.e. coexistence of ordered and disordered phases) in surfactant from warm-active dunnarts suggesting that there was a perturbation to the packing of the surfactant film (Fig. 4a). On the other hand, under the same in vitro conditions, surfactant from torpid dunnarts demonstrated phase coexistence throughout film compression up to $30\,mN\,m^{-1}$, indicating stable packing into liquid-condensed regions (Fig. 4b). Moreover, the area of coverage of probe-excluded liquid-condensed regions increased throughout compression and was greater than for surfactant from warm-active dunnarts (Fig. 4c). Atomic Force Microscopy (AFM) confirmed the presence of large circular liquid-condensed domains in surfactant from torpid dunnarts with a mean diameter approximately 5 times greater than those found in surfactant from warm-active dunnarts (Fig. 4d). The height profiles of both types of domains were similar to each other and similar to those that would be obtained with a pure DPPC film (Panda et al. 2004), thereby supporting the fluorescence data by concluding that these were liquid-condensed, DPPC-enriched domains.

Hence, in terms of film structure and behaviour during compression, surfactant from torpid dunnarts was more effective at 23°C than surfactant from warm-active dunnarts (Orgeig et al. 2007). In general, the function and structure of surfactant films from torpid dunnarts were more similar to those expected of natural surfactant isolated from traditional mammalian models that are classed as 'good' surfactants. As these studies were performed at 23°C, a temperature more similar to the body temperature of torpid dunnarts, this supports our previous finding that surfactant function is optimised to function best at that temperature at which it was isolated from the animal. However, future structural studies performed at in vitro temperatures that match the in vivo temperatures are required to confirm this hypothesis (Orgeig et al. 2007).

3 Pressure

We have recently investigated the possibility that high hydrostatic pressure can act as an evolutionary selection pressure resulting in specific adaptations to the surfactant system. Hence, we explored the molecular, biochemical, cellular and physiological characteristics of the surfactant system of diving mammals to determine whether it possesses specific characteristics (adaptations) to cope with the repeated collapse and re-inflation of the lung that occur during diving, in order to provide resistance against the compressive effects of the high hydrostatic pressures (Miller et al. 2004, 2005, 2006b; Miller 2005; Foot et al. 2007). We analysed each component and organisational level of surfactant individually for adaptations (Foot et al. 2006). This series of studies is presented here as an integrated case study for the effect of pressure on all aspects of the pulmonary surfactant system.

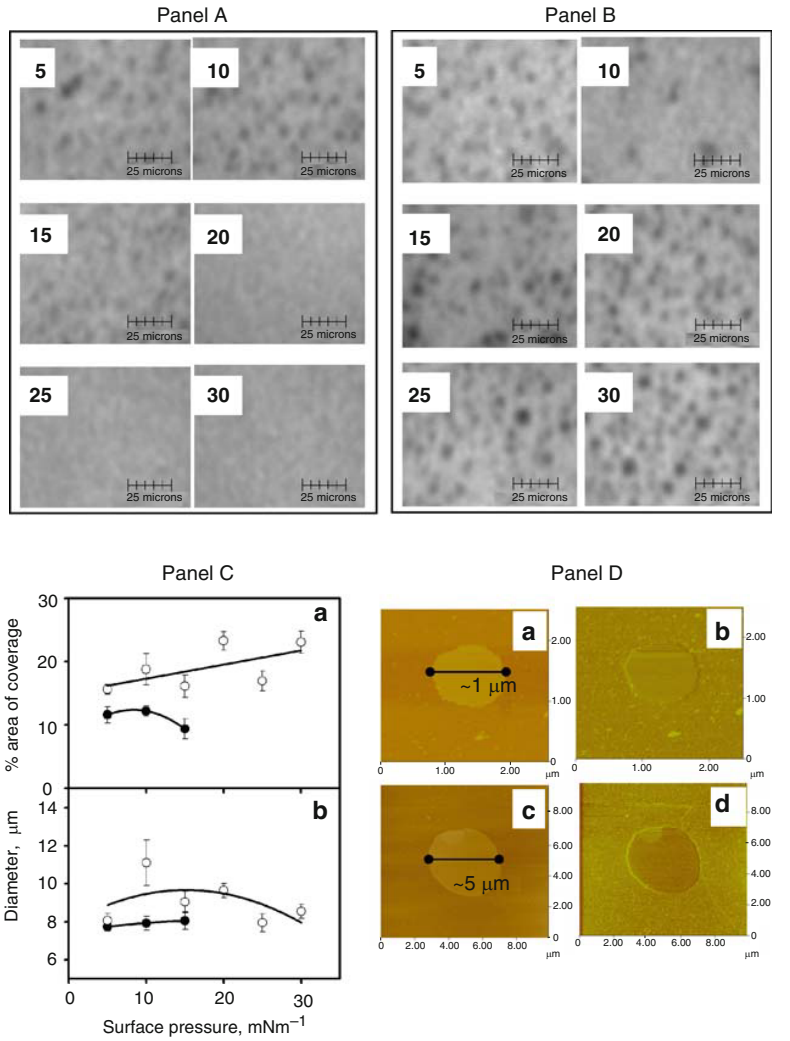

Fig. 4 Fluorescence images taken with a Karl Zeiss epifluorescence microscope of solvent-spread surfactant films containing 1 mol% of the fluorescent PL probe NBD-PC (1-palmitoyl-2-[12-{(7-nitro-1,3-benzooxadiazol-4-yl) amino}dodecanoyl]-sn-glycero-3-phosphocholine) from warm-active (*Panel A*) and torpid (*Panel B*) dunnarts, compressed on a Langmuir-Wilhelmy balance at 23°C to 5, 10, 15, 20, 25 and 30 mN/m surface pressure (indicated inside the images). Subphase: 0.15 M NaCl, 1.5 mM $CaCl_2$, 1.0 mM TRIS-HCl buffer at pH = 7.0. *Dark domains* represent liquid-condensed probe excluded domains. *Panel C*: Quantitative fluorescence image analysis of solvent-spread surfactant films of warm-active (*solid symbols*) and torpid (*open symbols*) dunnarts at different surface pressures. **a** Percentage of area of coverage of the probe-excluded regions. **b** Diameter (μm) of the probe-excluded regions. *Panel D*: Atomic force microscopy images of solvent-spread surfactant films from warm-active (*a*,*b*) and torpid (*c*,*d*) dunnarts at 30 mN m^{-1} surface pressure. The films were transferred onto freshly cleaved mica by the Langmuir–Blodgett transfer technique. Area of scan: *a*,*b* 2.5 × 2.5 μm^2; *c*, *d* 10 × 10 μm^2. *a* and *c* were taken in height mode (the *lighter areas* indicate a greater height profile), while *b* and *d* were taken in phase mode. *LE* liquid expanded phase; *LC* liquid condensed phase. Figure reproduced from Orgeig et al. 2007 with permission from Oxford University Press

3.1 Selection at the Molecular Level

We tested the hypothesis that sequence variations in the SP-C protein of marine mammals have resulted in functional adaptations to cope with the repeated collapse and reinflation of the lung during diving. We examined the ratio (ω) of non-synonymous (amino acid-changing, d_N) to synonymous (silent, d_S) substitution rates among nucleotide and inferred amino acid sequences of the SP-C gene (*sp- c*) (Foot et al. 2007). An excess of non-synonymous over synonymous nucleotide substitutions indicates positive selection (Nielsen 1998; Yang et al. 2000). We also examined the biophysical properties that are associated with the amino acid substitutions in the diving lineages (Foot et al. 2007). To control for lack of phylogenetic independence, we made the comparisons in a series of independent mammal contrasts consisting of semi-aquatic species (i.e. can forage in water, but do not usually dive to depths of more than 5 m) or diving species (i.e. can dive deeper than 70 m) and their nearest terrestrial relatives (i.e. do not forage in water).

SP-C is a highly conserved protein with significant selection constraints, but there was evidence of positively selected sites particularly in the N-terminal domain of SP-C in diving mammals (Foot et al. 2007) (Fig. 5a). Using phylogenetic analysis by maximum likelihood (Yang 1997), site models strongly identified positive selection at different sites in the polar N-terminal extramembrane domain of SP-C in the three diving lineages: site 2 in the cetaceans (whales and dolphins), sites 7, 9 and 10 in the pinnipeds (seals and sea lions) and sites 2, 9 and 10 in the sirenians (dugongs and manatees) (Fig. 5a). Analysis of the biophysical properties that were influential in determining the amino acid substitutions showed that isolelectric point, chemical composition of the side chain, polarity and hydrophobicity were the crucial determinants. Particular sites that demonstrate evolutionary lability are sites 2 and 10, at which there is a tendency for more polar and/or more charged residues, as they are involved in polar interactions with the hydrophilic head groups of the phospholipid layer (Fig. 5b). Stronger binding of the N-terminal domain to the phospholipid layer would be highly desirable during the extreme compression of the lung that occurs during deep diving. The improved binding would prevent squeeze-out of the more fluid USP and neutral lipids, e.g. Chol, which is presumed to occur during normal lung compression on expiration (Fig. 5b). The removal of these lipids from the monolayer results in an enrichment in DSP and increases the surface activity of the monolayer, which is important in preventing the adhesion of respiratory surfaces in terrestrial mammals. However, a monolayer of pure DSP does not respread easily upon reinflation. For the true diving mammals, complete lung collapse is essential for diving. Hence, a more fluid lipid layer may be critical for the rapid reinflation of the lungs during the short excursions to the water surface. In addition, at site 9 there is a tendency for more hydrophobic residues particularly in the pinnipeds, as this site may be involved in hydrophobic interactions with the palmitoylated cysteines (Fig. 5b) and may enable SP-C to adsorb more readily to the air–liquid interface. Such a property would be beneficial when the lungs need to be reinflated rapidly upon resurfacing after a dive. Hence, positive selection in the N-terminal domain

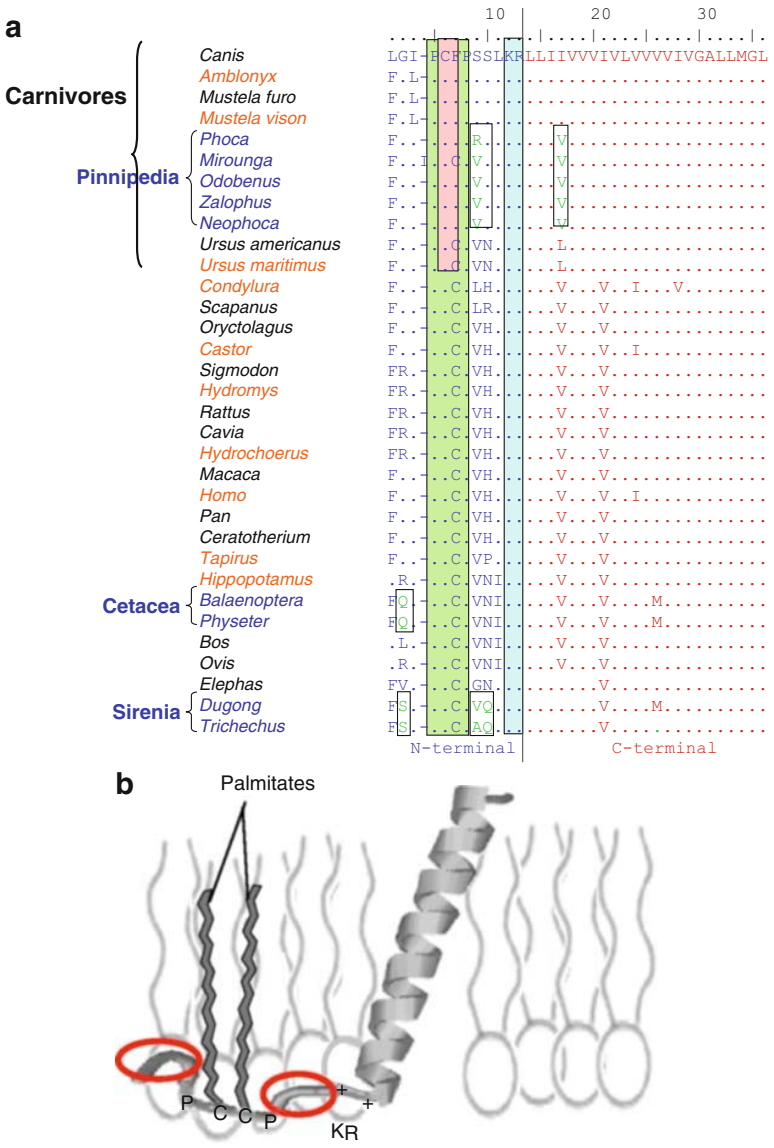

Fig. 5 **a** Aligned amino acid sequences of the mature SP-C protein inferred from translation of the nucleotide sequences published in (Foot et al. 2007). Species indicated in *black* are terrestrial, in *orange* are semi-aquatic and in *blue* are divers. *Dots* indicate conserved amino acids between the sequence of interest and *Canis*. *Dashes* indicate an alignment gap due to the insertion of an extra amino acid in *Mirounga*. Note that due to this insertion, the sequences listed in this figure have one additional amino acid (total 36), and the amino acid position number (from position 4 onwards) is greater by one amino acid relative to the sequences listed in Fig.3. The *light blue box* indicates the two positively charged amino acids, lysine and arginine at the boundary of the N- and C-terminal domains. The *light green box* indicates the PCCP motif, which in the case of the carnivores is a PCFP domain (*pink box*). Amino acids indicated in *light green* with a *black box* surrounding them

of SP-C in diving mammals may reflect adaptations to the repeated collapse and reinflation of the lung upon diving and resurfacing (Foot et al. 2007).

3.2 Selection at the Compositional Level

We hypothesised that the composition of both lipids and proteins will be different in diving mammals compared with terrestrial mammals, to enable the rapid and potentially traumatic expansion of the collapsed lung. We compared the lipid and protein composition of surfactant from different pinniped species (California sea lion, Northern elephant seal and Ringed seal) with those of surfactant isolated from similar-sized terrestrial mammals (cow and sheep) (Miller et al. 2006b). In addition we hypothesised that there will be selection during development, with changes seen in the composition of the surfactant system of a diving mammal as it develops from a terrestrial pup to an aquatic adult. To test this hypothesis we analysed the composition of adult and newborn surfactant from California sea lions (Miller et al. 2005).

3.2.1 Phospholipids and Cholesterol

The relatively minor differences in the phospholipid classes included a reduced level of the anionic surfactant phospholipids, PG and PI, in diving mammals compared with terrestrial mammals (Miller et al. 2006b) (Table 2). Given the potentially

Fig 5 *(Continued)* are those that were identified as being under positive selection in the site models. Positively selected amino acids are only indicated in the diving species of each contrast. Figure reproduced from Foot et al. 2006 with permission from Elsevier. **b** Schematic diagram of an SP-C molecule in a surfactant phospholipid film. The α-helix within the C-terminal transmembrane domain is embedded at an angle in the phospholipid fatty acid tails, and the N-terminal extramembrane domain is associated with the hydrophilic phospholipid head groups. The two palmitate groups that are covalently linked to two cysteine residues (*C*) are anchored within the phospholipid fatty acid tails. On either side of the cysteine residues is a proline residue (*P*) which changes the orientation of the protein chain, enabling correct orientation of the palmitates and the N-terminal segment with the head groups. The *two plus signs* indicate two positively charged residues, Lys (*K*) and Arg (*R*) at the boundary between the more polar N-terminal segment and the hydrophobic C-terminal segment. The *two red circles* indicate the locations of site 2 and sites 9 and 10, which are evolutionarily labile and are under positive selection in the diving mammals. In cetaceans and sirenians, the charge and polarity of these residues is important, possibly leading to stronger binding of the N-terminal tail to the phospholipid headgroups, leading to greater stability of the lipid–protein complex during the high compression forces during diving. In pinnipeds, the hydrophobicity at sites 9 and 10 is crucial, possibly leading to greater interactions with the palmitic acid residues linked to the cysteines (*C*), and possibly aiding in the adsorption of SP-C to the air–liquid interface upon resurfacing after a dive. Figure reproduced from Foot et al. 2006 with permission from Elsevier

destabilizing forces that diving mammal lungs are exposed to, the reduction in PG and PI would appear counterintuitive. However, a reduction in the anionic phospholipids may also lead to an impaired surface activity, which may be better suited to the hypothesised anti-adhesive function of surfactant in these animals. In relation to development within the California sea lion we found that PI is dominant in newborn animals, whereas PG becomes dominant in mature animals, a scenario commonly reported during development of other mammalian species (Hallman and Gluck 1975; Benson et al. 1983; Egberts et al. 1987). In addition, surfactant from the newborn California sea lion was even lower in total anionic phospholipids (i.e. PG + PI) than that from the adult, i.e. 2.9% and 6.8%, respectively.

There were no differences in surfactant phospholipid saturation between diving mammals and their terrestrial counterparts (Miller et al. 2006b). However, surfactant fluidity may also be increased by decreasing the fatty acid chain length (Chapman 1975). Indeed, we discovered that the molecular species of the major phospholipid class, PC, demonstrated a greater percentage of short chain fatty acids (PC16:0/14:0, PC16:0/16:1 and PC16:0/16:0), and a corresponding decrease in longer chain molecular species (PC16:0/18:1) in three species of diving mammal compared with the terrestrial species (Miller et al. 2006b) (Table 2). Similarly, Spragg et al. (2004) demonstrated an increase in fluidic phospholipid species in pinnipeds compared with human and pig surfactant. Hence, this could potentially indicate a diving adaptation in the marine mammals, serving to increase fluidity and therefore enabling surfactant to perform an anti-adhesive function during lung collapse (Miller et al. 2006b).The cholesterol levels were highly variable between diving species, which may be related to the dynamic role of cholesterol (Orgeig 2001) (Table 2). In relation to development within the California sea lion, we described higher levels of the short chain molecular species, PC16:0/14:0, in the adult and an increase in cholesterol relative to the newborn (Miller et al. 2005). These changes in conjunction with the increase in the anionic phospholipids would increase the fluidity of surfactant in the adult, allowing for rapid adsorption to the air–liquid interface, but would result in a poorly surface-active material.

3.2.2 Surfactant Proteins

There were no differences in SP-A levels relative to either total phospholipid or total protein between any of the marine and terrestrial species (Miller et al. 2006b) (Table 2). Similarly, Spragg et al. (2004) reported no significant differences in SP-A between elephant seals and humans. As SP-A does not have a significant role in regulating the biophysical function of surfactant, but is instead involved in host defence functions (Haagsman and Diemel 2001), this finding is not surprising. Although not consistent across all marine mammal species, there was a tendency for a reduced amount of SP-B in the surfactant of diving mammals (Foot et al. 2006) (Table 2). It is likely that the low SP-B levels in some pinniped species may be responsible for the low surface activity and poor equilibrium surface tensions that we have observed (see below). On the other hand, Spragg et al. (2004) observed an increase in SP-B

in elephant seal surfactant relative to human surfactant when analysed by densitometric scanning of electrophoretic gels. However, when analysed by immunologic methods, as in the study by Miller et al. (2006b) and normalised to protein or phospholipid, there was no difference in either SP-B or SP-C (Spragg et al. 2004). Of the three diving species analysed by Miller et al. (2006b), elephant seal also demonstrated the highest levels of SP-B. Hence, it appears that differences in surfactant proteins are not consistent across all marine mammals.

In relation to development within the California sea lion, we described a higher level of SP-B in surfactant from the adult compared with the newborn (Miller et al. 2005). As SP-B functions to facilitate respreading and reformation of the surface film (Possmayer et al. 2001), an increased concentration of SP-B, especially in conjunction with the elevated PC16:0/14:0 and the elevated anionic phospholipids, may ensure that the collapsed surfactant film may respread more easily on lung expansion, a function required by the diving adult, but not the terrestrial newborn.

3.3 Selection at the Functional Level

The function of surfactant must be tailored to the unusual lung structure and breathing mechanics of diving mammals. In marine mammals, the lungs undergo complete collapse during deep dives (Kooyman 1989), resulting in epithelial surfaces coming into contact for extended periods of time. We hypothesised that diving mammals require predominantly an anti-adhesive pulmonary surfactant to prevent adherence of alveolar surfaces following lung collapse. In addition, the surfactant monolayer needs to rapidly reform over the hypophase for efficient lung function. Both of these properties require a highly fluid surfactant mixture, which would demonstrate increased spreadability and adsorption to the air–liquid interface. However, simultaneously such a mixture would demonstrate reduced surface activity compared with terrestrial mammals, as surface tension must only be reduced sufficiently to prevent adhesion of epithelial surfaces, instead of the very low surface tensions that are required to stabilise minute alveoli during a regular inflation/deflation cycle. To further strengthen this hypothesis we analysed the biophysical function of adult and newborn surfactant from California sea lions (Miller et al. 2005) to determine whether there are changes in the function of the surfactant system of a diving mammal as it develops from a terrestrial pup to an aquatic adult.

Supporting this ‘anti-adhesive’ hypothesis drawn from Kooyman’s analysis we discovered that the surfactants of Northern elephant seal, Northern fur seal and Ringed seal were unable to reach typical equilibrium surface tension (γ_{eq}) values ($\sim 25\,mN\,m^{-1}$) after 5 min adsorption (Miller et al. 2006a) (Table 3). However, surfactant from the California sea lion was able to reach the levels of the cow and sheep ($<25\,mN\,m^{-1}$). None of the pinnipeds were able to obtain the very low minimum surface tensions (γ_{min}) achieved by cow ($<2\,mN\,m^{-1}$) (Table 3). Hence, reducing surface tension to very low values is not likely to be the primary

function of surfactant in pinnipeds as it is in terrestrial mammals, but rather a highly fluid anti-adhesive surfactant is likely to be more important to enable the lungs to reopen following collapse during deep diving. These conclusions are further supported by the findings of differences in biophysical function between newborn and adult California sea lions. Although, relative to terrestrial mammals, both adult and newborn surfactant from California sea lions display poor surface-activity (Miller et al. 2006a), it appears that the adult surfactant has better adsorptive properties, as γ_{eq} is lower compared with surfactant from the newborn (Miller et al. 2006a). These increased adsorptive properties would be required for an anti-adhesive type of surfactant and may be better suited to cope with regular lung collapse of diving adults. On the other hand, newborn surfactant had better surface tension lowering properties (Miller et al. 2006a), which appear to be better suited for an alveolar stability function and may reflect the type of tidal volume breathing that occurs in terrestrial mammals.

There are a number of chemical contributors to the poor surface activity in pinniped surfactant, including the overall reduction in the anionic phospholipids, PG and PI, the reduction of SP-B in some pinniped species and an increase in the short chain phospholipid molecular species, PC16:0/14:0 and PC16:0/16:1. All these factors may help explain the very poor surface activity (i.e. γ_{eq} and γ_{min}) and the high surface area compressions that are required to reduce surface tension to γ_{min} (Miller et al. 2006a). Moreover, the relatively greater adsorptive properties of the adult (i.e. the lower γ_{eq} values) compared with the newborn may be explained by the higher level of anionic phospholipids (i.e. PG + PI), the higher levels of the short-chain molecular species, e.g. PC16:0/14:0, higher levels of SP-B, and an increase in cholesterol. These changes may ensure rapid film reformation after lung collapse in the diving adult. On the other hand, these changes would result in a poorly surface-active material, as indicated by the relatively higher γ_{min} compared with the newborn.

Hence, while one particular compositional change in itself may not be highly significant, it is the combination of all of these changes (e.g. lipid and protein composition, as well as protein sequence and structure) that contribute highly significantly to changes in surfactant function in diving compared with terrestrial mammals. Finally, the surfactant of newborn California sea lions is more similar to that of terrestrial newborn mammals, whereas the adult has a ‘diving mammal’ surfactant that can aid the lung during deep dives. Perhaps as the young animals enter the water and begin to forage (and hence dive) for themselves, their surfactant system develops to function more efficiently under these different physiological circumstances.

4 Hypoxia

Despite the fact that hypoxia has significant health consequences, either in adults that ascend to high altitudes, or in the fetus that develops in a compromised uterine environment, the influence of hypoxia on the function of the pulmonary surfactant

system either in adults or during development has not been widely addressed. There is a range of available experimental animal models and experimental protocols that can be utilised to test the effect of hypoxia on the surfactant system. Here we summarise the sketchy information that is available on the role of hypoxia in shaping the pulmonary surfactant system of adult and developing lungs.

4.1 Hypoxia and Altitude: Effects on the Adult Pulmonary Surfactant System

Several studies have examined the effect of hypoxia on lung mass and lung growth, as well as lung function in terms of compliance and alveolar fluid clearance, either in adult or young postnatal animals. In general, hypoxia stimulates lung growth (Sekhon and Thurlbeck 1995, 1996) and lung mass (Hammond et al. 2001), and impairs transalveolar fluid transport (Suzuki et al. 1999) and lung compliance in newborn rats following prenatal hypoxia exposure (Cheung et al. 2000). On the other hand, the information available on the effect of hypoxia on the adult surfactant system, either natural hypoxia at altitude, or induced experimentally, is extremely scant. Acute hypoxia induced experimentally leads to changes in the surfactant lipids (Liamtsev and Arbuzov 1981), including a decrease in phosphatidylcholine (Prevost et al. 1980; Zaitseva et al. 1981) and an increase in lysophosphatidylcholine (Zaitseva et al. 1981) and lysocompounds (Prevost et al. 1980) with a concomitant increase in phospholipase activity (Prevost et al. 1980). Surface activity is impaired (Liamtsev and Arbuzov 1981; Zaitseva et al. 1981; Belov et al. 1985), and edema of the interalveolar septa can occur (Zaitseva et al. 1981). While PC levels of alveolar surfactant decreases, the incorporation of ^{32}P into lung PC and PE increased (Chander et al. 1975), suggesting that the reduced alveolar content may be due to changes in secretion and/or local inactivation, and not due to synthesis (Orgeig et al. 2004).

One interesting but poorly researched area is the response of the surfactant system to chronic hypoxia caused by altitude. Rats raised at an altitude of 3,500 m demonstrated a reduction in the number of macrophages and in the amount of alveolar and lung tissue phospholipid, especially PC (Hegde et al. 1980). Llamas on the other hand, born at an altitude of 4,720 m, demonstrated numerous prominent Clara Cells with large 'apical caps', many of which had been extruded into the terminal bronchioles (Heath et al. 1976). Although the extruded material was not analysed, it is possible that the hyperactivity of the Clara Cells was an adaptive response to chronic hypoxia (Heath et al. 1976). These tantalizing snippets of information should encourage future research into this area (Orgeig et al. 2004).

4.2 Fetal Hypoxia and Growth Restriction: Effects on the Pulmonary Surfactant System in Mammals

Fetal growth restriction (FGR) is a major cause of low birthweight infants and increases the risk of respiratory distress and death in both term and preterm infants

(Tyson et al. 1995; Minior and Divon 1998). Causes of FGR include maternal undernutrition, hypertension, anemia, placental infarction and tobacco smoking. Growth-retarded fetuses suffer from fetal hypoxemia and hypoglycemia, elevated levels of circulating catecholamines and cortisol (Nicolaides et al. 1989; Gagnon et al. 1994; Harding et al. 2001), and decreased levels of insulin-like growth factors and their binding proteins (Owens et al. 1994).

It is generally accepted that in the normal fetus during late gestation, activation of the hypothalamo-pituitary axis (HPA) leads to an increase in the basal levels of adrenocorticotropic hormone (ACTH) and circulating cortisol which functions to facilitate lung maturation (Gross and Ballard 1997). Since the observation by Liggins that fetal exposure to glucocorticoids induced premature delivery and his proposal that glucocorticoids specifically induce surfactant synthesis (Liggins 1969), there has been a plethora of studies investigating the timing, dosage and mechanisms of this pathway (reviewed by Jobe and Ikegami 2000). Although there have been many conflicting results owing to differences in timing, dosage, species choice and whether it is the fetus or the mother that is treated, it does appear that antenatal glucocorticoid treatment reduces the incidence of respiratory distress syndrome by $\sim$50%; it improves fetal lung mechanics after very short treatment-to-delivery times and changes the surfactant system, but only after more prolonged and multiple dosage treatments (Jobe and Ikegami 2000).

Early studies indicated the possibility that growth-restricted infants demonstrated accelerated lung maturation (Gross et al. 1981), suggesting that they may be at lower risk of respiratory distress. As cortisol is increased during periods of physiological stress, such as fetal hypoxemia, it is possible that this represented the mechanism for the stimulation of lung maturation. However, other studies since then have shown that there is no evidence that infants that have been stressed by events such as fetal growth retardation or pre-eclampsia demonstrate a lower incidence of RDS (Tyson et al. 1995; Jobe and Ikegami 2000). However, in addition to such multivariate epidemiological studies, many different experimental animal studies have been performed, using both different species (e.g. sheep, rabbits, guinea pigs, mice) and experimental protocols (placental restriction, undernutrition, knockout) to induce FGR (reviewed by Jobe and Ikegami 2000). Of these only a relatively small number of studies have concentrated on lung and surfactant function, and these have yielded conflicting information.

The hypothesis that FGR leads to enhanced lung maturation stems from the observation that the phosphatidylcholine to sphingomyelin ratio (PC/SM) increases in amniotic fluid of FGR fetuses (Gross et al. 1981). However, this finding was not supported in fetal sheep in which FGR was induced by carunclectomy (Rees et al. 1991); here a decrease in the total PL concentration of luminal liquid occurred. Similar findings were also made in FGR induced by maternal undernutrition in neonatal guinea pigs (Lechner et al. 1986; Lin and Lechner 1991), where substantial reductions in total surfactant phospholipids (including disaturated PC) in lavage and lung tissue occurred, although PL composition, the number of lamellar bodies per type II cell and lung compliance remained normal.

In the fetal sheep, prolonged hypoxemia induced by maternal hypoxia (48 h at gestational days 126–130 or days 134–136) lead to an elevated plasma cortisol level, which was more pronounced later in gestation and correlated with elevations in lung tissue SP-A and SP-B mRNA levels (Braems et al. 1998). SP-C mRNA level was unaffected. The alterations were dependent on the age of the fetus, as only older fetuses (134–136 days) responded to hypoxemia (Braems et al. 2000). Hypoxemia-induced increases in SP-A and -B mRNA occurred in an ovine model of chronic placental insufficiency and FGR (Gagnon et al. 1999). Chronic fetal hypoxemia, maintained for 21 days during late gestation (∼109–130 days) decreased fetal growth and lung growth proportionately, and decreased lung DNA content. Fetal cortisol levels increased, and correlated significantly with increases in SP-A and –B mRNA (but not SP-C mRNA). Although lung morphology and function were not assessed, the decrease in lung DNA content and concentration, with an increase in SP mRNA synthesis, suggests that there was a switch from lung cell proliferation to fetal lung cell maturation (Gagnon et al. 1999).

In direct contrast, chronic placental insufficiency during late gestation (120–140 days) did not change the SP-A, -B or -C mRNA or SP-A protein levels in the lung tissue of fetal sheep (Cock et al. 2001). There was also no correlation between SP mRNA or SP-A protein levels and cortisol levels. Furthermore, although DNA content decreased, relative to lung weight, the DNA concentration was higher in the growth retarded sheep. As DNA concentration decreases during normal gestation (Nardo et al. 1995), the increase found here suggests that the lungs of the growth retarded fetuses were structurally immature.

There is still a relative paucity of information on the effect of fetal hypoxia and growth retardation on the surfactant system, and the data on the levels of SP mRNA expression are inconsistent. The primary difference between the study of Gagnon et al. (1999) and that of Cock et al. (2001) lies in the timing of sampling relative to gestation. Cock et al. (2001) induced FGR between days 120 and 140 days of gestation compared to ∼109–130 days in the study by Gagnon et al. (1999). It is possible that the levels of SP mRNA in the older fetuses had already reached their maximal expression and could not be stimulated further by cortisol. Age exerted a specific effect on SP mRNA expression and cortisol levels after 48 h of mild hypoxia (Braems et al. 2000), so it appears that there is a very narrow window in which surfactant maturation can be perturbed by environmental factors (Orgeig et al. 2004). While this robustness may represent an adaptive advantage during normal development, it may also explain the variable outcomes described (Jobe and Ikegami 2000) in therapeutic interventions such as glucocorticoid administration. Therefore, in order to optimise treatment strategies of fetuses and infants at risk of FGR-related postnatal respiratory complications, it is essential that the mechanisms and timing of lung and surfactant maturation during late gestation are understood, especially in relation to environmental factors which lead to FGR (Orgeig et al. 2004).

4.3 Fetal Hypoxia: Effects on the Pulmonary Surfactant System in Non-mammals

Although the overall pattern of surfactant maturation is similar between vertebrate species, the onset and completion of the development of the surfactant system differ dramatically between species (Johnston and Daniels 2001) (Fig. 6a). These differences do not appear to be directly related to phylogeny, as the most closely related species often have the most different patterns. Instead it appears that the developmental process is determined by birthing strategy, which in turn correlates with the development of relative hypoxia as an embryo develops. For example, green sea turtles, which bury their eggs under wet sand (which can exhibit a high bacterial and fungal load so that the eggs are routinely exposed to low oxygen concentrations during development), the viviparous lizard *Tiliqua rugosa*, as well as placental mammals all complete their surfactant development much earlier (75–80%) than most oviparous species (90–95%) that lay their eggs in a manner that exposes them to normal air (Johnston and Daniels 2001) (Fig. 6a). Therefore, we have proposed that one of the factors that drive the development of the vertebrate surfactant system may be hypoxia (Johnston and Daniels 2001).

When sea turtle eggs were incubated in mild, but prolonged hypoxia (17% O_2) (Johnston et al. 2001), there was no effect on the development of the surfactant, suggesting that the pulmonary surfactant system is unresponsive to mild decreases in PO_2. As sea turtle eggshells are more permeable to gases and water vapour than avian eggs (Ackerman and Prange 1972), the lack of effect probably results from the high conductance of chelonian eggshells. It is probable, therefore, that turtle embryos are tolerant of increasingly hypoxic conditions as they are able to acquire oxygen more readily than other shelled embryos and, as a result, oxygen may not have been limiting in the study by Johnston et al. (2001). Moreover, eggs of *Chelonia mydas* incubated in large numbers experience oxygen tensions as low as 9% O_2, which leads to increased mortality of near-term embryos and abnormal yolk size in hatchlings (Wood and Wood 1979). However, if embryos are incubated in smaller numbers such that they experience ambient oxygen tensions of 14% O_2, development progresses normally (Wood and Wood 1979). Clearly, the severity of hypoxia in this experiment was not great enough to alter the course of development in the sea turtles (Johnston et al. 2001).

On the other hand, under similarly mild hypoxic (17% O_2) conditions, overall development of chicken embryos is accelerated, with hatching brought forward by 24 h (Starrs et al. 2001; Blacker et al. 2004). Moreover, we demonstrated a significant acceleration in the development of the surfactant lipid profile in chicks developing under hypoxic incubation from day 10 of incubation (Blacker et al. 2004). Specifically, hypoxia induced both the early release of PL, DSP and Chol as well as an enhanced maturation of the surfactant lipid ratios (i.e. %DSP/PL, Chol/PL and Chol/DSP), indicating upregulation of the synthetic and secretory pathways of surfactant lipids from day 16 of incubation (Fig. 6b). This premature maturation correlated with the early pipping and hatching, but did not alter the

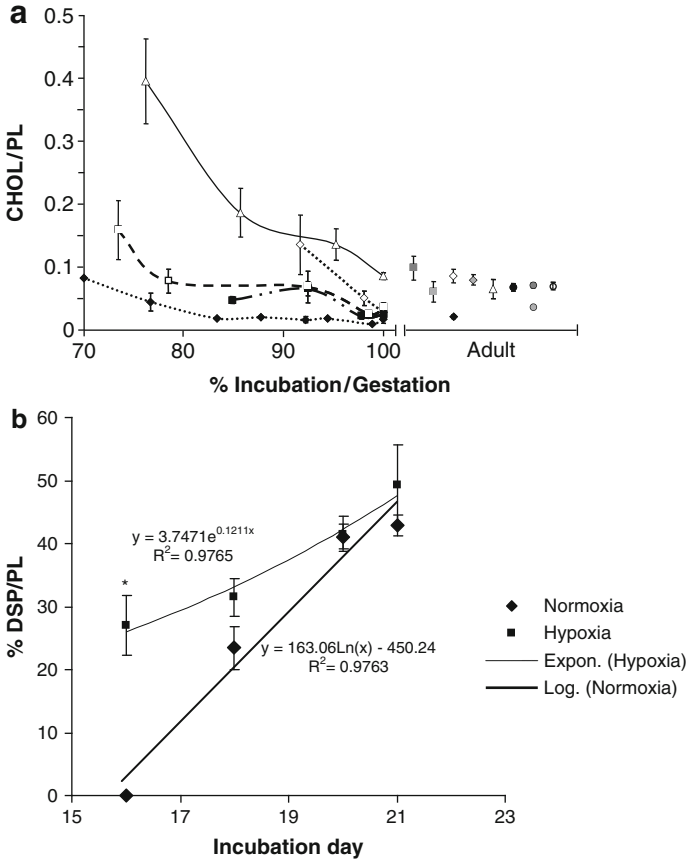

Fig. 6 **a** Cholesterol expressed as a ratio to total phospholipid (μg/μg) from lavage of the developing (*dark line with open triangle*) and adult (*open triangle*) chicken, *Gallus gallus*, the developing (*dotted line with open diamond*) and adult (*open diamond*) bearded dragon, *Pogona vitticeps* (Wood et al. 1995; Johnston et al. 2000), the developing (*dotted line with filled diamond*) and adult (*filled diamond*) sleepy lizard, *Tiliqua rugosa* (Johnston et al. 2002b), the developing snapping turtle, *Chelydra serpentina* (*dark dashed line with filled square)* (Johnston et al., 2002a), the developing green sea turtle, *Chelonia mydas* (*dark dashed line with open square*) (Johnston et al. 2001), the mature leatherback sea turtle, *Caretta caretta* (*dark filled square*), the mature flatback sea turtle, *Natator depressus* (*pale filled square*) (Daniels et al. 1996), the adult central netted dragon, *Ctenophorus nuchalis* (*pale filled diamond*) (Daniels et al. 1990), the adult fat-tailed dunnart, *Sminthopsis crassicaudata* (*dark filled circle*) (Langman et al. 1996), the adult rat, *Rattus norvegicus* (*filled circle with dark edge*) (Orgeig et al. 1995), the newborn rat, *R. novegicus* (*pale filled circle*) (Chol/PC) (Suzuki et al. 1978) and the adult human, *Homo sapiens* (*open circle*) (Doyle et al. 1994). Note: The axis for the adult data points does not represent a timescale, but has been expanded for clarity. Figure reproduced from (Johnston and Daniels 2001) with permission from Elsevier. **b** Effect of incubation under normoxic (21% O_2) or hypoxic (17% O_2) conditions on the percentage of phospholipids (PL) that are disaturated (DSP) (*%DSP/PL*) in lung lavage from embryonic chickens at incubation days 16, 18 and 20 (post-pip) and within the first 24 h of hatching (day 21). Data are expressed as means ± SEM. *Asterisk* = significant differences in %DSP/PL, between normoxic and hypoxic conditions ($P < 0.05$). Figure reproduced from Blacker et al. 2004 with permission from the American Physiological Society

end point, i.e. the lipid composition at hatching was the same in hypoxic and normoxic embryos. The mechanism by which hypoxia could act to trigger surfactant maturation is most likely hormonal. In the chick embryos, plasma corticosterone levels increased in response to hypoxia early in embryonic development. Moreover, in a separate experiment in which we treated developing embryos with the glucocorticoid agonist, dexamethasone, maturation of the surfactant system was also accelerated (Blacker et al. 2004). It is likely, therefore, that the maturation process caused by hypoxia is mediated by glucocorticoids.

5 Conclusions and Future Directions

In this review we have summarised numerous evolutionary and mechanistic studies which show that the evolution of the pulmonary surfactant system has been shaped by environmental selection pressures which include temperature, pressure and hypoxia. Changes in body temperature induce changes in surfactant lipid composition both in the short term, i.e. acute changes within an individual and on an evolutionary time scale between vertebrate groups. However, the evidence to date indicates that changes in either surfactant protein composition or surfactant protein sequence and structure do not appear to be common or consistent. Hence, it is presumably the changes in lipid composition that are responsible for the significant changes in surface activity and surfactant film structure that we have observed with changes in body temperature.

The causal relationship between changes in various lipid components and changes in film structure and behaviour needs to be further characterised. Furthermore, the possibility that the relative expression of SP-C varies with changes in body temperature needs to be examined, as does the possibility that animals with variable body temperatures express an SP-B protein with a different primary sequence. Moreover, the influence of alterations in body temperature on the development of the pulmonary surfactant system has not been examined.

With respect to pressure, we have summarised a series of modifications at numerous organisational levels including modified proteins, lipid components, biophysical function and developmental pattern within a group of mammals experiencing a unique environmental factor, viz. elevated hydrostatic pressure. It appears that diving mammals require an anti-adhesive surfactant with greater fluidity and rapid expansion capabilities to cope with the repeated collapse and reinflation of the lung and large increases in hydrostatic pressure. These functional adaptations are supported by molecular modifications in a key protein (SP-C) and lipid and protein compositional changes. The production of a ‘diving’ type of surfactant may be triggered in part, and in some diving groups, by the onset of diving, lending support to the idea that pressure is the driving force behind the differences observed between terrestrial and diving mammals. An interesting developmental system that deserves investigation from a pulmonary surfactant perspective and that involves changes in pressure, is the metamorphic transition of aquatic larval amphibians to terrestrial adult amphibians.

At this stage there are no clear hypotheses regarding the likely response of the adult surfactant system to hypoxia, which presumably explains and reflects the scarcity of experimental data in this area. Clearly, in the case of developing embryos, hypoxia is a major evolutionary selection pressure, as it appears to trigger various mechanisms to increase maturation, enabling the newborn to escape the potentially dangerous hypoxic environment as quickly as possible. While this is frequently at the expense of the overall growth of the organism, it appears that the lungs and the surfactant system are not only spared, but instead demonstrate accelerated development. This intraspecific plasticity in timing of onset and/or rate of development has recently been termed physiological heterokairy (Spicer and Burggren 2003; Blacker et al. 2004), which has been proposed as a potential mechanism by which environmental factors are able to influence the development of a system within individuals, and which may in some instances over evolutionary timescales result in differences between taxa which are the result of heterochrony (Spicer and Rundle 2007). It will be important to establish whether the heterokairous mechanism involving environmental hypoxia and glucocorticoid hormones is a universal vertebrate phenomenon, hence strengthening the potential link between heterokairy, i.e. intraspecies plasticity and heterochrony, i.e. interspecies differences.

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Midbrain Structures and Control of Ventilation in Amphibians

L.H. Gargaglioni and L.G.S. Branco

Abstract Despite recent advances, the mechanisms of neurorespiratory control in anuran amphibians are far from understood. Among key brainstem structures believed to play a major role in the ventilatory control of amphibians is the nucleus isthmi (NI) and *Locus coeruleus* (LC). It has been suggested that the NI acts to inhibit hypoxic and hypercarbic drives to breathing. The putative mediators for these responses are glutamate and nitric oxide. As to the LC, it has now been reported that this nucleus is a CO_2-sensitive receptor site in amphibians, which mediates the ventilatory response to hypercapnia. This chapter reviews the available data on the role of the NI and LC in the control of ventilation in amphibians.

1 Introduction

Amphibians and mammals share several common features of control of breathing. In both classes, the rhythmogenic and pattern-forming elements are particularly adapted to approach the demands determined by the environment: behavior, metabolic needs, and breathing mechanisms. Studies on amphibians document an involvement of the chemical drive to breathing and the receptors (for review see Gargaglioni and Milsom 2007). Hypoxic ventilatory responses are elicited by peripheral arterial chemoreceptors (Van Vliet and West 1992), whereas hypercarbic ventilatory responses are predominantly mediated by central chemoreceptors that are sensitive to increasing H^+ concentrations in the extracellular fluid that surrounds the fourth ventricle (Smatresk and Smits 1991; Branco et al. 1992; Reid et al. 2000). Recently, we have provided evidence that *Locus coeruleus* (LC) is a chemosensitive area of the central nervous system. To our knowledge there is no data available on the role of LC in hypoxia-induced hyperventilation in amphibians. Thus, this seems

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to be a ripe topic still to be assessed. We will discuss the role of LC as a key site involved in the control of breathing, more specifically to the ventilatory responses to hypercarpnia, later in this chapter.

Studies in tadpoles and adult amphibians indicate that the endogenous respiratory rhythm is generated within the medulla, as observed in mammals (McLean et al. 1995; Reid et al. 2000; Torgerson et al. 1998; Wilson et al. 2002). Therefore, a possible homology between the amphibian lung rhythm generator and the mammalian pre-Bötzinger area has been proposed, and this region may be phylogenetically conserved as a neural structure that permitted development of air breathing in bimodal respiration (Torgerson et al. 1998).

Despite these similarities, the breathing patterns of the two classes are different. Mammals and birds maintain homeostasis of arterial blood gases by rhythmic and continuous breathing. Some teleost fish also ventilate continuously, especially at high temperatures or during hypoxic conditions (Soncini and Glass 2000). Conversely, amphibians, reptiles, and most air-breathing fish display an intermittent pattern of aerial respiration, characterized by lung ventilations that occur in single events or are grouped into episodes of several breaths separated by nonventilatory periods (apnea) of variable duration (Milsom 1991). Mammals may breathe episodically when their metabolic needs are low, e.g., during hibernation, whereas amphibians and reptiles may also breathe continuously (if the respiratory drive is elevated), which suggests a common mechanism for breathing in vertebrates (cf. Kinkead et al. 1997). Episodic breathing occurs even when the respiratory drive is constant, i.e., without fluctuations of arterial blood gases or chemoreceptor input. Therefore, it seems that this pattern is an intrinsic property of the central respiratory control system that is independent of oscillations of arterial blood gases (Kinkead 1997).

Oka (1958a, b) found that the clustering of the breaths into distinct episodes could be completely eliminated only by a transection behind the optic lobes (i.e., behind the midbrain), just in front of the cerebellum. This suggested that a more rostral site was essential for the creation of breathing episodes, and subsequent studies employing in vitro (Reid et al. 2000) and in situ preparations (Gargaglioni et al. 2007) identified areas in caudal half of the midbrain of the bullfrog (*Rana catesbeiana*) that appear responsible for the production of episodic breathing patterns (Milsom et al. 1999; Reid et al. 2000; Gargaglioni et al. 2007).

Kinkead et al. (1997) suggested that the nucleus isthmi (NI), a mesencephalic structure located in the amphibian brain between the roof of the midbrain and the cerebellum, might be such a structure (Fig. 1). Thus, structures within the midbrain of anurans may both provide a tonic drive to breathing and produce specific breathing patterns. In addition, Kinkead et al. (1997) proposed that, from a neuroanatomical perspective, the NI could be compared to the pontine respiratory group, which in mammals contributes to the control of the breathing pattern in a fashion that mimics the effects of pulmonary vagal feedback.

The NI was first described in anurans by Gaupp in 1897 (cf. Wang 2003), and has now been described for all vertebrates, except for cyclostomes and mammals (Hoffmann 1973). Some studies have shown that the NI is a visual center that

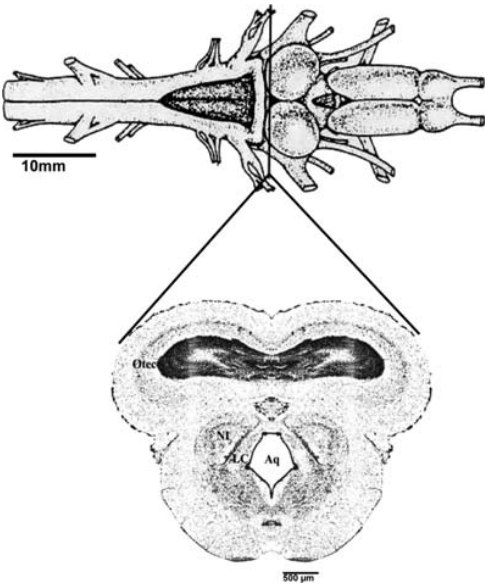

Fig. 1 Dorsal view of the anuran brain, indicating the level of midbrain section illustrated bellow. Abbreviations: *Aq* Aqueduct of Sylvius. *LC Locus coeruleus*, *NI* nucleus isthmi, *Otec* optic tectum

receives information from the optic tectum but is not an auditory center, as previously suggested, since no auditory responses were recorded from amphibian NI (cf. Wang 2003). In anuran amphibians, the NI consists of a cortex and a medulla. While the cortex is rich in cells, the medulla shows scattered cells and numerous nerve fibers (Larsell 1924). The dendrites of NI neurons generally lie in the horizontal plane and extend towards the medulla. The NI consists of approximately 8,000 neurons in frogs and 4,800 neurons in toads (Wang 2003). Interestingly, the NI differentiates during metamorphosis, a period that marks the initiation of pulmonary ventilation in bullfrogs (Burggren and Infantino 1994) and the transfer of central chemoreceptive influence from gill to lung ventilatory control (Torgerson et al. 2001). This evidence is consistent with the notion that the NI plays a role in neurorespiratory control in amphibians. Below we address studies on the involvement of the NI in the control of breathing in amphibians, based on different experimental approaches.

2 Lesion Studies

2.1 Basal Respiratory Drive

As previously mentioned, studies by Oka (1958a, b) showed that transection at the level of the NI removes episodic breathing. However, it was not until 1997 that Kinkead et al. (1997) investigated the role of the NI in anuran ventilatory control.

The authors reported that, following microinjection of kainic acid into the NI (1.5 h prior to the experiments), the respiratory motor output in the Vth and Xth cranial nerves of bullfrogs often shifted from an episodic to evenly spaced pattern. Breathing episodes were, however, still observed in some animals after microinjections of kainic acid, and the reduction in eucapnic ventilation was proportional to the reduction of chemosensitivity. Therefore, it was suggested that the NI is not directly responsible for turning breathing episodes on and off, but that it provides a tonic excitatory input to the respiratory centers. The participation of the NI in the control of breathing in unanesthetized toads (*Chaunus schneideri*, formerly known as *Bufo paracnemis*) has also been tested by performing an electrolytic lesion in this area (Gargaglioni and Branco 2000). The episodic breathing pattern was not eliminated following electrolytic lesion, in agreement with the study by Kinkead. In our study, however, we did not observe a reduction in the resting pulmonary ventilation as demonstrated by Kinkead et al. (1997). It is well-known that both electrolytic and kainic lesions present technical limitations. Electrolytic lesions cause damage both to cell bodies and axons of passage, and kainic acid can result in lesions at sites distant from the injection (Maglóczky and Freund 1995). Moreover, the literature reports that kainic acid stimulates glutamatergic synapses for a few hours after its administration, an event followed by gliosis and neuronal lesions of the glutamatergic neurons (Winn 1991). To address this issue, we conducted chemical lesions (ibotenic acid) in the NI and performed the experiments 3 days later, when chemical lesions were expected to have occurred. According to Jarrard (1989), ibotenic acid appears to be the excitotoxin of choice when specific lesions of neurons that do not affect fibers of passage are required. In our study, we used the method of Klüver-Barrera (Kiernan 1990) to evaluate the intensity of perikarya damage. Histochemical analysis of neuronal cell components in the midbrain tissue lesioned with ibotenic acid revealed that this neurotoxin exclusively destroyed neuronal somata, while neuronal axons and dendrites were preserved. We were then able to further prove that neither cell bodies nor fibers of passage, in fact, do not participate in the resting control of ventilation (Gargaglioni et al. 2002), as previously shown with electrolytic lesions (Gargaglioni and Branco 2000).

Lesions of NI cell bodies did not affect the instantaneous breathing frequency (Kinkead et al. 1997; Gargaglioni and Branco 2000; Gargaglioni et al. 2002), which has been used as a reliable indicator of the endogenous respiratory rhythm in intermittent breathers. Thus the available data are consistent with the notion that the NI does not participate in the generation of the respiratory rhythm. In conclusion, the NI seems not to be involved in the production of episodic breathing in anuran amphibians, but it does participate in the ventilatory response to hypoxia and hypercarbia, as discussed below.

2.2 Responses to Hypoxia

It is now well established that the hyperventilation induced by hypoxia in adult anurans primarily results from activation of peripheral chemoreceptors, located in the aortic arch and carotid labyrinth (Van Vliet and West 1992). Chemoreceptors may,

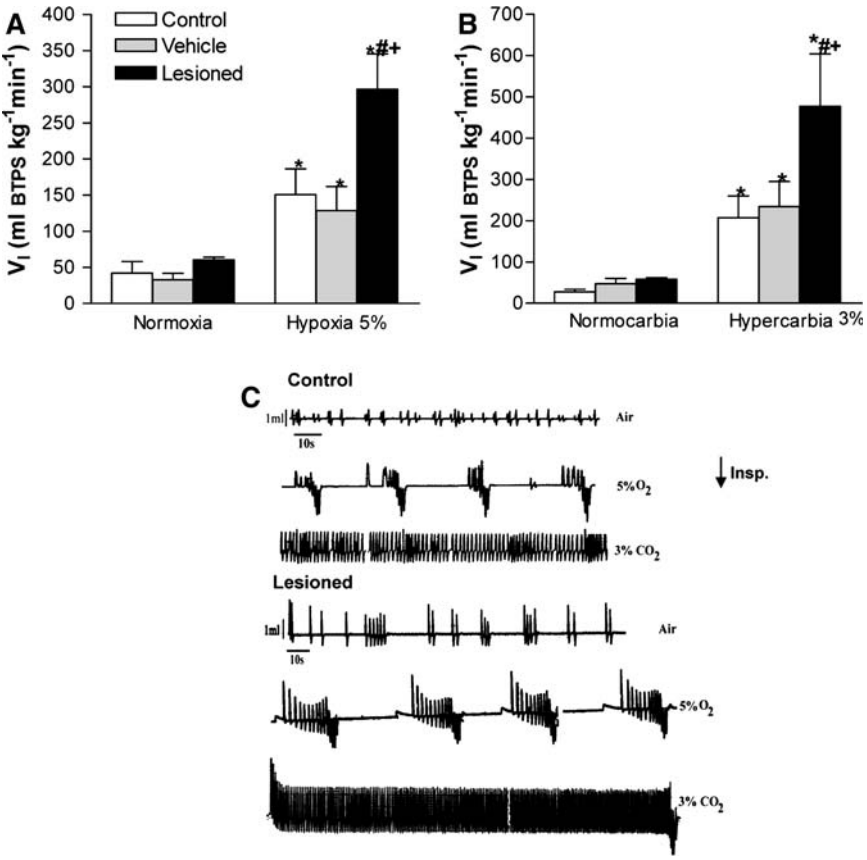

Fig. 2 **a** Ventilation (V_I) of the control, vehicle and ibotenic acid lesioned groups during normoxia and hypoxia (5% O_2). **b** Ventilation (V_I) of the control, vehicle and ibotenic acid lesioned groups during normocarbia and hypercarbia (3% CO_2). **c** Pulmonary ventilation recordings obtained for the control and ibotenic acid lesioned groups during air, hypoxia (5% O_2).and hypercarbia (3% CO_2). *Asterisk* indicates a significant effect of hypoxia or hypercarbia compared to normoxic/normocarbic value, *hash* indicates a significant difference between the control and lesioned groups, *plus* indicates a significant difference between the vehicle and lesioned groups. (Adapted from Gargaglioni et al. 2002)

however, be present on the pulmocutaneous trunk (Hoffmann and de Souza 1982). The peripheral arterial chemoreceptors are analogous, and perhaps homologous, to the chemoreceptors of mammals (Van Vliet and West 1992).

Studies from our laboratory (Gargaglioni and Branco 2000, 2004; Gargaglioni et al. 2002) established that both electrolytic and chemical lesions of the NI cause an increased ventilatory response to hypoxia (Fig. 2), which suggests that NI inhibits hypoxic ventilatory response in toads.

As a key point, the increased ventilation during hypoxia in NI-lesioned toads is consistent with previous studies on our laboratory rats. This shows that both electrolytic lesion of the Locus coeruleus (a noradrenergic pontine nucleus) (Fabris et al. 1999) and chemical lesion of the pontine Nucleus raphe magnus (NRM, a serotoninergic cell group) (Gargaglioni et al. 2003) also caused an elevated respiratory response to hypoxia, due to increases in tidal volume. This suggests an interesting parallel between LC, NRM and NI in ventilatory control during hypoxic conditions.

2.3 Responses to Hypercarbia

Central respiratory CO_2 chemoreceptors have clearly been documented in adult anuran amphibians and are thought to be distributed throughout the medulla, surrounding the fourth ventricle (Smatresk and Smits 1991 and Branco et al. 1992). In intact anaesthetized toads, the central chemoreceptors contribute about 80% of the hypercapnic respiratory drive (Branco et al. 1992), suggesting a dominant role in the ventilatory acid–base regulation. In mammals, these receptors were once thought to be located only on the surface of the ventral medulla but seem to be distributed more widely. Recently, sites have been identified in the ventrolateral medulla, nucleus of the solitary tract, ventral respiratory group, locus coeruleus, caudal medullary raphe, and fastigial nucleus of the cerebellum (for a review, see Nattie 2001). However, it remains uncertain if the same is true for amphibians. In addition to central chemoreceptors, olfactory receptors sensitive to CO_2 powerfully inhibit breathing in unanesthetized bullfrogs (Coates and Ballam 1990). Moreover, in adult anurans, pulmonary stretch receptors are also CO_2-sensitive, since their firing rates decrease in response to elevated CO_2 levels (Milsom and Jones 1977).

In bullfrogs, the NI differentiates during metamorphosis in which the transition to pulmonary ventilation occurs (Burggren and Infantino 1994). At this point, the central chemoreceptors assume control of lung ventilation (Torgerson et al. 2001; Wilson et al. 2002). These changes suggest that the NI may be involved in hypercarbic drive to breathing. In agreement with this notion, Kinkead et al. (1997) and Gargaglioni et al. (2002) have established that CO_2 chemosensitivity is indeed altered following NI lesions. In our study (Gargaglioni et al. 2002), we found that toads with chemical NI lesions show an enhanced ventilatory response to hypercarbia (3% inspired CO_2). On the other hand, Kinkead et al. (1997), demonstrated that microinjection of kainic acid into the NI attenuates the increase in fictive breathing induced by hypercapnia. We believe that these differences may reside in the fact that kainic acid is known to stimulate glutamatergic synapses a few hours after its administration, representing a long-lasting excitatory effect (Watanabe et al. 1987). Since Kinkead et al. (1997) performed the experiments only 1.5 h after the administration of kainic acid, NI neurons may have been activated, rather than chemically lesioned. Another possible explanation is that ibotenic acid produces more selective and limited lesions, compared to kainic acid. According to Guldin and Markowitsch (1982), ibotenic acid is to be preferred relative to kainic acid, when local neuronal

lesions are desired. Therefore, we used ibotenic acid; the production of more selective lesions may promote a more consistent understanding of the role played by the NI in ventilatory control.

3 Putative Mediators Within the NI

As stated above, the NI inhibits pulmonary ventilation in toads exposed to hypoxic and hypercarbic conditions. This indicates that NI may be considered an important site for the control of breathing, although the mediators/modulators involved are poorly understood. We have studied two putative mediators, glutamate and nitric oxide (NO), based on previous data showing that this nucleus contains glutamate receptors and expresses nitric oxide synthase (Wang et al. 1995; Muñoz et al. 1996), and that both neurotransmitters/neuromodulators (glutamate and NO) are important in the control of ventilation (Haxhiu et al. 1995; Gozal et al. 1996).

3.1 Glutamate

L-glutamate receptors are known to be present in the NI (Wang et al. 1995). Such receptors can be divided into two major classes, ionotropic and metabotropic receptors. Ionotropic L-glutamate receptors are ligand-gated ion channels. They can be classified into three types, named after their most selective agonists, NMDA (*N*-methyl-D-aspartate), kainic acid, and DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA). In contrast to the ionotropic glutamate receptors, the metabotropic glutamate receptors are G protein-coupled receptors that modulate second messenger systems.

A growing number of studies report a participation of L-glutamate in the control of breathing. In rats submitted to hypoxia, inhibition of L-glutamate receptors by the NMDA antagonist, MK-801, reduces the magnitude of the hyperventilatory response to hypoxia, whether the antagonist is applied directly to the ventral surface of the medulla (Ang et al. 1992) or administered systemically (Soto-Arape et al. 1995). As to the CO_2 response, Harris and Milsom (2001) found that intracerebroventricular injection of MK-801 to the ground squirrel during hypercapnia caused an increase in the respiratory frequency and a decrease in tidal volume. In amphibians, L-glutamate is known to be one of the neurotransmitters in the rostral brainstem involved in the control of ventilation in *Rana catesbeiana* (McLean et al. 1995). Therefore, we investigated the role of glutamatergic neurotransmission in the NI on the hypoxic or hypercarbic drives to breathing in amphibians (Gargaglioni and Branco 2003). In this context, we microinjected the L-glutamate receptor antagonist, kynurenic acid, into the NI, before exposing the toads to hypoxia and hypercarbia. This drug antagonizes all ionotropic L-glutamate receptor types (Birch et al. 1988).

Microinjection of kynurenic acid into the NI had no effect on normal breathing, which indicates that L-glutamate plays no role under resting conditions. This blocker, however, increased the ventilatory response to hypoxia (7% and 5% inspired O_2) and hypercarbia (3% inspired CO_2), due to an increased tidal volume. Taken together, these data indicate that L-glutamate exerts an inhibitory influence on hyperventilation induced by hypoxia and hypercarbia. L-glutamate has been referred to as the primary central excitatory neurotransmitter involved in the control of ventilation, and we found an increased ventilatory response to hypoxia and hypercarbia after kynurenic acid microinjection. Similar results were observed by Dillon et al. (1991), who reported that microinjection of kynurenic acid into the rostral ventrolateral medulla of rats caused an increase in the ventilatory response to hypoxia and hypercapnia. In addition, rats microinjected with KYN in the locus coeruleus also exhibit an increased ventilatory response to hypoxia, which results from an increased tidal volume (Ferreira et al. 2004). However, the hyperventilation observed in Dillon's study was due to a significant increase in respiratory frequency and in tidal diaphragm activity. On the other hand, our results differ from those obtained by Ohtake et al. (1998), who performed intravenous administration of the NMDA receptor antagonist MK-801 in rats. They suggest that L-glutamate may not be involved in the ventilatory response to hypercapnia. These contradictory results may be related to the route of administration of the antagonists, in as much as systemic drug administration usually elicits different results from those obtained from the central drug administration. Furthermore, MK-801 antagonizes only NMDA receptors, whereas kynurenic acid blocks both NMDA and non-NMDA receptors. Therefore, it is possible that the hypercapnic ventilatory response may involve non-NMDA receptors. Future studies are needed in order to clarify this matter. To summarize, data indicate that the glutamatergic receptors in the NI do not participate in the control of ventilation during normoxia, but that they keep the level of hyperventilation to a minimum during hypoxia and hypercarbia.

3.2 Nitric Oxide

Since the discovery of the biological actions of NO, this gas has been found involved in several functions (Jaffrey and Snyder 1995). NO is a free radical gas that mediates important physiological regulatory events in cell regulation, cell–cell communication and signaling, functioning as an intracellular messenger, neurotransmitter, and hormone (Murad 1999). It is synthesized from L-arginine by a family of enzymes, the NO synthases (NOS) (Garthwaite and Boulton 1995), of which three types have been identified: the neuronal (nNOS), the endothelial (eNOS) and the inducible (iNOS) forms (Bredt et al. 1991). The importance of NO can be demonstrated by inhibiting its effect (Rees et al. 1990), using L-arginine analogs, such as L-NAME a nonselective inhibitor of NOS that acts on both the constitutive and inducible isoforms of the enzymes.

Recently, it became clear that NOS is expressed in the isthmic region of amphibians (Muñoz et al. 1996). Although NO synthases were originally identified in mammalian tissues, there is evidence that NO may be a neuronal messenger of early phylogenetic origin and conserved through the evolution (Muñoz et al. 1996). Accordingly, a substantial number of studies have indicated that NO is involved in a wide variety of central and peripheral processes in vertebrates and invertebrates (cf. Garthwaite and Boulton 1995). Consistently, a recent study (Hedrick et al. 1998) suggested the importance of endogenous NO for the neurotransmission and/or neuromodulation of the respiratory drive to breathing in the bullfrog brainstem.

Based on these facts, we examined the role of the NO pathway in the hypoxic and hypercarbic ventilatory responses in the NI of toads by microinjecting L-NAME (Gargaglioni and Branco 2001). With regard to the effect of NO in hypoxia-induced hyperventilation, microinjection of L-NAME into the NI elicited an increased response to 5% inspired O_2, due to an increase in tidal volume. These data suggest that NO may also mediate the inhibitory effect of the NI in the ventilatory response to hypoxia, as previously demonstrated for glutamate. In mammals, NO has been shown to play an important role by mediating central hypoxic ventilatory reflexes (Haxhiu et al. 1995; Gozal et al. 1996). Previous studies by Gozal et al. (1996) demonstrated that systemic administration of L-NAME induces markedly reduced ventilatory responses to hypoxia. Likewise, Haxhiu et al. (1995) documented that oxygen deprivation leads to activation of the NO pathway in the central nervous system, contributing to hypoxia-induced increase in ventilation. The authors suggested that NO may inhibit inhibitory pathways which, together with excitatory pathways, are triggered by hypoxia. In agreement with these findings, studies from our laboratory have shown that central NO plays a major role in the ventilatory response to hypoxia, since i.c.v. injection of L-NAME abolished the ventilatory response to hypoxia in conscious rats (Fabris et al. 1999). On the other hand, more recent studies by Fabris et al. (2000) and Nucci et al. (2004) demonstrated that microinjection of L-NAME into the LC and NRM, respectively, caused an increase in the ventilatory response to hypoxia, which suggests that in these pontine structures NO acts as an inhibitory neurotransmitter during hypoxia-induced hyperventilation. From these studies it is evident that NO is an important messenger molecule in peripheral and central neuronal structures, which are associated with the control of breathing during hypoxia. Moreover, NO may mediate both excitatory and inhibitory components of hypoxic ventilatory responses.

With regard to the response to CO_2, microinjection of L-NAME into the NI causes a significant increase in ventilatory response to hypercarbia, due to a higher tidal volume. Therefore, the data available support the notion that NO in the NI may exert an inhibitory influence on the integration of the CO_2-drive to breathing. Compared to hypoxia, the effects of NO in the ventilatory response to CO_2 are poorly understood. A previous study (Teppema et al. 1997) reported that the ventilatory CO_2 sensitivity in the peripheral and central chemoreflex loops is depressed after intravenous administration of the NOS inhibitor (N^{ω}-nitro-L-arginine [L-NNA]) in anesthetized cats. More recently, Barros and Branco (1998) demonstrated that the ventilatory response to hypercapnia does not change by administering

L-NNA to rats. In addition, these authors observed an unusual ventilatory breathing pattern 2 h after NOS inhibition. This pattern consisted of episodes of many breaths, separated by episodes of few breaths, similar to the patterns of some ectothermic vertebrates. This indicates that this respiratory control pattern is highly conserved in vertebrates, and that NO plays a role in normal respiratory function in rats.

We may therefore conclude that NO has no role in the NI under resting conditions, but that it exerts an inhibitory modulation on the hypoxic and hypercarbic drives to breathe, which acts on the tidal volume. The present observations, along with other studies on the presence of NO synthase in amphibians (Muñoz et al. 1996), indicate a considerable degree of phylogenetic conservation of the NO pathway amongst vertebrates.

4 Locus Coeruleus

As mentioned above, the first description of the *Locus coeruleus* (LC) was published by Reil (1809) but the term *Locus coeruleus* was proposed by the anatomists Wenzel and Wenzel (1812), reviewed by Russel (1955). The LC nucleus is a well-delineated cluster of noradrenaline neurons (A6 cell group), located adjacent to the fourth ventricle in the pontine brainstem (Dahlström and Fuxe 1964). Apart from noradrenaline the cell bodies also contain several neuropeptides (Sutin and Jacobowitz 1991). It has been described in a large number of animals, ranging from frogs and birds to primates. In the rat, each LC consists of about 1,500 densely packed neurones, all of which are immunoreactive for tyrosine hydroxylase, while LC provides noradrenergic projections to the forebrain, cerebellum, brainstem, and spinal cord.

It is estimated that ∼50% of all the noradrenergic projections in the central nervous system originate in the LC (Aston-Jones et al. 1995; Berridge and Waterhouse 2003). Consequently, LC is implicated in the control of many homeostatic functions including the maintenance of attention, motivation and arousal states (Svensson and Thorén 1979; Bhaskaran and Freed 1988), sleep (Aston-Jones and Bloom 1981), fever response (Almeida et al. 2004; Ravanelli et al. 2007), control of breathing (Erickson and Millhorn 1984; Guyenet et al. 2005; Oyamada et al. 1998; Dawid-Milner et al. 2001; Hilaire et al. 2004; Viemari et al. 2004) and cardiovascular function (Sved and Felsten 1987; Ward and Darlington 1987; Miyawaki et al. 1993; Murase et al. 1993; Anselmo-Franci et al. 1998).

In anurans, González and Smeets (1991) distinguished a large tyrosine-hydroxylase (TH) immunoreactive, but dopamine negative, group of cells at the isthmus region, which lies at the rostral end of the hindbrain (Fig. 1). This isthmic cell group contains noradrenaline (González and Smeets 1991, 1993, 1994) and innervates the spinal cord, cerebellum and telencephalon (Parent 1975; Tohyama et al. 1975; González and Smeets 1991, 1993, 1994). This area is considered to be homologous to the LC of mammals, and this homology is based on its position, noradrenergic content, and projections to both the telencephalon and spinal cord (González and Smeets 1993; González et al. 1994; Marin et al. 1996). Different from rats, the

LC in *Rana* is constituted by 100–140 TH immunoreactive cells (TH-ir) on each side (Marin et al. 1996). Despite considerable efforts and recent advances in studies of respiratory control in amphibians, the role of LC is not completely understood. In this section we present some data on the involvement of LC in the control of breathing in anuran amphibians.

4.1 Basal Respiratory Drive

In mammals, some studies indicate that LC neurons display a respiratory-related activity, i.e., they have direct access to information about the timing of the respiratory output from the medullary respiratory centers (Oyamada et al. 1998, 1999; Andrzejewski et al. 2001). Moreover, LC contributes to the adaptation of adult breathing to physiological needs, and provides a tonic excitatory drive that contributes to a normal breathing rate in rats (Guyenet et al. 1993; Oyamada et al. 1998; Dawid-Milner et al. 2001; Li and Nattie 2006) and mice (Shirasawan et al. 2000; Hilaire et al. 2004). In this context, Hilaire et al. (2004) pointed out that LC noradrenergic neurons provide a tonic excitatory stimulus that maintains breathing frequency, and are necessary for the development of a normal respiratory rhythm. Recently, Li and Nattie (2006) showed that substantial lesions of brainstem catecholaminergic neurons (including LC) slow breathing frequency during air breathing, and that this effect is present in both wakefulness and in NREM sleep. Taken together these data may indicate that LC noradrenergic neurons provide a tonic drive to breathe. However, recent data from our laboratory demonstrated that selective lesion of the LC using 6-OHDA (a toxin that selectively eliminates catecholaminergic neurons) did not change basal ventilation (Biancardi et al. 2008), which suggests that noradrenergic neurons are located in the LC play no role in respiratory control under resting conditions.

Recently, Gargaglioni et al. (2007) showed that electrical stimulation believed to be in the vicinity of the LC of frogs (*Rana catesbeiana*) caused an increase in respiratory frequency, whereas the chemical stimulations (glutamate) had no effect. These differences may be due to the fact that electrical stimulation excites both fibers of passage and cell bodies, whereas chemical stimulation activates only cell bodies. Additionally, we have tested the participation of noradrenergic LC neurons in the control of breathing in unanesthetized toads (*Chaunus schneideri*) by using 6-OHDA lesion (Noronha-de-Souza et al. 2006). The LC lesion did not change the pulmonary ventilation of toads under resting conditions. Moreover, lesions of LC noradrenergic neurons did not affect the instantaneous breathing frequency, which has been used as a reliable indicator of the endogenous respiratory rhythm in intermittent breathers. This suggests that the LC neurons do not participate in the generation of the respiratory rhythm.

4.2 Responses to Hypercapnia

Several studies on mammals have demonstrated that c-Fos techniques can be used to identify neurons involved in responses elicited by hypercapnia (Haxhiu et al. 1996; Teppema et al. 1997; Berquin et al. 2000). Although neuronal function cannot be inferred from Fos expression, these studies brought new insight into the anatomical distribution of putative intrinsically chemosensitive neurons within chemoreflex pathways (Berquin et al. 2000). In mammals, studies under in vivo conditions showed that CO_2 stimulation increases the expression of c-Fos gene in LC neurons (Haxhiu et al. 1996; Teppema et al. 1997). In addition, extracellular recordings from LC neurones in both neonatal and adult rat show that they respond to systemic hypercapnia with an increase in spike frequency under in vivo conditions (Elam et al. 1981; Stunden et al. 2001). Local acidification of noradrenergic neurons of LC increases respiratory frequency and phrenic nerve discharge in cats (Coates et al. 1993).

The LC neurons are of particular interest in CO_2 challenge since >80% of neurons are found to be chemosensitive, responding to hypercapnia with an increased firing rate (Pineda and Aghajanian 1997; Oyamada et al. 1998; Filosa et al. 2002). Recently, Li and Nattie (2006) lesioned the catecholaminergic (CA) neurons of the rat brainstem and found that the ventilatory response to 7% CO_2 was significantly decreased in sleep and wakefulness, suggesting that brainstem CA neurons participate in central chemoreception in vivo during both NREM sleep and wakefulness. In their study, approximately 84% of LC–CA neurons were eliminated, indicating that LC is an important site for hypercapnic ventilatory response. Recently, we have investigated the participation of LC noradrenergic neurons in relation to the CO_2 drive to breathe. Our data indicate that LC noradrenergic neurons modulate the hypercapnic ventilatory drive, since chemical lesion of this structure reduced the hypercapnic ventilatory response, due to a decreased V_T (Biancardi et al. 2008). We found that a reduction of approximately 80% of noradrenergic neurons of LC was associated with a large decrease in the response to CO_2 of approximately 64%, indicating that this nucleus exerts an important influence on CO_2-drive to breathing.

Until recently, there were no data available for the role of LC in the central chemoreception of amphibians. Therefore, we have investigated if LC is a CO_2/H^+ chemoreceptor site in anuran amphibians. Initially, we provided morphologic evidence, i.e., the expression of *c-fos* in neurons of the LC after hypercarbic challenge. The LC is one of the brainstem cell groups thought to be involved in physiological responses to hypercarbia, since a marked increase in *c-fos* positive cells in this nucleus was induced after 3 h of exposure to a hypercarbic gas mixture (Noronha-de-Souza et al. 2006).

In addition, we have used selective chemical lesion of LC catecholaminergic neurons to verify a possible involvement of this nucleus in the respiratory responses to hypercarbia. Our data show that chemical lesions of the LC with 6-OHDA resulted in an attenuation of hypercarbia-induced hyperventilation (Fig. 3), whereas this effect was absent under resting conditions (Noronha-de-Souza et al. 2006). This finding, associated with the fact that the isthmic catecholaminergic cell group of amphibians (where LC is placed) does not contain dopaminergic or adrenergic cell

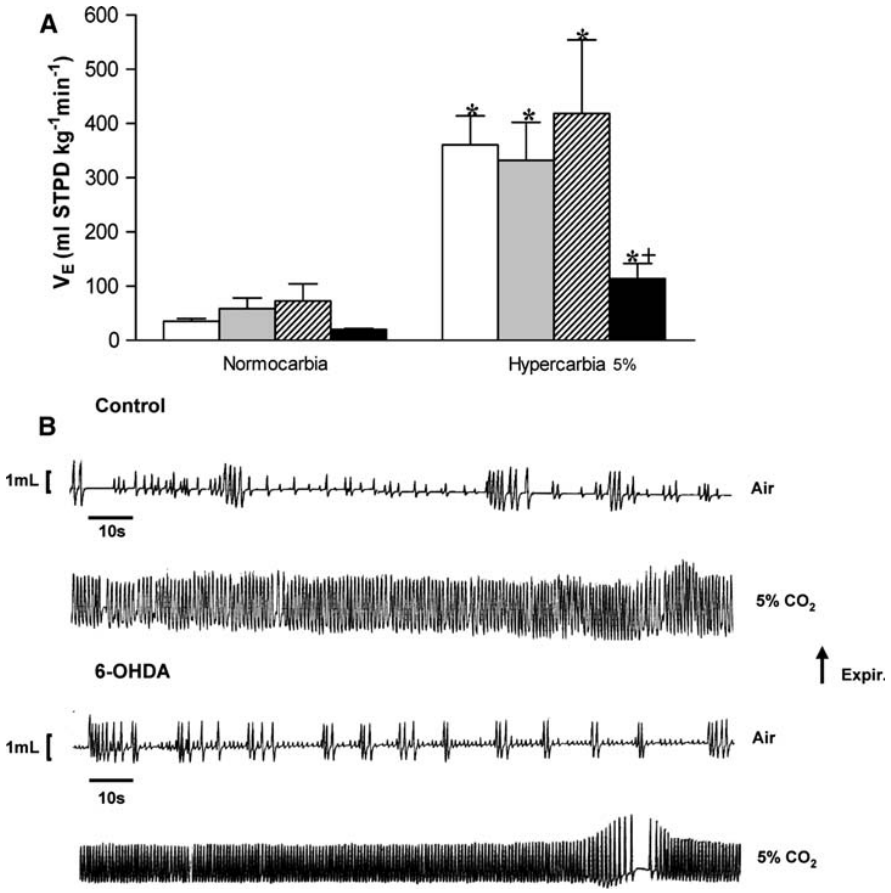

Fig. 3 **a** Ventilation (V_E) of the control, vehicle, peri and 6-OHDA groups exposed to normocarbia and hypercarbia (5% CO_2). **b** Pulmonary ventilation recordings obtained for the control, vehicle, peri and 6-OHDA groups during normocarbia and hypercarbia (5% CO_2). *Asterisk* indicates a significant effect of hypercarbia compared to the normocarbic value, *plus* indicates significant differences between 6-OHDA and all other groups during hypercarbia. (Adapted from Noronha-de-Souza et al. 2006)

bodies (González and Smeets 1993), strongly suggests that noradrenergic LC neurons are involved in processing or modulating central chemoreceptor information in amphibians.

We further investigated whether LC neurons are intrinsically pH-sensitive. To test this hypothesis we performed local acidification by microinjecting mock CSF with different pH values (7.2, 7.4, 7.6, 7.8 and 8.0). Mock CSF perfusion is a well-established method for studying the central chemoreceptor drive to breathe (Hitzig and Jackson 1978; Hitzig et al. 1985; Branco et al. 1992; Sanchez et al. 2001).

We performed local acidification of the LC by microinjection of mock CSF with different pH values. Notably, pulmonary ventilation increased after local reduction of the pH (mock CSF of 7.2, 7.4 and 7.6), which suggests that LC is a chemosensitive site in the CNS of amphibians.

Richerson (1999) and Putnam et al. (2004) proposed two essential criteria that a central chemoreceptor neuron must possess: (1) it must respond to changes in CO_2 that occur under nonpathological conditions in vivo, and this response must be due to mechanisms intrinsic to the specific cell; (2) it must have the capability of increasing respiratory output in response to an increase in CO_2, which could be accomplished if the neuron is part of the respiratory network or projects to respiratory neurons. The data of Noronha-de-Souza et al. (2006) fit the first criterion since lesions of the LC resulted in an attenuation of the hypercarbia-induced hyperventilation, and local tissue acidosis increased the ventilation of the toads. With regard to the second criterion, it is well known that the LC of mammals projects to respiratory neurons such as ventral medullary and solitary tract nuclei (Van Bockstaele et al. 1998, 1999). There is neuroanatomical evidence suggesting that LC of amphibians is homologous to the LC of mammals primarily on the basis of its position, noradrenergic content, and projections to brainstem structures (González and Smeets 1993; González et al. 1994; Marin et al. 1996). Furthermore, under in vivo conditions, in mammals, LC neurons appear to be activated by systemic hypercapnia, as judged by increased expression of *c-fos* gene product (Haxhiu et al. 1996; Teppema et al. 1997). The c-*fos* expression technique has been extensively used as a marker of neuronal activity, induced by a number of stimuli including hypercarbia. We found that an increased inspired-CO_2 concentration (5% CO_2) induces Fos-like immunoreactivity in the LC of toads, reinforcing the idea that the LC of amphibians is homologous to the LC of mammals. Further analysis is needed to understand the specific functional connections of the LC with the neuronal circuitry involved in the control of respiration in amphibians, but we can speculate that the LC of amphibians might be a central chemoreceptor site.

5 Final Remarks and Perspectives

The understanding of the neurorespiratory control mechanisms in amphibians has recently advanced considerably. The generation of episodic breathing is an intriguing topic. This pattern is currently accepted as an intrinsic property of the central respiratory control system. It was previously suggested that the NI might be responsible for generating the episodic breathing in anurans (Kinkead et al. 1997). Nevertheless, manipulation of the NI (by lesion or drug microinjection) failed to eliminate the episodic pattern, which suggests that the NI is not directly responsible for turning breathing episodes on and off (Kinkead et al. 1997; Gargaglioni and Branco 2000, 2001, 2003; Gargaglioni et al. 2002). Nonetheless, the accumulated data suggest that the NI acts to inhibit hypoxic and hypercarbic drives to breathe by restricting increases in tidal volume. The inhibitory role of the NI is similar to that of certain

pontine sites (LC and NRM) in rats (Fabris et al. 1999; Nucci et al 2004). The LC and NRM of mammals and the NI of amphibians are not homologous, but the analogous effects of these lesions are remarkable. There may be a number of putative mediators for these responses, but so far only glutamate and nitric oxide have been described.

Since we found an alteration in tidal volume after NI lesion and microinjection of drugs, it is possible that NI acts as a relay site for pulmonary stretch receptor information. According to Wang et al. (1999), these receptors are sensitive to lung volume, and provide a feedback for lung inflation, which limits tidal volume. In general, activation of stretch receptors suppresses inspiration and enhances expiration (Hering-Breuer reflex). Additionally, a recent study by Reid et al. (2000) showed that pulmonary stretch receptor feedback has a crucial role in the integration of CO_2 chemosensory information during hypercapnia in *Bufo marinus*. The NI appears to provide an inhibitory input to respiratory sites, which limits breathing amplitude when the respiratory drive is elevated. This is supported by McLean et al. (1995) who verified that microinjection of excitatory and inhibitory amino acids in isolated adult frog brainstem influenced the frequency and amplitude of lung-burst activity. Further experiments are necessary to investigate the relationship between brainstem modulating regions and the NI.

The interaction between L-glutamate and NO in the NI is also an important topic for future studies. In this context, we observed that the effect of L-NAME on ventilation resembled the effect observed following kynurenic acid microinjection into the NI, suggesting a possible interaction between L-glutamate and NO. In fact, it has been reported that activation of brain L-glutamate leads to synthesis and release of NO (Garthwaite et al. 1989). Previous studies demonstrated that NO formation mediates L-glutamate-induced cGMP accumulation (Bredt and Snyder 1989) and NMDA-evoked neurotransmitter release in the brain (Montague et al. 1994). L-glutamate released from a presynaptic terminal, acting on NMDA receptors, promotes an increase in intracellular calcium that can stimulate constitutive NOS, generating NO. The NO formed diffuses to the presynaptic terminal, where it stimulates guanylate cyclase and elevates cyclic GMP concentration, leading to further increase in the release of L-glutamate and augmentation of synaptic transmission (Garthwaite 1991).

Relatively less is known about LC. A recent study supports the notion that the noradrenergic neurons of toads are a site of central chemoreception (Noronha-de-Souza et al. 2006). Based on these data, we can suggest that the presence of widespread central chemoreceptor sites evolved early, since they are present in amphibians. Very probably, the transition from water to air breathing was associated with demands for a more flexible and sensitive CO_2 control system, which brought the development of multiple central sites for CO_2/H^+ detection. Our findings emphasize further the similarities between anuran and mammalian LC, and support the proposed homology of this nucleus in both groups.

In summary, midbrain structures such as NI and LC are essential in the mediation of CO_2 drive to breathing. Despite of recent significant advances in this field, further studies are urgent in an attempt to shed light on the neurochemical

mechanisms responsible for these vital responses to environmental stress. For instance, the putative role of LC mediating the hypoxic ventilatory responses is still pending.

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Comparative Aspects of Hypoxia Tolerance of the Ectothermic Vertebrate Heart

H. Gesser and J. Overgaard

Abstract This chapter reviews cardiac contractile performance and its regulation during hypoxia/anoxia with regard to cellular metabolism and energy state, in particular hypoxia-tolerant ectothermic vertebrates. Overall the contractile performance of the hypoxic isolated heart muscle varies in a way that relates to the occurrence of hypoxia/anoxia in the natural life of the animal. The hypoxic/anoxic performance of the heart muscle correlates positively with the glycolytic capacity relative to the aerobic capacity, and this performance also tends to be high in hearts having a low aerobic or maximal working capacity. Indirect evidence suggests a particular role for creatine kinase, and that mechanical efficiency may increase in some species. Despite the restricted energy production, hypoxic/anoxic performance is often strongly stimulated by agents such as adrenaline. Frequently, mechanical performance is reduced less by the oxygen lack itself than by factors commonly associated with it, such as increases in extracellular K^+.

1 Introduction

It is generally accepted that survival of vertebrates depends critically on continuous cardiac activity. Cardiac activity and performance may, however, vary considerably between species as well as with endogenous and exogenous conditions, such as temperature, activity, and oxygen availability. This chapter is focused on how myocardial function is affected by severe hypoxia with special focus on ectothermic vertebrate species that display key adaptations to this condition. Examples include diving air-breathers such as turtles, frogs or crocodilians; fish experiencing severely deoxygenated waters; or hagfishes burrowed in carcasses. Hypoxia is often associated with other challenges such as acidosis and elevated extracellular

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potassium concentrations, and the additive effects of such disturbances are therefore also discussed here with regard to myocardial hypoxic performance.

In adult mammals and birds the heart is considered to be almost exclusively aerobic, although myocardial hypoxia may occur under pathological conditions or under special circumstances such as high altitude, diving, burrowing, or parturition (e.g., Jarmakani et al. 1978; Ostadal et al. 1999; Mühlfeld et al. 2005). In contrast, the risk of myocardial oxygen lack is considerably higher in ectothermic vertebrates due to a number of anatomical, behavioral, and environmental reasons. The mammalian and bird myocardium consists of a compact tissue with densely packed muscle cells supplied with blood through an extensively developed coronary system. In contrast, many ectothermic vertebrates have hearts where the atria and parts of the ventricle wall are formed by a spongy myocardium consisting of thin cell bundles forming a network with only a sparse or absent coronary supply. The ventricular wall often consists of two tissue types where an exterior layer of compact tissue, resembling that in ventricles of mammals and birds, surrounds the inner spongy tissue. The proportion of spongy versus compact tissue in the myocardial wall varies according to species and developmental stage. Thus, the compact tissue may constitute as much as 70% in some tuna species, whereas it is about zero in most amphibians and many fish species (Tota 1983; Davie and Farrell 1991; Driedzic and Gesser 1994; Ostadal et al. 1999). Much of the oxygen to the spongy part of the heart is supplied by the blood pumped through the cardiac chamber rather than by a coronary supply. In fish, this is of particular importance as the heart is the last organ before the gills, and the spongy part of the myocardium is therefore perfused by deoxygenated blood returning from the systemic circulation. This has the paradoxical consequence that during intense activity the heart may in fact only receive poorly oxygenated blood although the demand on cardiac activity is increased (Farrell and Clutterham 2003). However, in other situations of severe oxygen deficiency, the demand on the cardiac function is often lower due to a concomitant decrease in the metabolic rate of the whole animal (Driedzic and Gesser 1994; Farrell and Stecyk 2007).

Cardiac output is the product of stroke volume and beat frequency, and both of these parameters are generally easily measured in vertebrates under in vivo, in situ or in vitro conditions. Nevertheless, many studies also use isolated cardiac preparations (e.g., cardiac strips) to address cellular processes. Although such preparations are more remote from the situation in the living animal, they offer some advantages with regard to the control of diffusion conditions and the recording of metabolic parameters. The present review primarily concerns such studies, with the consequence that important aspects of the control and integration of the whole heart, and its function in the living animal, are neglected. In studies of isolated tissue, the effects of challenges such as oxygen deficiency on twitch-force development and relaxation in isolated tissue are often taken as a proxy of the corresponding effect on stroke volume (Fig. 1). A decreased peak force and an impaired relaxation with an elevated resting tension would correspond to an increased end-systolic and a decreased end-diastolic volume, resulting in a reduced stroke volume in the living animal.

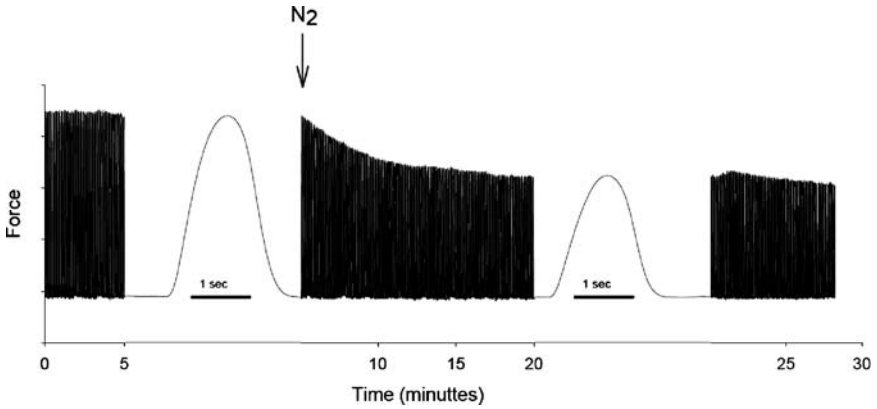

Fig. 1 Representative trace of isometric force development in heart muscle preparation from Red-eared slider (*Trachemys scripta elegans*). Stimulation rate: 0.3 Hz = 18/ min, temp: 20°C, pH: 7.6 (2% CO_2, ~25 mM HCO_3). *Arrow* indicates the transition from oxygenated to hypoxic/anoxic conditions

2 Comparative Hypoxia Tolerance of Cardiac Function

As shown in Fig. 2, the effects of severe hypoxia/anoxia on maximal cardiac contractile force can vary greatly between vertebrate species. Most examples shown in Fig. 2 are from the same laboratory, because even small differences in experimental conditions can markedly influence performance of the oxygen-deficient heart muscle. This is exemplified by cardiac tissue from carp, where force development varies greatly with the total concentration of extracellular bicarbonate–CO_2, or in hypoxic cardiac strips from trout, where adrenergic stimulation or high extracellular Ca^{2+} remove most of the reduction in force generation during severe hypoxia (Gesser 1977; Gesser et al. 1982; Nielsen and Gesser 1983).

Not surprisingly, freshwater turtles are among the best at preserving contractility during anoxia, whereas, for example, mammalian species typically show drastic decreases in twitch force. Also among fish, the best preserved performance seems to occur in species known to have a high hypoxia tolerance such as carp, eel and hagfish (Fig. 2). Thus the risk of an animal species experiencing oxygen lack appears to be a major factor correlating with the anoxic mechanical performance of the isolated heart muscle. Furthermore, hypoxia tolerance seems to be higher in amphibian and reptile species from temperate zones, such as the European viper and the edible frog, whereas tolerance is lower in tropical species such as the marine toad, the alligator and the python (Fig. 2). Other parameters such as gender may also be of importance, as twitch force during severe hypoxia decreased less, or was better recovered upon reoxygenation, in myocardial preparations from female than from male trout and rat (Ostadal et al. 1999; Battiprolu et al. 2007). Moreover, different stocks/lines within a species may vary considerably, as noted for rainbow trout and rat (Baker et al. 2000; Faust et al. 2004).

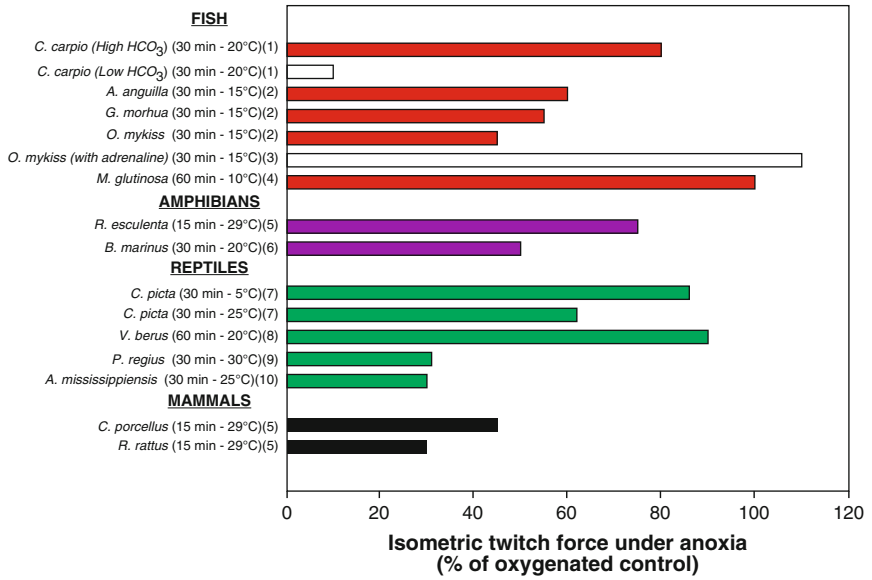

Fig. 2 Anoxic twitch force relative to that under oxygenation (%) in cardiac preparations from: common carp (*Cyprinus carpio*); European eel (*Anguilla anguilla*); Atlantic cod (*Gadus morhua*); rainbow trout (*Oncorhynchus mykiss*); Atlantic hagfish (*Myxine glutinosa*); edible frog (*Rana esculenta*); marine toad (*Bufo marinus*); painted turtle (*Chrysemys picta*); European viper (*Vipera berus*); ball python (*Python regius*); American alligator (*Alligator mississippiensis*); guinea pig (*Cavia porcellus*); rat (*Rattus rattus*). All cardiac preparations are from isolated and electrically paced cardiac strips with the exception of *M. glutinosa* where force is measured with a force transducer coupled to the *in situ* heart preparation. Data for *C. carpio* are shown with high and low $CO_2 - HCO_3$ concentrations (constant pH), and data for *O. mykiss* are shown with and without adrenergic stimulation. All other preparations are run in a physiological solution without pharmacological supplements. Data are obtained from: *(1)* Gesser (1977), *(2)* Hartmund and Gesser (1996), *(3)* Gesser *et al*. (1982), *(4)* Hansen and Sidell (1983), *(5)* Joseph et al. (2000), *(6)* Andersen et al. (2004), *(7)* Overgaard et al. (2005), *(8)* Gesser and Poupa (1978), *(9)* Zaar et al. (2007), and *(10)* Gesser and Wang (unpublished)

3 Cellular Energy Metabolism During Hypoxia/Anoxia

Adenosine triphosphate (ATP) is the primary energy currency in vertebrates. When cellular oxygen availability is high, almost all ATP is provided by oxidative phosphorylation, where 1 mol glucose leads to formation of 26–38 mol ATP (Brand 2005). When ATP production from oxidative phosphorylation is insufficient, ATP can be produced anaerobically by glycolysis, but in this case, 1 mol of glucose units yields only 2 mol ATP with glucose and 3 mol ATP with glycogen as substrate (e.g., Lehninger 1970) (Fig. 3). In addition to the low ATP yield, anaerobic glycolysis is also associated with a fast depletion of energy stores and an accumulation of lactic acid, which causes acidification. Nevertheless, anaerobic metabolism is the sole source of ATP during complete anoxia, but specialized anoxia-tolerant animals

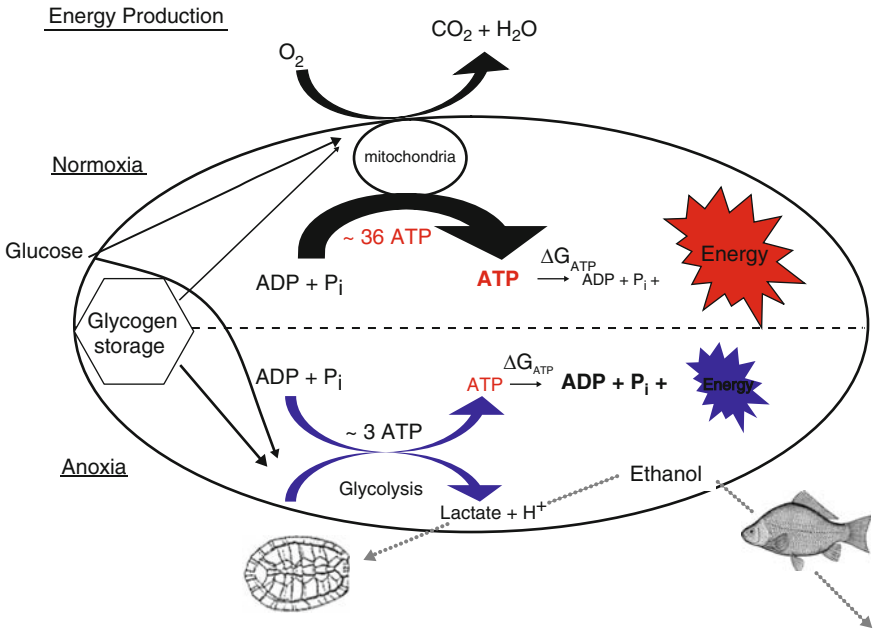

Fig. 3 Schematic presentation of the major effects of anoxia on cellular energy production and energy state: *Top*: normoxic conditions where ~36 mol ATP are produced per mol glucose, and waste products in the form of CO_2 and water diffuse to the extracellular space. Under these conditions, the energetic state (ΔG_{ATP}) is high, with a high ATP/ADP ratio and low levels of free phosphate. *Bottom*: anaerobic conditions where fermentation of glucose only produces 2–3 mol ATP per mol glucose. The waste product lactic acid accumulates intra- and extracellularly, causing intra- and extracellular acidification. However, the anoxia-tolerant crucian carp can excrete the waste products in the form of ethanol, and anoxia-tolerant turtle species can alleviate the acidification by large buffer reserves in the shell and bone. During anoxia, energy state (ΔG_{ATP}) decreases, and the free phosphate level increases

can attenuate the associated adversities. Thus, the ability of freshwater turtles and crucian carp to survive anoxia relies, in part, on their extraordinary ability to handle anaerobic waste products. Freshwater turtles utilize the large buffer capacity of bone and shell to attenuate acidification due to lactic acid (Jackson 2000, 2002), while ethanol, which is excreted through the gills, is the anaerobic end-product in crucian carp (Van Warde 1991) (Fig. 3).

3.1 Metabolic Depression of the Whole Animal and the Heart

The cardiac demand varies with whole body energy turnover, which is typically low in vertebrates enduring long-term anoxia. According to Farrell and Stecyk (2007), a low cardiac power output is attained through different strategies. Thus, some anoxia-tolerant species, such as crucian carp and hagfish, have an inherently low

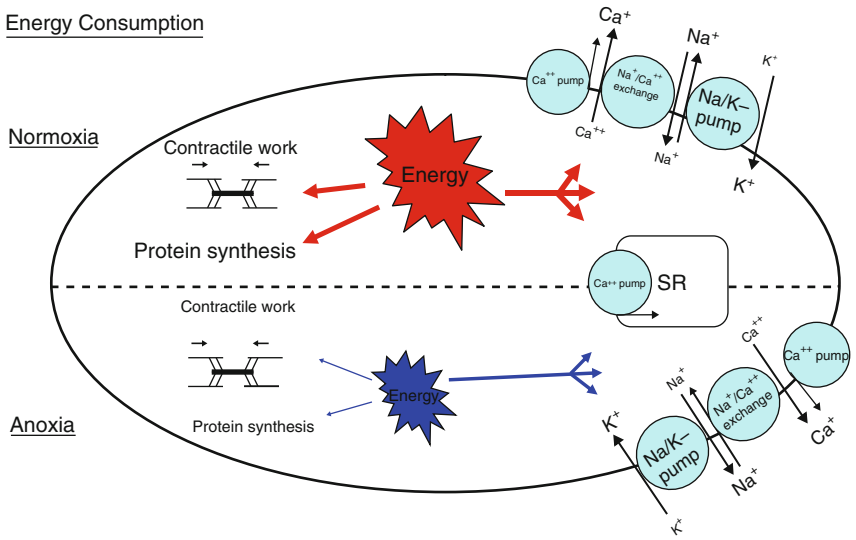

Fig. 4 Schematic presentation of the effects of anoxia/hypoxia on cellular energy consumption in cardiac cells. *Top*: normoxic conditions where energy is predominantly used for protein synthesis, contractile work, and ion motive pumps such as the Na^+/K^+ ATPase and the Ca^{2+} ATPase. The activity of these pumps maintains transmembrane ion homeostasis, which is essential for the excitability and the ability to maintain Ca^{2+} transport during excitation–contraction. *Bottom*: Anaerobic conditions are associated with a general metabolic depression due to translational arrest, spike and channel arrest (*see text*)

routine cardiac power output, while, for example, freshwater turtles severely depress cardiac power output during anoxia (Farrell and Stecyk 2007). This is generally in accordance with whole body metabolic rate, where the crucian carp maintains activity and displays a moderate down regulation of metabolic rate during anoxia (Nilsson and Renshaw 2004), whereas anoxic freshwater turtles reduce whole body metabolism by 80–90% (Jackson 1968). Furthermore, this reduction is amplified at low temperature during winter hibernation, when the turtles may enter a coma-like state (Lutz and Nilsson 1997; Storey and Storey 1990; Boutilier 2001; Jackson 2002).

In addition to an upregulation of anaerobic energy production, the transition from oxygenated conditions to anoxia involves a reorganization of cellular energy-consuming processes (See Fig. 4), which may depress overall metabolic requirements (Hochachka 1986; Driedzic and Gesser 1994; Hochachka et al. 1996; Lutz and Nilsson 1997; Boutilier and St-Pierre 2000; Boutilier 2001). Overall, protein synthesis is reduced dramatically during anoxia, although synthesis of a few specific proteins, including heat shock proteins, may be upregulated in anoxic turtles (Chang et al. 2000). Protein synthesis normally accounts for a large part of cellular energy turnover so "translational arrest" renders a larger proportion of the available ATP for ion pumping (Hochachka et al. 1996). Depending on the tissue type, ion motive pumps may account for 25–75% of total metabolism during normoxia (see Hochachka et al. 1996; Boutilier and St-Pierre 2000; Boutilier 2001).

However, since overall ATP-turnover is reduced by more than 75% in some species the activity of ion motive pumps is obviously bound to be reduced, despite the fact that an enlarged fraction of ATP is available to ion motive pumps during anoxia in turtles (Hochachka et al. 1996; Boutiler and St-Pierre 2000). The overall reduction in activity of ion motive pumps affects ionic gradients and consequently cellular integrity, unless it is accompanied by a simultaneous reduction in the passive transmembrane ion transport that is associated with electrical activity, membrane co- and countertransport and leakage. Such reductions do occur, and they have been referred to as "channel arrest" and "spike arrest". Channel arrest is caused by a reduction in the number and permeability of the membrane ion channels. Channel arrest has been reported for Na^+, K^+ and Ca^{2+} channels in turtle brain, and for the Na^+ channel in hepatocytes (see Hochachka et al. 1996; Boutilier and St-Pierre 2000; Jackson 2000). Spike arrest, in which the electrical activity of excitable tissue is reduced, is afforded by reduced channel activity, and also by increased levels of inhibitory neuro-transmitters and a decreased neural activity (Hochachka et al. 1996; Lutz and Nilsson 1997; Boutilier and St-Pierre 2000; Boutilier 2001; Jackson 2002). Despite the marked reorganization of energy-consuming processes, a new steady state is not necessarily attained during long-term anoxia. Thus, continued anoxic exposure is associated with a gradual increase in extracellular potassium ($[K^+]_0$) in turtles, where $[K^+]_0$ increases from $\sim$3 mmol l^{-1} to values exceeding 10 mmol l^{-1} in both painted turtle and snapping turtle (Jackson and Ultsch 1982; Ultsch et al. 1999; Reese et al. 2002) (See Fig. 4). It should also be noted that channel arrest does not always occur during long-term anoxia, as exemplified by the heart of crucian carp (Paajanen and Vornanen 2003).

3.2 Aerobic Metabolism and Hypoxia

The ability to sustain cardiac function during hypoxia depends not only on the glycolytic capacity but also on the ability to maintain oxidative phosphorylation at low oxygen availabilities. This aspect seems somewhat neglected, possibly due to experimental problems relating to the variation in the degree of hypoxia that different cells will experience in multicellular preparations. The maintenance of aerobic ATP production includes the cellular means for supplying mitochondria with oxygen, as well as the ability to maximize the degree of coupling (i.e., the P/O ratio – ATP per oxygen molecule). In recent years, the picture of the regulation of this coupling has changed considerably, as reviewed by Brand (2005). Thus, in both endo- and ectothermic vertebrates, the P/O ratio seems more variable and typically below the value of 6 provided by the standard biochemical textbook. For example, a low activity level in a tissue well supplied with oxygen may result in a reduced P/O ratio mainly because of a proton leak through the inner mitochondrial membrane (Brand 2005). Hypoxia can augment the P/O ratio as it reduces the mitochondrial proton gradient and thereby the proton leak, as well as increasing the substrates for oxidative phosphorylation (ADP and inorganic phosphate) (Gnaiger 2001). Moreover,

an increased efficiency of ATP consuming processes would favor energy balance during severe hypoxia, especially in organs such as the heart, which maintain function during anoxia. Indeed we have observed an apparent decrease in the cost of contraction during hypoxia in ventricular strips of freshwater turtle, which may be related to an improved P/O ratio. However, the underlying mechanisms for this apparent improvement of energy economy are still unclear, and a reduced expenditure to noncontractile processes may also be involved (Overgaard and Gesser 2004). However, in a direct study of the cost of cross-bridge formation in mammalian heart muscle, there was no evidence for an improved efficiency during hypoxia, and cost of cross-bridge formation actually tended to increase in hypoxia-sensitive species (Joseph et al. 2000).

3.3 Enzyme Complement and the Relation Between Contractility and Energy State

Glycolytic ATP delivery is particularly important during hypoxia/anoxia, and it has been suggested that a high glycolytic enzymatic capacity relative to the aerobic enzymatic capacity is an indicator of the ability to maintain anaerobic cardiac performance. Indeed, a comparison of isolated ventricular preparations from different teleost species provided a positive correlation between the maintenance of hypoxic contractility and the ratio of the glycolytic to the aerobic capacity assessed respectively by activities of pyruvate kinase and cytochrome oxidase recorded in vitro (Gesser and Poupa 1974) (Fig. 5). Similarly, heart muscle from freshwater turtle, crucian carp and hagfish (*myxine glutinosa*), which are known for the ability to maintain contractility during anoxia, displayed the highest ratios of pyruvate kinase to cytochrome oxidase among 13 vertebrate species (Christensen et al. 1994). However, the high ratio found in anoxia-tolerant species is to a large extent due to a low aerobic capacity (a low cytochrome oxidase activity) rather than to a high glycolytic capacity (a high pyruvate kinase activity) (Christensen et al. 1994). Accordingly, a comparison of heart muscle from 15 vertebrate species shows that the glycolytic capacity first increases and then levels off as the aerobic capacity increases (Driedzic et al. 1987). Thus, hypoxic tolerance seems predominantly to relate to a low energy turnover, rather than to a high glycolytic flux per se (Driedzic et al. 1987; Overgaard and Gesser 2004; Farrell and Stecyk 2007).

Glycolysis is also of importance in the oxygenated heart where it protects contractility and electro-mechanical coupling in a way not necessarily reflected in metabolic capacity data (Lorenz and Paul 1997; Gesser 2002; Kockskamper et al. 2005; Farrar et al. 2006, Battiprolu et al. 2007). In heart muscle from both turtle and rainbow trout glycogen appears to be the main substrate during severe hypoxia (Reeves 1963; Battiprolu et al. 2007), whereas access to glucose seems to improve contractility during oxygenation (Battiprolu et al. 2007). The situation may, however, vary with species as well as conditions. Hence, in the anoxic turtle heart a shift towards glucose utilization appears as the contractile performance approaches its maximum (Reeves 1963), and the cardiac glucose uptake increased fivefold during

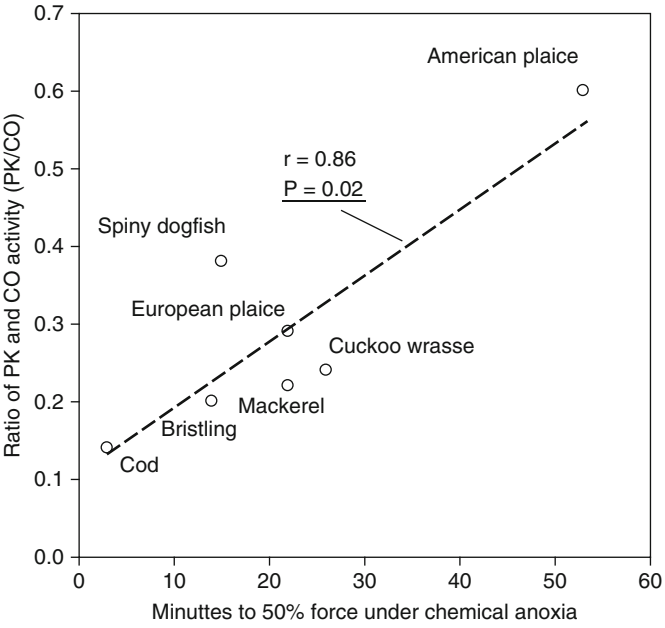

Fig. 5 Relationship between the relative glycolytic capacity and hypoxia tolerance in seven species of marine fish: cod (*Gadus morrhua*), bristling (Sprattus sprattus), mackerel (*Scomber scomber*), spiny dogfish (Squalus acanthias), European plaice (Pleuronectes platessa), cuckoo wrasse (*Labrus ossifragus*), and American plaice (*Hippoglossoides platessoides*). The relative glycolytic capacity is calculated from the ratio of pyruvate kinase and cytochrome oxidase activity (PK/CO). Here it is assumed that pyruvate kinase activity is a proxy for glycolytic capacity, and cytochrome oxidase activity is a proxy for oxidative capacity. Data are from Gesser and Poupa 1974

hypoxia in sculpins in which the receptors for acetylcholine and adenosine were blocked (MacCormack and Driedzic 2007).

Another remarkable feature of the enzymatic complement of the heart muscle from hypoxia-tolerant species is the high activity of creatine kinase (Christensen et al. 1994). Creatine kinase may be crucial due to its role in the cellular energy distribution relating to cellular compartmentation. Accumulating evidence indicates that the cell contains separated units, in which processes consuming and producing energy are tightly coupled (e.g., Saks et al. 2006; Birkedal and Gesser 2006). Thus, the creatine kinase reaction functions as a "spatial buffer" because it facilitates the maintenance of sufficient diffusion rates of ATP and ADP between ATP-consuming and -producing regions within the myocyte. A high creatine kinase activity will therefore limit local reductions in energy state at the ATPase sites. Similarly, the creatine kinase reaction can also counteract local acidification at the ATPase sites as the rephosphorylation of ADP to ATP binds one H^+ (Wallimann et al. 1992). Indirect evidence for the significance of both creatine kinase and glycolysis under hypoxia was the elevated myocardial activities of both creatine kinase and pyruvate kinase in turtles that had been acclimated to anoxic conditions (Birkedal and Gesser 2004).

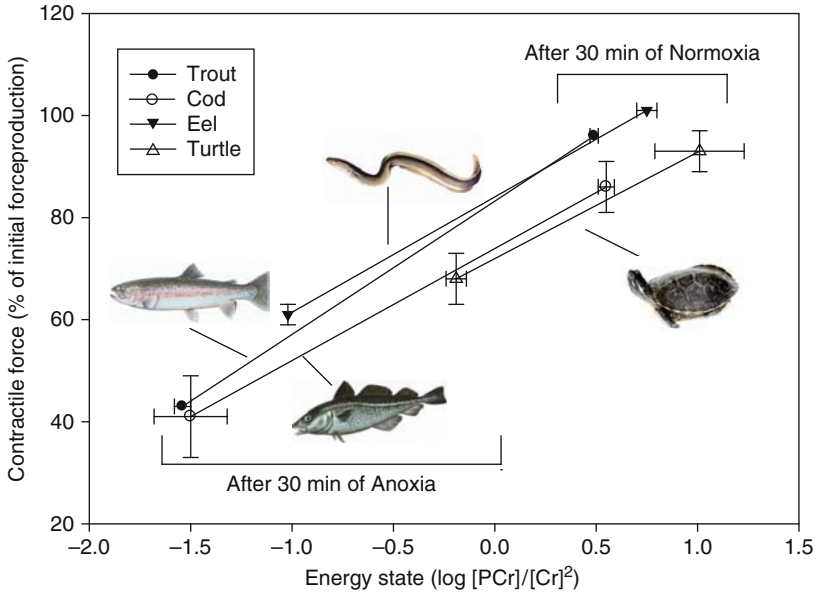

Fig. 6 Relationship between contractile force and energy state in ventricular preparations from four vertebrate species (cod, eel, trout and turtle). Data are from Hartmund and Gesser, 1996. The ventricular preparations were exposed to either 30 mins of normoxia (*right side*) or 30 min of anoxia (*left side*). Force is expressed relative to that produced at the onset of the experiment, and the energy state is approximated by the ratio of $\log([PCr]/[Cr]^2)$ (*see text for details*). The figure illustrates that the hypoxia-tolerant species (turtle and eel) are just as sensitive to decreases in energy state as the hypoxia-sensitive species. However, the tolerant species are characterised by the ability to maintain energy state significantly higher during anoxia, and therefore they retain the ability to produce contractile force under these adverse conditions

4 Cellular Energy State and Contractile Force

When cellular respiration is impaired by removal of oxygen or application of cyanide (chemical anoxia), the myocardium typically responds with a decrease in force production and/or contraction frequency. Resting tension (i.e., force during diastole) either increases or stays unchanged, as exemplified in Fig. 1. These changes follow upon decreases in cellular energy state, ΔG_{ATP}. Indeed, contractility during severe hypoxia correlates closely with the cellular energy state as exemplified in Fig. 6. Thus, in a study of four vertebrate species, contractile force and the estimated ΔG_{ATP} fell together along a regression line that did not differ significantly for turtle, eel and cod, whereas the regression line for trout tended to be steeper (Hartmund and Gesser 1996).

Energy state (ΔG_{ATP}) is defined as the free energy released by hydrolysis of ATP to ADP and P_i:

$$\Delta G_{\mathrm{ATP}} = \Delta G^{0}_{\mathrm{ATP}} - RT \ln \frac{[\mathrm{ATP}]}{[\mathrm{ADP}][P_{\mathrm{i}}]}.$$

The calculation of ΔG_{ATP} is based on the free concentrations of the reactants and, except for ADP, they can for example be measured by NMR. The free concentration of ADP can not be directly measured by available techniques, and ΔG_{ATP} is therefore often assessed using the creatine kinase reaction:

$$P\mathrm{Cr} + \mathrm{ADP} + \mathrm{H}^{+} \rightarrow \mathrm{Cr} + \mathrm{ATP}.$$

Assuming that the creatine kinase reaction is close to equilibrium; that [ATP], [*P*Cr] and [Cr] are "free"; and that the bulk of cellular P_{i} comes from the hydrolysis of creatine phosphate, the term $\frac{[\mathrm{ATP}]}{[\mathrm{ADP}][P_{\mathrm{i}}]}$ may be approximated by the expression $K\mathrm{eq} \times \frac{[P\mathrm{Cr}]}{[\mathrm{Cr}]^2}$ (Meyer 1988).

The creatine kinase reaction is strongly directed to ATP formation as shown by the apparent equilibrium constant, *K*eq, being >100 (Teague et al. 1996). It is clearly important, therefore, not to evaluate the energy state from [ATP] alone, as $\frac{[\mathrm{ATP}]}{[\mathrm{ADP}][P_{\mathrm{i}}]}$ determines the amount of energy released by ATP hydrolysis. Hence, the ATP concentration may decrease by only 10–20% during anoxia while the energy state may be severely reduced through large fractional increases in "free" [ADP] and phosphate (Wasser et al. 1990; Jackson et al. 1995; Hartmund and Gesser 1996; Overgaard and Gesser 2004).

Since the contractility of heart muscle from different vertebrates seems to be affected similarly with decreasing energy state (Fig. 6) (Hartmund and Gesser 1996), it can be argued that it is the maintenance of energy state rather than the tolerance to a low energy state that differs between anoxia-tolerant and -intolerant species (van den Thillart 1989; Hartmund and Gesser 1996). As long as the energy consumption exceeds the energy production (See Figs. 3 and 4) in the hypoxic myocardium, the energy state will decrease while ADP and phosphate will increase. Increases in ADP and phosphate, both of which are important substrates in glycolysis and oxidative phosphorylation, will stimulate the anaerobic metabolism (Pasteur effect) so that a balance between energy-consuming and -producing processes is approached (Hardie 2000; Andrienko et al. 2003). Thus, the truly hypoxia-tolerant vertebrates are not necessarily characterized by a high anaerobic capacity, but rather by the ability to match energy production and consumption at a relatively high energy state (Figs. 4, 5, and 6) (van den Thillart 1989; Wasser et al. 1990; Buck et al. 1998; Overgaard and Gesser 2004).

Cardiac contractility and its regulation are probably less affected by a lowering of the energy state itself than by factors associated with it, such as increases in free inorganic phosphate and decreases in pH due to a net hydrolysis of creatine phosphate and lactic acidosis (Fig. 7). Both these changes lower myofibrillar Ca^{2+} sensitivity (Driedzic and Gesser 1994; Fukuda et al. 2001; Crampin and Smith 2006) and inhibit the power stroke step in the myosin–actin cycle (Cooke and Pate 1985; Godt and Nosek 1989; Westerblad et al. 2002). It is worthy of note that the cellular content of phosphocreatine and thus the release of free phosphate may

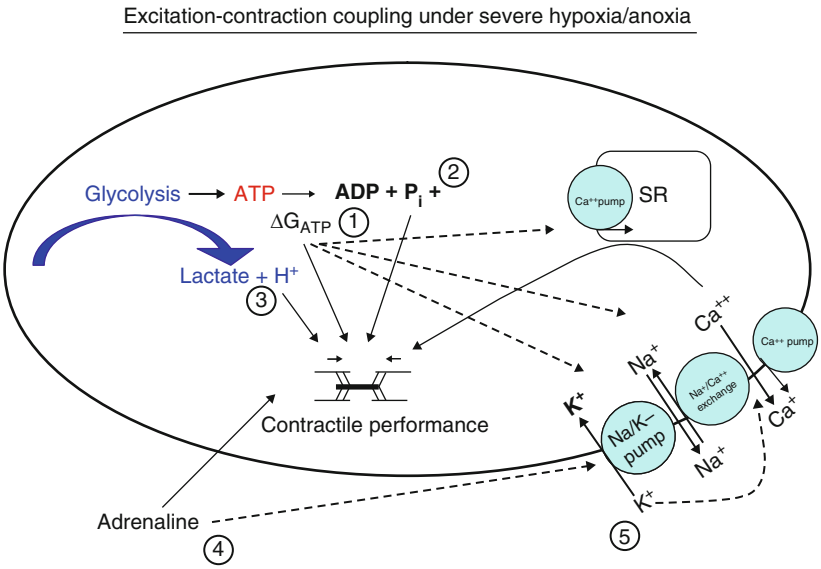

Fig. 7 Direct and indirect effects of hypoxia on excitation–contraction coupling in cardiac cells. A decrease in energy state under hypoxia may directly affect the ATP hydrolysis associated with the actin–myosin interaction, or it may act indirectly by affecting the activity of ion-motive pumps in the sarcolemma or sarcoplasmatic reticulum (*1 in circle*). Increased concentrations of ADP and P_i associated with hypoxia will also directly reduce the power stroke of the actin myosin interaction (*2 in circle*). The increased concentration of protons associated with anaerobic metabolism will also reduce the contractile performance through competitive binding of hydrogen ions to the Ca^{2+} binding sites of troponin C (*3 in circle*).These effects may be counteracted by a hypoxia-induced release of adrenaline, which increases the ATP regeneration and the Ca^{2+} transient, and stimulates Na^+/K^+ ATPase and the maintenance of transsarcolemmal ion balances (*4 in circle*). Nevertheless, a progressive increase in K^+ may develop during long-term anoxia in turtles in particular, and this will depolarise the membrane and shorten the ventricular action potential, whereby Ca^{2+} influx and contractility are depressed (*5 in circle*)

differ among species. For instance, the phosphocreatine store in heart tissue from the hypoxia-tolerant freshwater turtle is relatively small compared to other ectothermic vertebrates (Christensen et al. 1994). As a consequence of the low phosphocreatine stores, a decrease in ΔG_{ATP} will only result in a relatively small release of phosphate. Furthermore it seems that the impact of phosphate on contractility is reduced at decreased cellular energy states in the freshwater turtle myocardium (Jensen and Gesser 1999).

5 Cardiac Excitation–Contraction (E, C) Coupling

The vertebrate heart muscle works as a syncytium, i.e., the contractile proteins of all its cells are activated simultaneously by elevations of the cytosolic Ca^{2+} activity of which the magnitude and duration determine the degree of activation. The Ca^{2+}

activity is governed mainly by the membrane ion transport mechanisms indicated in Fig. 7. An inward Na^+ current drives the membrane potential to positive values and opens L- type Ca^{2+} channels through which Ca^{2+} enters the cell down its electrochemical gradient. This inward current is balanced by an outward K^+ current so that the membrane potential stabilizes at a plateau. Here the Na^+–Ca^{2+} exchange (Fig. 7) may contribute to the Ca^{2+} influx due to a reversal of the combined gradients for Na^+ and Ca^{2+} (Vornanen 1999; Hove-Madsen et al. 2003; Galli et al. 2006b). The action potential is terminated when an increased outward K^+ current repolarizes the cells and closes the L-type Ca^{2+} channels (Bers 2002). The Ca^{2+} entered across the sarcolemma elevates the cytosolic Ca^{2+} activity and causes the Ca^{2+} transient, which activates the contractile proteins either alone or after having been amplified by a Ca^{2+} induced Ca^{2+} release from the sarcoplasmatic reticulum (SR) (Fig. 7). The SR provides most of the activator Ca^{2+} in the mammalian and bird cardiac cells, while the Ca^{2+} entered across the sarcolemmal typically predominates in ectothermic vertebrates, and in many ectothermic species it is uncertain if the SR contribution is of any significance (Tibbits et al. 1990; Thomas et al. 1996; Bers 2002). The Ca^{2+} activating contractility enters the cytosol passively along its electrochemical gradient. In contrast, its removal and in turn relaxation depend on energy-requiring processes such as the Na^+–Ca^{2+} exchanger and Ca^{2+}–ATPases in the sarcolemma and the SR membrane (Fig. 7) (Bers 2002). Here the $Na^+ - Ca^{2+}$ exchanger is driven by the Na^+ gradient provided by the Na^+/K^+- ATPase. Mainly because of the energy requiring Ca^{2+} removal, the E–C coupling has been estimated to consume up to 20–30% of the energy supporting the contractile activity (Kammermeier 1997; Gibbs 2003).

The E–C coupling is the main determinant of mechanical activity. Studies of action potential and membrane currents in heart muscle from ectothermic vertebrates such as crucian carp, goldfish and freshwater turtle suggest that the influence of hypoxia on the E–C-coupling is complex and species-dependent. As in the case of endothermic vertebrates (Vleugels et al. 1976), the action potential was shortened under hypoxia in myocardium from goldfish. This shortening probably reduces activation of the contractile apparatus and has been ascribed to an opening of sarcolemmal ATP dependent potassium channels (K_{ATP}) mediated by nitric oxide, NO, and c-GMP following a lowered ATP/ADP ratio (Cameron et al. 2003; Chen et al. 2005).

A lowered energy state is most probably the prime cause for the opening of the K_{ATP} channels in both the sarcolemma and the inner mitochondrial membrane (Noma 1983; MacCormack and Driedzic, 2002; Cameron et al. 2003; Chen et al. 2005). However, the role of the mitochondrial K_{ATP} channels is not settled (e.g., Dröse et al. 2006). It has been suggested that they are involved in increasing the viability of hypoxic myocardial cells (Chen et al. 2005), and mitochondrial K_{ATP} channels have also been linked to hypoxic preconditioning (Yellon et al. 1998). In yellowtail flounder myocardium, anoxia induced an increase in twitch force, which was abolished in the presence of diazoxide, an inhibitor of the opening of mitochondrial K_{ATP} channels (MacCormack and Driedzic 2002). When mitochondrial K_{ATP} channels open, they allow a flux of K^+ into the mitochondrial matrix, which

depolarizes the mitochondria and causes Ca^{2+} release (Holmuhamedov et al. 1998). Thus, it has been suggested that mitochondrial Ca^{2+} release occurs during hypoxia and/or acidosis in ectothermic vertebrates (Gesser and Poupa 1978; Bowser et al. 1998; MacCormack and Driedzic 2002), and such a release may also be behind the finding (Vornanen 1999) that the action potential of ventricle cells from the anoxia-tolerant crucian carp was prolonged instead of shortened, upon inhibition of the cell respiration. This prolongation appeared to be due to an inward current through the Na^{+}–Ca^{2+} exchanger following a release of some intracellular Ca^{2+} store.

In turtles kept under prolonged anoxia at 21°C, the duration of action potential is prolonged, and the density of the inward sarcolemmal Ca^{2+} current decreased in ventricular but not atrial cells. No effects appeared for cells from turtles subjected to prolonged anoxia at 5°C, which is closer to the temperature experienced by the turtles during winter hibernation in ice-covered ponds. Low temperature itself, however, entailed a substantial increase in the resting membrane potential to less negative values; a several-fold prolongation of the action potential; a decreased density of the calcium current through the L-channels; and a lowered spontaneous contraction rate. These changes may represent a preacclimation to the poor availability of oxygen and energy during hibernation. Thus, the prolongation of the action potential seems to correlate with a decreased frequency of spontaneous contraction and thus with a lowered energy demand (Stecyk et al. 2007). The functional significance of these effects associated with hypoxia on the duration of action potential and on ion currents is unclear, although they may protect the cellular energy state by lowering both twitch-force development and heart rate, and the energy expenditure of both myosin ATPase and ion transport (Stecyk et al. 2007).

6 Factors Influencing Cardiac Contractility During Anoxia

Decreased ATP production in the hypoxic myocardium may reduce contractility directly through changes in energy state, $[P_i]$ or [ADP], but hypoxic/anoxic conditions may also affect contractility indirectly through changes in the intra- and extracellular compartments. Increased $[H^{+}]$ associated with lactic acid formation will, for example, directly influence contractility, and acidification may also have indirect effects by increasing plasma Ca^{2+} in some ectothermic vertebrates (Ruben and Bennett 1981; Jackson and Heisler 1982). In both turtle and trout, severe hypoxia is associated with a large increase in circulating catecholamines, which may enhance contractility (Wasser and Jackson 1991; Perry and Reid 1994). Finally, prolonged metabolic depression in hibernating freshwater turtles may increase extracellular potassium, which will depolarize the plasma membrane and shorten the cardiac action potential (Ultsch and Jackson 1982; Nielsen and Gesser 2001; Overgaard et al. 2005). Hyperkalemia may also arise during intense activity, which for the teleost heart may entail hypoxia. Thus, hypoxia or anoxia is associated with a number of changes in the intra- and extracellular compartments that may alter

mechanical activity in the heart. The isolated and combined effects of these intra- and extracellular disturbances have recently been reviewed with respect to the freshwater turtle myocardium (Overgaard et al. 2007), and below we briefly discuss the general influence of these factors on myocardial contractility.

6.1 Acidosis

Anoxia is typically associated with a progressive extra- and intracellular acidosis (Allen et al. 1985; Wasser et al. 1990; Jackson et al. 1995). Acidosis affects several parts of the E–C coupling and is generally believed to exert negative inotropic effects on mammalian cardiac muscle which relate to a depression of myofilament Ca^{2+} responsiveness (Orchard and Kentish 1990; Crampin and Smith 2006). This decrease in Ca^{2+} responsiveness is caused by the competitive binding of hydrogen ions to the Ca^{2+} binding sites of troponin C (see Crampin and Smith 2006).

Acidosis markedly decreases cardiac twitch force in most fish, while it induces more modest effects in many air-breathing ectotherms such as, for example, freshwater turtles (Poupa et al. 1978; Gesser and Poupa 1983; Overgaard et al. 2005; 2007). In a few species, such as flounder and the marine toad, acidosis has even been reported to exert positive inotropic effects (Gesser and Jørgensen 1982; Andersen et al. 2004). Thus, it has been suggested that the negative effects of acidosis are counteracted by an increase in intracellular Ca^{2+}, possibly from mitrochondrial stores (Gesser and Poupa 1978; Driedzic and Gesser 1994). The effects of acidosis may also be influenced by their interaction with other factors; thus, lactic acidosis did not influence contractility in ventricular preparations from freshwater turtles at 25°C, but it did lead to a ~30% reduction in twitch force at 5°C. Hence, it is possible that acidosis impairs contractility more during overwintering than at higher temperature. Furthermore, anoxia and acidosis in combination typically reduce heart muscle performance much more than they do when acting separately. Thus, severe acidosis may inhibit glycolysis and thereby contribute to a reduction in cellular energy state. (Wasser et al. 1990; Jackson et al. 1995; Shi and Jackson 1997; Shi et al. 1999; Bobb and Jackson 2005). Finally, it should be noted that the type of acidosis is of importance: metabolic acidosis seems to have less dramatic effects on contractility than respiratory acidosis (Driedzic and Gesser 1994).

6.2 Hyperkalemia

Anoxia and hypoxia may be linked to increased levels of extracellular potassium ($[K^+]_0$) in some species. Hyperkalemia may occur during intense activity where, for example, teleost hearts may also experience hypoxia. However, the detrimental effects of hyperkalemia are mainly of relevance for animals such as freshwater turtles, where increasing $[K^+]_0$ reflects an inability of the Na^+/K^+ pump to maintain a normal transcellular cation distribution during long-term anoxia (Jackson and Ultsch 1982, Ultsch et al. 1999; Reese et al. 2002). In these situations $[K^+]_0$

may increase above 10 mM, which will severely depress twitch force (Nielsen and Gesser 2001; Kalinin and Gesser 2002; Overgaard et al. 2005; Gesser 2006). Elevated $[K^+]_0$ depolarizes the membrane potential and shortens the ventricular action potential, which reduces Ca^{2+} influx and contractility (Paterson et al. 1993; Nielsen and Gesser 2001). In painted turtles, an elevation of $[K^+]_0$ from 2.5 to 10 mM reduces twitch force of ventricular strips by 50–95% (Overgaard et al. 2005), and it also results in unstable contractions and lowering of the maximal frequency at which regular contractions may be produced (Nielsen and Gesser 2001; Kalinin and Gesser 2002; Overgaard et al. 2005; Gesser 2006). In fact, hyperkalemia appears to be of major importance as it seems to be the most potent depressor of cardiac force development in cold anoxic turtles. Thus, it is possible that a progressive increase in potassium levels may ultimately compromise sustained cardiac activity in overwintering turtles (Overgaard et al. 2005).

6.3 Regulation of Hypoxic Contractility by Adrenaline and Ca^{2+}

Myocardial contractility can be stimulated by catecholamines and/or elevations of extracellular Ca^{2+}, which increase the intracellular Ca^{2+} transients during the myocardial action potential (e.g., Kitazawa 1984; Bers 2002). For the mammalian myocardium this response seems to be considerably depressed by hypoxia, but the situation may be different for the myocardium of ectothermic vertebrates. Hence the myocardium from both eel and rainbow trout displayed a pronounced increase in isometric force in response to adrenaline under both oxygenated and anoxic conditions (e.g., Gesser et al. 1982). Similarly, an elevation of extracellular Ca^{2+} from 1.25 to 5 mM stimulated force development and glycolysis during anoxia in trout heart muscle (Nielsen and Gesser 1983). Likewise, the combination of adrenaline and elevations of extracellular Ca^{2+} stimulated force development in anoxic myocardial preparations from freshwater turtle (Overgaard et al. 2005). Clearly, there is room for considerable improvements of contractility during anoxia which indicates that anoxia does not necessarily elicit the full anaerobic capacity in the heart muscle of many ectothermic vertebrates.

Adrenergic stimulation increases Ca^{2+} currents through L-type Ca^{2+}-channels in the sarcolemma, and thereby exerts a positive inotropic effect (Bers 2002). Indeed, adrenergic stimulation increases the duration of the action potential in turtles, although this was only significant when the action potential had previously been compromised by hyperkalemia (Nielsen and Gesser 2001), and adrenaline increases twitch force in ventricular strips from *Trachemys* (Ball and Hicks 1996; Nielsen and Gesser 2001; Overgaard et al. 2005; Galli et al. 2006a). These effects are consistent with the rise in stroke volume, which occurs upon adrenergic stimulation in vivo (Hicks and Wang 1998; Overgaard et al. 2002). The positive effects on contractility induced by catecholamines and elevations of extracellular Ca^{2+} are of physiological interest. Hence the spongy myocardium of fish would be expected to receive less oxygen and experience oxygen lack in situations with high

animal activity, which typically involves catecholamine stimulation. Furthermore, the freshwater turtle hibernating with low or no access to oxygen has been shown to have increased levels of both catecholamines (Wasser and Jackson 1991; Keiver et al. 1992) and large increases in extracellular Ca^{2+}. Here Ca^{2+} is released as a result of the progressive buffering by calcium-carbonate stores in the carapace (see Jackson 2002). An increase in plasma Ca^{2+} may also occur in other species (Ruben and Bennett 1981). For both adrenergic stimulation and hypercalcemia, the potential positive effects may be more prominent in alleviating other negative inotropic agents associated with anoxia, tending to reduce sarcolemmal Ca^{2+} influx. Indeed, hypercalcemia and in particular adrenaline have been shown to alleviate negative inotropic effects of hyperkalemia and acidosis in freshwater turtles and several teleost species (Yee and Jackson 1984; Jackson 1987; Nielsen and Gesser 2001; Overgaard et al. 2005; Gesser 2006).

7 Conclusion

The mechanical performance of the heart muscle under hypoxia/anoxia varies largely among species in a way that mostly, but not always, can be related to the environment and behavior of the animal. It is positively correlated to the glycolytic capacity relative to the aerobic capacity and tends to be high in hearts having a low aerobic or total working capacity. Despite the restricted energy production, hypoxic/anoxic performance often responds strongly to positive inotropic agents such as adrenaline. Frequently, mechanical performance appears to be less reduced by the oxygen lack itself than by other factors such as increases in extracellular K^+ commonly associated with hypoxia/anoxia in the living animal. The relation between mechanical performance and the excitation–contraction coupling during hypoxia/anoxia appears ambiguous.

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Control of the Heart and of Cardiorespiratory Interactions in Ectothermic Vertebrates

E.W. Taylor and T. Wang

Abstract The functional anatomy of the respiratory and cardiovascular systems and the neuro-anatomy and neuro-physiology of the systems that are implicated in the co-ordination of cardiac output with ventilation are reviewed in fish, including air-breathing fish, amphibians and reptiles. Recent data is reviewed in the light of previous studies on mammals. This account focuses on the roles of the autonomic nervous system in both feed-forward and feedback control of the respiration-related, beat-to-beat changes in heart rate that accompany continuous, rhythmical breathing as well as the marked changes in heart rate associated with bouts of discontinuous breathing. The control of cardiac shunting in species with incompletely separated systemic and pulmonary blood flow is also described.

1 Introduction

Vertebrates supply oxygen to the tissues and remove metabolically produced CO_2 through a concerted action of the respiratory and cardiovascular systems. An appropriate coordination of these systems is, therefore, important for effective gas exchange, and it is essential that both systems can respond in a coordinated manner when metabolic demands change or when gas composition in the environment is altered. It is not surprising, therefore, that the cardiovascular and respiratory systems of all vertebrates are functionally linked. For example, changes in heart rate with ventilation occur in all vertebrate groups and, in spite of the large anatomical differences, it is believed that the essential components of the cardiorespiratory control systems are similar among the different vertebrate taxa (e.g. Taylor et al. 1999). For example, in mammals, heart rate rises during inspiration, a phenomenon termed

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respiratory sinus arrhythmia (RSA), while fish may show periods of synchrony between heart beat and ventilation (CRS).

As a common feature of vertebrates, the respiratory rhythm is generated in the brainstem, while the heart beat is initiated by pacemaker cells at the sinoatrial node, or more diffusively within the vena cava. Cardiorespiratory interactions, therefore, arise from coordination of activities in the central respiratory rhythm and the cardiac pacemaker. Ventilatory and cardiovascular responses are initiated by feedback from peripheral and central chemo- and/or mechanoreceptors that sense, for example, changes in blood gas composition or lung stretch. Central integration of the afferent feedback from these receptors ultimately modifies motor output to the respiratory muscles and the autonomic regulation of the heart and blood vessels. In addition to reflexes that are initiated or mediated by afferent receptor input, cardiorespiratory interactions can be generated within the central nervous system (CNS) and, thus, serve as a feed-forward mechanism. These interactions occur in mammals in the nucleus ambiguus (NA), an area of the brainstem situated ventrolaterally from the dorsal motor nucleus of the vagus (DVN), where the bulk of cardiac vagal preganglionic neurons (CVPN) are situated (Jordan and Spyer 1987). Clear evidence has been obtained for two functional populations of CVPN in dogfish (Barrett and Taylor 1985c) and then in mammals (Daly and Kirkman 1989) and exploration of similar distributions and roles for CVPN in other non-mammalian vertebrates is a central theme of this review, briefly considered by Taylor (1993). Roles for reflex and central mechanisms are, of course, not mutually exclusive and it is entirely possible that both occur simultaneously in all groups of vertebrates. Although many ectothermic vertebrates exhibit changes in heart rate and blood flow during ventilation that are much larger than commonly observed in mammals, the underlying mechanisms are better understood in mammals than in other groups of vertebrates. Thus, in a series of studies on cross-perfused dogs, where the effects of lung stretch receptor feedback could be dissociated from central irradiation of ventilatory activity to the cardiovascular center, Anrep et al. (1936a, b) firmly established that both mechanisms contribute significantly to RSA in mammals. Now it is widely recognized that the following factors contribute to RSA in mammals:

(1) Central communication between the respiratory centres and the cardiac vagal motoneuron pools in the medulla, acting as feed-forward control
(2) Activation of pulmonary stretch receptor stimulation during ventilation acting as a feedback signal
(3) Modulation from CO_2- and O_2-sensitive chemoreceptors
(4) Mechanical effects on the heart and associated blood vessels due to ventilatory movements of the thoracic cavity

Here we will review the interactions between ventilation, heart rate and central vascular blood flows in the major groups of ectothermic vertebrates (i.e. excluding birds and mammals). A general model of these interactions is given in a schematic diagram in Fig. 1. Apart from giving a general introduction to the range of mechanisms underlying the control of cardiorespiratory interactions in each group, we have

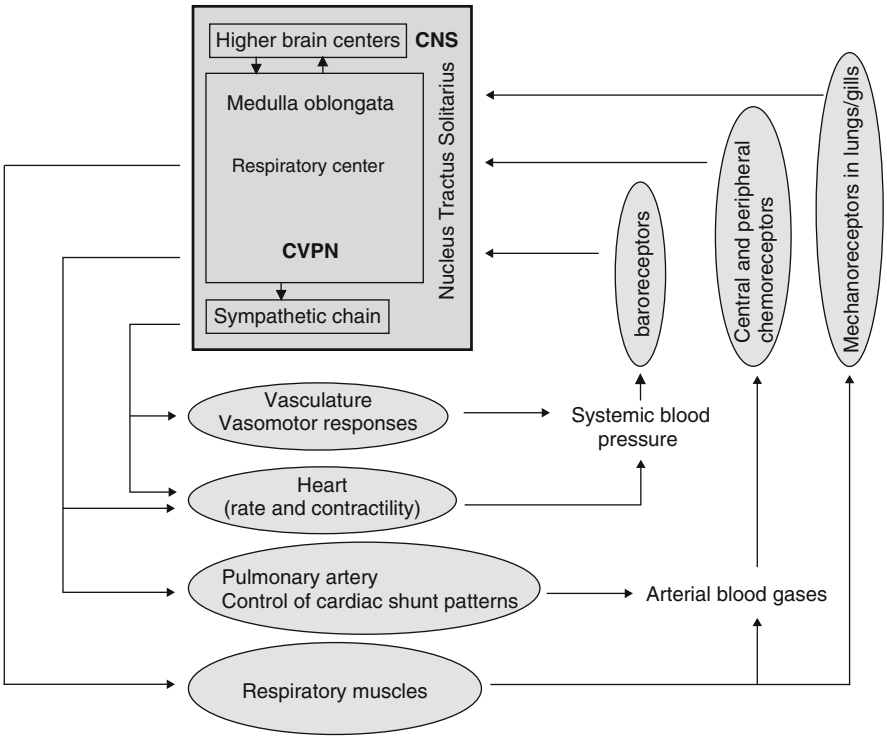


Fig. 1 Diagram of the major components mediating cardiorespiratory interactions in vertebrates. The respiratory rhythm is generated within the medulla oblongata, and leads to subsequent activation of respiratory muscles. Ventilation stimulates mechanoreceptors in the respiratory apparatus (gills or lungs), which, together with chemoreceptors sensing ambient and blood gas levels, provide feedback to the respiratory oscillator. Cardiovascular changes accompanying ventilation may arise from central interactions between the respiratory and cardiovascular centers within the central nervous system (CNS), as well as from feedback from central and/or peripheral chemoreceptors. In amphibians and reptiles, control of blood gases is also influenced by changes in pulmonary blood flow and cardiac shunt patterns. Variations in arterial blood pressure, resulting from altered cardiac output and changes in vasomotor tone, are sensed by baroreceptors. All of these inputs are integrated in the nucleus tractus solitarius (NTS). The heart and the pulmonary artery are innervated by the vagus, which exerts an inhibitory action on heart rate and causes a constriction of the pulmonary artery that increases pulmonary vascular resistance and induces right-to-left cardiac shunts. The sympathetic innervation normally increases both rate and contractility of the heart, and may lead to a minor relaxation of the sphincter surrounding the pulmonary artery

chosen a few examples to illustrate these mechanisms. Tonic and rapid phasic control of the heart that is likely to generate cardiorespiratory interactions is predominantly parasympathetic (Bootsma et al. 1994; Campbell et al. 2004, 2006). Accordingly, this synoptic review will concentrate on cardiac control from the parasympathetic nervous system, via the Xth cranial nerve, the vagus, and its interactions with activity in the nerves supplying the cranial respiratory muscles in fish and amphibians and the thoracic respiratory apparatus in reptiles. Some information

derived from mammalian studies is included to clarify our current understanding of certain mechanisms. For more detailed accounts of this topic the reader is directed to recent reviews (Taylor et al. 1999, 2001; Wang et al. 1999, 2001b).

2 Efferent Innervation of the Vertebrate Heart

The innervation of the heart has undergone marked evolutionary changes within vertebrates and may be viewed as a gradual transition from an aneural heart in hagfish, which are the most primitive extant craniates, to the dual innervation that is characteristic of all higher vertebrates (Fig. 2). Thus, while the heart of hagfish (myxinoids) does not appear to be innervated (Augustinsson et al. 1956; Jensen 1965), and seems insensitive to the classic neurotransmitters, the lamprey heart receives vagal innervation. In contrast to all vertebrates, however, this vagal innervation is excitatory, and is mediated by acetylcholine acting on nicotinic cholinoceptors (Taylor et al. 1999).

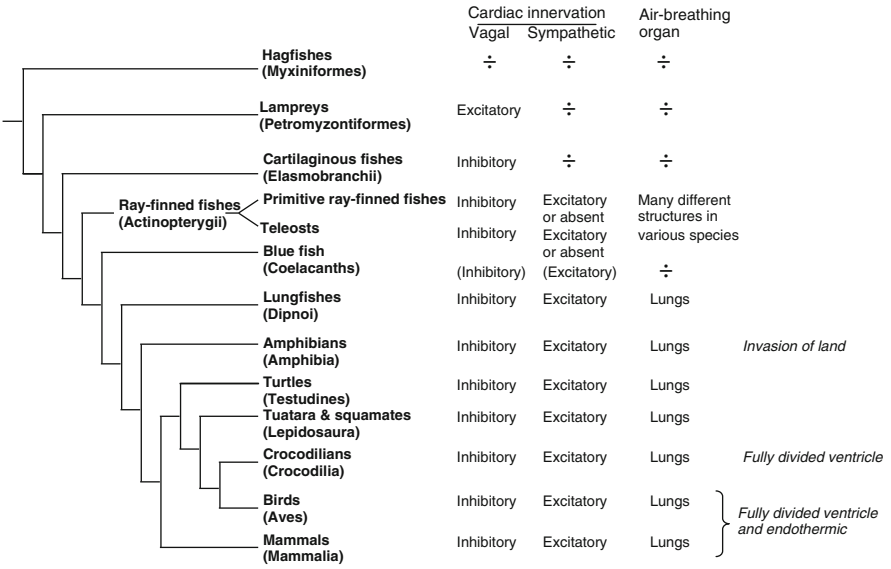


Fig. 2 Phylogeny of the cardiac innervation and air-breathing organs in the major groups of living craniates. The heart of hagfishes is aneural, while the heart of lampreys receives an excitatory vagal innervation. An inhibitory vagal innervation that relies on activation of muscarinic receptors on the heart appeared in cartilaginous fishes and was retained in all other vertebrates. The excitatory sympathetic innervation is present in most but not all ray-finned fishes, and all other higher vertebrates. Air-breathing organs evolved independently in several groups of ray-finned fishes, while true lungs first appeared within lungfishes and are found in all tetrapods. Endothermy seems to have evolved independently in birds and mammals

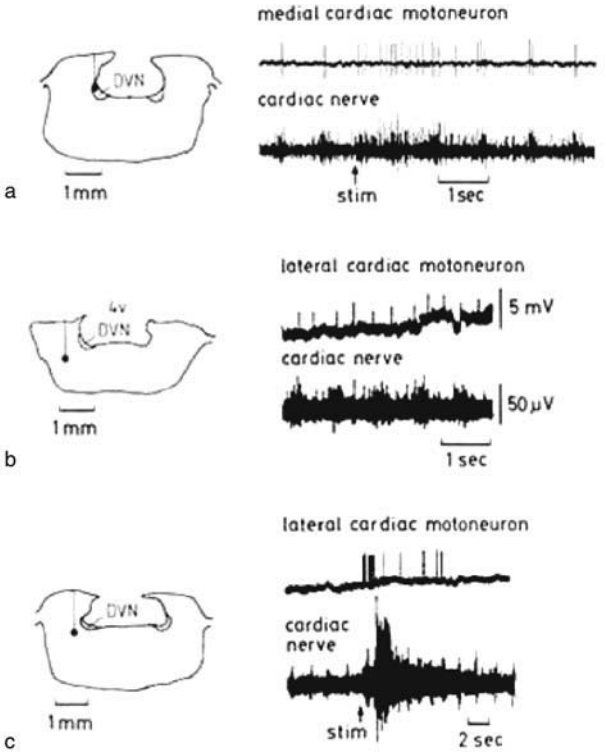
Elasmobranchs are the earliest group known to have an inhibitory vagal innervation of the heart, but there is no direct sympathetic innervation. Circulating catecholamines, however, are important in cardiac control (Randall and Taylor 1991). A dual innervation of the heart appeared in ray-finned fishes (Actinopterygii), and while sympathetic innervation may have been subsequently lost in some cases such as sturgeons, the combination of vagal inhibition and sympathetic stimulation is characteristic of all higher vertebrates. The inhibitory parasympathetic input from the tenth cranial nerve (the vagus) uses acetylcholine (ACh) as neurotransmitter that acts on muscarinic receptors and can be blocked by the muscarinic receptor antagonist atropine. This innervation predominantly affects heart rate. The excitatory sympathetic input from spinal nerves, on the other hand, stimulates both rate and force of contraction, and uses adrenaline or noradrenaline as neurotransmitter. These catecholamines act on β-adrenergic receptors that can be blocked by antagonists such as propranolol or sotalol.

There are plenty of studies showing that infusion of ACh or catecholamines mimic the effects of vagal or sympathetic stimulation respectively, but it is also well-established that the autonomic nervous system of many lower vertebrates uses a number of other agents in addition to the classic neurotransmitters. There is evidence for purinergic control of the heart in dogfish, while neuropeptide Y modulates its cardiac responses to noradrenaline (Xiang et al. 1994). In amphibians, there is a particular prevalence of non-adrenergic-non-cholinergic (NANC) factors (see Morris and Nilsson 1994), and in snakes a NANC factor seems to accelerate heart rate following a meal (Wang et al. 2001a). Within the context of cardiorespiratory interactions, the existence of these transmitter substances implies that the control exerted via the autonomic nervous system is subject to modulation by endocrine and endogenous factors.

2.1 Elasmobranchs

The well-developed autonomic nervous system of sharks and rays is clearly differentiated into parasympathetic and sympathetic components (Taylor 1992), but the sympathetic nervous system does not extend into the “head” (Young 1950). As a result, there is no direct sympathetic innervation of the heart or the gills, and nervous control of the heart and cardiorespiratory interactions is restricted to the inhibitory vagal innervation. Although variations in cholinergic vagal tonus on the heart are the sole source of nervous cardioregulation in elasmobranchs (Butler and Taylor 1971; Taylor et al. 1977), circulating catecholamines have been shown to modulate the sensitivity of the heart to parasympathetic control (Agnisola et al. 2003).

Injection of neural tracers to identify vagal preganglionic neurons (VPN) in the brainstem of the dogfish, *Scyliorhinus canicula* revealed that about 90% were located in the dorsal motor nucleus of the vagus (DVN). A clearly distinguishable group of cells with scattered distributions outside of the DVN that comprised 8% of the total population of VPN were identified as cardiac VPN (CVPN), innervating the heart via the branchial cardiac nerve (Fig. 3). They constitute about 45% of CVPN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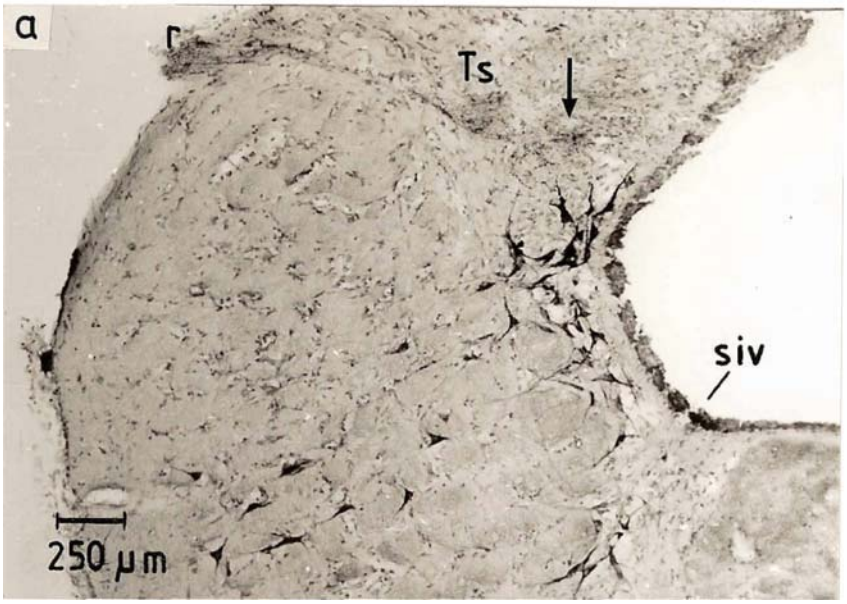
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with the rest located in the DVN, where they have an overlapping rostro-caudal distribution with neurones supplying respiratory muscles in the gill arches (Barrett and Taylor 1985b; Taylor 1992; Taylor et al. 2009c) This dual location of CVPN has been shown to have important functional implications (see below).

2.2 Teleosts

The sympathetic chains extend into the head in teleosts, where they contact cranial nerves, and form vagosympathetic trunk with the vagal fibers that exert cardioinhibitory control (e.g. Gannon and Burnstock 1969). Thus, teleosts may be considered the earliest group of vertebrates with both sympathetic and parasympathetic control of the heart (Taylor 1992 and see Fig. 2). However, the influence of the sympathetic innervation on the fish heart varies between species and is often virtually absent, so that parasympathetic influences predominate, particularly in the generation of cardiorespiratory interactions (Fig. 2).

A similar distribution of VPN and CVPN to that described above for the dogfish has been described in the cod, *Gadus morhua* and trout, *Oncorhynchus mykiss* (Withington-Wray et al. 1987). About 12% of VPN were located outside the DVN and, although most of these were CVPN, some of them innervated the gills, possibly supplying vasomotor input to branchial blood vessels (Taylor 1992). A current study of pacu, *Piaractus mesopotamicus* (E.W. Taylor and C.A.C. Leite, unpublished observations) revealed CVPN distributed in separate nuclei within the DVN, a ventral group containing about 60% of cell bodies, and a dorsal group containing about 40% of cell bodies. In addition, there were a small number of cell bodies scattered laterally outside of the DVN, constituting only about 2% of the total number of CVPN. So the neuranatomical basis for control of the heart and CRI is not consistent within the teleost fishes. In pacu, the CVPN had overlapping rostro-caudal distributions with motoneurons having their axons in the VIIth, IXth and Xth cranial nerves, supplying respiratory muscles, but not with those supplying the mandibular branch of the Vth cranial nerve (Fig. 4 and see Taylor et al. 2009b).

Fig. 3 (Continued) **a** A transverse section taken through the brainstem of the dogfish, *Scyliorhinus canicula*, to show cardiac vagal preganglionic neuron cell bodies (CVPN) stained black with horseradish peroxide, after its application to a cardiac nerve. They are located in the DVN close to the 4th ventricle (grouped directly below the *vertical arrow*) and in scattered locations ventrolateral to the DVN (*r*, rootlet of vagus nerve; *siv*, sulcus intermedius ventralis in the wall of the fourth ventricle; *Ts*, sensory projection from the cardiac vagus). **a–c** Extracellular recordings from CVPN in the brainstem of the dogfish, identified by antidromic stimulation of the cardiac nerve followed by dye injection (the course of the electrode and the recording position are shown by the *line* and *filled circle*); together with simultaneous recordings of activity in the ipselateral cardiac nerve. A unit located in the DVN (**a**) fired in bursts that contributed to those recorded from the cardiac nerve and responded to mechanical stimulation of a contra-lateral gill septum (stim). Units recorded from lateral locations, outside the DVN, fired regularly (**b**), sporadically, or were silent (**c**), except when responding to mechanical stimulation of a gill septum (stim). (Taken from Taylor 1992)

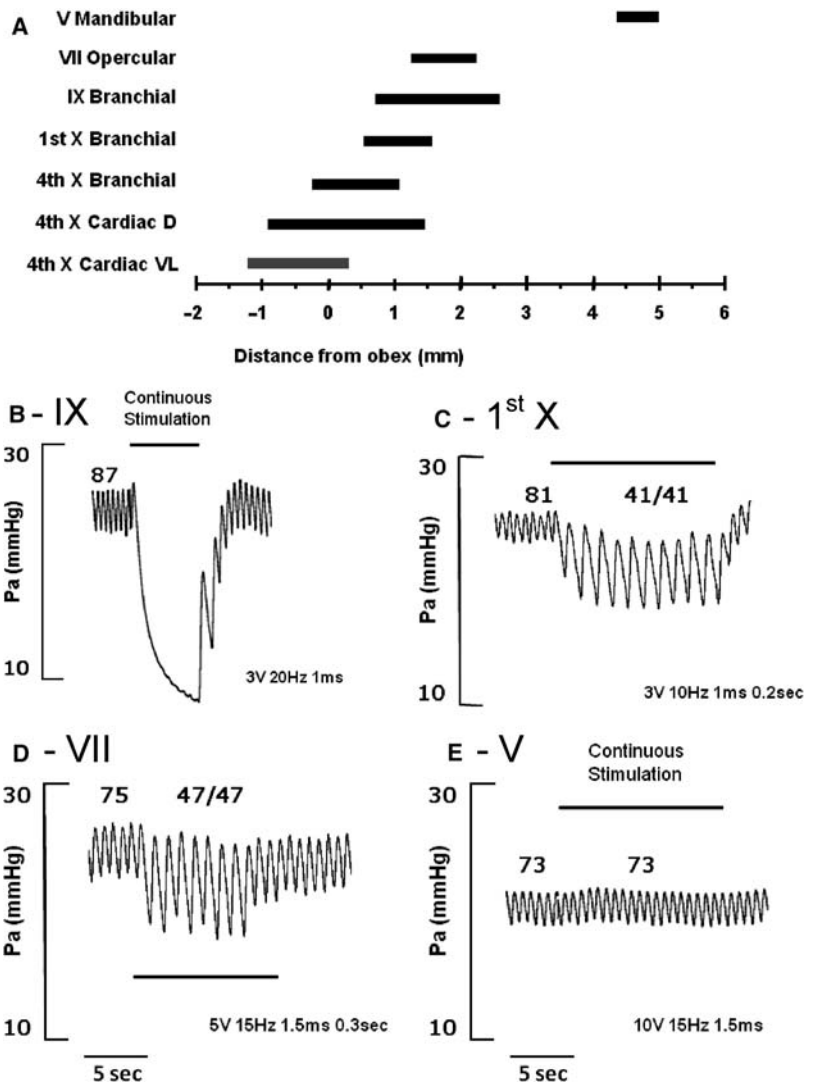

Fig. 4 **a** The rostro-caudal distribution in the brainstem of the pacu, *Piaractus mesopotamicus,* of cell bodies of respiratory visceral motor neurones (RVMN) supplying axons to the mandibular Vth, opercular VII, branchial IX, first and fourth branchial X, together with the CVPN in the DVN (Cardiac D) and ventro-lateral group (Cardiac VL). Data are taken from fish with the best fills (largest number of labelled cell bodies). Effects on heart rate, measured as blood pressure, of electrical stimulation of the central cut ends of: *B*, the glossopharyngeal IXth; *C*, the branchial Xth; *D*, the facial VIIth; and *E*, the mandibular Vth cranial nerves. The period of stimulation is indicated by the *horizontal bar above* or *beneath* each trace and the stimulation parameters are given *below* each trace. The initial, intrinsic heart rate (bpm), followed by the bursting rate of the electrical stimuli and the consequent heart rate are given *above* each trace. Continuous stimulation of the IXth caused cardiac arrest, but stimulation of the Vth was without effect. Phasic stimulation of the VIIth, XIIth or Xth recruited heart rate to the applied bursting frequency. (Taken from Taylor et al. 2009c)

2.3 Air-breathing Fish

Application of a neural tracer to branchial branches of the vagus nerve in the bowfin, *Amia calva*, labelled cell bodies in the dorsal motor nucleus of the vagus (DVN) and in lateral locations outside the DVN, as described in teleosts (e.g. cod) and all tetrapod vertebrates (Taylor et al. 1999). The nerve supplying the glottis and ABO had cell bodies in a ventrolateral location in the brainstem and the ventral horn of the anterior spinal cord. From their location it was possible to identify them as cell bodies which typically supply axons to the hypobranchial nerve that provides nerves to elements of musculature normally associated with feeding movements. In water-breathing fish these are used to gulp air at the surface. In addition, the ABO was innervated by a group of cell bodies in the DVN that may provide efferent axons to smooth muscle in the swimbladder wall, comparable with the vagal efferents controlling reflex broncho-constriction in the mammal (Taylor et al. 1996).

2.4 Amphibians

In the aquatic amphibian *Xenopus laevis*, the vagal motonucleus is located in the medulla oblongata over a rostro-caudal distance of about 4 mm, either side of obex, with over 90% of neuron cell bodies rostral of obex. A sensory projection of the vagus was revealed, by anterograde transport of HRP along afferent axons, with diffuse labelling of the dorsal visceral sensory nucleus over a rostro-caudal distance of about 2 mm, rostral of obex in an area identified as the nucleus of the solitary tract (NTS) (Wang et al. 1999). All branches of the vagus in *Xenopus* are supplied with efferent axons by neurons with their cell bodies, either in a medial nucleus, within the central grey, equivalent to the mammalian dorsal vagal motonucleus (DVN), or in a ventro-lateral nucleus, outside the central grey, which may be the amphibian equivalent of the nucleus ambiguus (NA). The cardiac vagus had 26% of its CVPM in the NA. Cardiac and pulmonary vagal motoneurons showed a largely overlapping distribution. In the axolotl *Ambystoma mexicanum*, the neotenous adult had no VPN outside of the DVN. Following metamorphosis, induced by injection of thyroxine, the number of VPN increased, and 15% were now located outside of the central grey matter. This ventro-lateral migration was accompanied by migration onto land and a switch to lung breathing (Taylor et al. 1999, 2001).

The neuranatomical evidence that cardiac and respiratory VPM are located in close proximity in the anuran brainstem implies that some cardiorespiratory interactions in anurans may be generated by synaptic interactions between central neurons generating efferent outflow to the heart and respiratory apparatus. This may be of particular importance in amphibians as their major respiratory muscles, as well as the airways, are innervated by cranial nerves, including the vagus, which have their cell bodies in the brainstem, in close proximity to CVPM. However, at present, there is no physiological evidence for such a synaptic interaction between

the populations of vagal preganglionic motoneurons controlling cardiovascular and respiratory function in amphibians.

As in mammals, the amphibian heart is innervated by spinal sympathetic stimulatory and cranial (vagal) parasympathetic inhibitory nerves. The sympathetic nerves join the vagus nerve before reaching the heart to form a vago-sympathetic trunk that innervates the atria, the ventricle and the sinus venosus. The vagosympathetic trunk also innervates a sphincter immediately distal to the point where the pulmocutaneous artery divides into the pulmonary artery proper and the cutaneous vessel (de Saint-Aubain and Wingstrand 1979; Morris and Nilsson 1994). This sphincter consists of concentrically arranged layer of smooth muscle cells, and provides a means to control pulmonary blood flow by redirecting cardiac output into the systemic circulation (Wang et al. 1999). Consistent with this pattern of innervation, injection of the muscarinic blocker, atropine, increases heart rate and pulmonary blood flow, while adrenergic blockade causes an immediate decrease in heart rate (Taylor and Ihmied 1995; Gamperl et al. 1999). Vagal tone on the heart dominates over-sympathetic tone in *Xenopus* (Taylor and Ihmied 1995) and the adrenergic regulation of the pulmonary circulation seems less important than the vagal tone. However, the extent to which withdrawal of vagal tone versus increased sympathetic tone accounts for the increased heart rate and pulmonary blood flows associated with ventilation in amphibians has not been determined. Numerous studies demonstrate the presence of other neurotransmitters than ACh and catecholamines in the autonomic nervous innervation of the cardiovascular system in amphibians, and atropine, for example, cannot fully block the decrease in heart rate as a result of vagal stimulation in *Bufo* (e.g. Preston and Courtice 1995). The existence of these NANC factors implies that it is technically difficult to study cardiovascular regulation in amphibians because traditional pharmacological approaches may not suffice.

2.5 Reptiles

The vagus nerve in reptiles runs to the heart, trachea, lungs, pulmonary and coronary vasculature, thymus, thyroid and gut, supplying preganglionic fibres. The vagus has an inhibitory effect on heart rate (Fig. 2), while effects on contractility appear to be rather small. The vagus also innervates smooth muscle surrounding the pulmonary artery, where increased vagal tone causes constriction and associated narrowing of the pulmonary artery. Thus, conditions of high vagal tone, characteristic of resting reptiles with low metabolic demands, are associated with low heart rates and low pulmonary blood flows, leading to right-to-left cardiac shunts where blood is recirculated into the systemic circulation. The vagal effects on both the heart and the pulmonary artery can normally be abolished with atropine, and while there is plenty of immunohistochemical evidence for the presence of NANC factors in the reptilian circulatory system, it seems that most of the efferent vagal control is mediated by ACh acting on the classic muscarinic receptors. The reptilian heart

also receives sympathetic innervation that exerts positive chronotropic and inotropic effects on both the atria and the ventricle. These effects can normally be blocked by β-adrenergic antagonists. In general, the vagus exerts larger effects on the pulmonary circulation than sympathetic innervation (Overgaard et al. 2002; Galli et al. 2007; Taylor et al. 2009), but this may depend on species, and may vary with metabolic state and certain types of behaviour.

The somatotopic representation of the vagus in reptiles is little-known and seems to vary between species. Early studies on reptiles described two divisions (medial and ventrolateral) of the vagal motor column in a variety of species, which were provisionally designated as the DVN and NA. The pattern of labelling is on the whole similar to that observed after applying neural tracers to the vagus nerve in other vertebrates with differences in the degree of representation of VPN in a lateral division. A "nucleus ambiguus" (NA) was identified adjacent to the DVN in the tortoise (Cruce and Niewenhuys 1974) with between 36 and 50% of VPN located in the NA of the terrapin (reported by Taylor et al. 1999). An initial HRP study of the vagal motor column in the agamid lizard *Uromastyx microlepis* revealed that the majority of VPN are in the DVN, with a small proportion (6%) ventrolaterally located in the NA. A putative NA was also described in a lizard and an alligator but was apparently absent from a snake (Taylor et al. 1999, 2001). However, in the rattlesnake *Crotalus durissus*, use of fluorescent markers revealed that the majority of VPN are in the DVN, with a small proportion (4%) ventrolaterally located in a putative NA. Despite the paucity of cells in the NA these reptiles showed clear respiratory modulation of heart rate (see below). The somatotropic representation of the cardiac and pulmonary innervation is virtually unknown in reptiles and further study is warranted.

The basis of the apparent variation in location of VPN is likely to be that the reptiles are not a homogeneous group, having wide evolutionary divisions separating the present-day reptiles (Taylor et al. 1999). The chelonians (turtles and tortoises) are anapsids, a group regarded as primitive, having arisen from close to the ancestral reptilian stock (evolved from primitive amphibians). The snakes and lizards are diapsids, from the same reptilian stock that produced the archosaurs. These in turn evolved into the ruling reptiles ("dinosaurs") represented today by the crocodiles and alligators and, on another evolutionary line, the birds. Birds have less than 5% of VPN outside of the DVN but 30% of CVPN are in a ventrolateral nucleus, and birds have been shown to exhibit RSA (Taylor et al. 2001). Mammals are recognised as having evolved from a separate, primitive reptilian stock, the synapsids. These were remote in evolutionary terms from the lines leading to the present-day reptiles and the birds, but may have been closer to their amphibian ancestors and to the primitive chelonians. In mammals the bulk of CVPN are located in the NA, where inhibitory influences from neighbouring inspiratory neurones are recognised as the major source of RSA (Taylor et al. 1999). Thus, the disposition of VPN may have phylogenetic as well as functional correlates, but there seems to be clear evidence that a dual location for CVPN is a factor in generating cardiorespiratory interactions in vertebrates.

3 Cardiorespiratory Interactions

3.1 Fish

In fish water and blood are delivered directly to either side of the respiratory gas exchange surfaces on the gills, and there is a close matching of respiratory water flow and cardiac output, according to their relative capacities for oxygen (the ventilation/perfusion ratio), which is thought to optimise respiratory gas exchange over the functional counter-current at the gills (Hughes and Shelton 1962; Piiper and Scheid 1977; Taylor 1992). As both water and blood flows over and within the gills are markedly pulsatile (e.g. Jones et al. 1974), close beat-to-beat temporal relationships between heart beat and ventilation or cardiorespiratory synchrony (CRS) have long been hypothesised as being important for the optimisation of respiratory gas exchange (Satchell 1960). More recent work has established direct evidence in some fishes for fine control of heart rate, including its beat-to-beat modulation by the respiratory cycle that generates cardiorespiratory interactions (CRI), culminating in CRS (Taylor 1992). In addition, more subtle modulation of heart rate by respiratory activity, termed cardiorespiratory coupling (CRC), has been demonstrated by power spectral analysis of cardiac intervals (Campbell et al. 2004; Taylor et al. 2006). Abolition of CRC by cardiac vagotomy was shown to affect oxygen uptake (Campbell and Egginton 2007).

In most fish it seems that variations in the inhibitory vagal tone, imposed by activity in cardiac vagal preganglionic neurones (CVPN) within the medulla oblongata, are the predominant factors generating cardiorespiratory interactions (Taylor et al. 1999, 2009–c). Below, we review the location of CVPN and the putative roles of feed-forward control from within the CNS and feed-back control from peripheral chemoreceptors and mechanoreceptors in determining their activity. Recordings of spontaneous efferent activity in cardiac vagi contain bursts of respiration-related activity. We have investigated the origins of this activity and its possible effects on the heart. Bursts of electrical stimuli delivered peripherally to the cardiac vagus or centrally to respiratory branches of cranial nerves VII, IX and X can recruit the heart at a range of frequencies (Taylor et al. 2006, 2009b). In elasmobranchs, phasic efferent activity in cardiac vagi that are the basis of cardiorespiratory interactions seems to originate primarily from central interactions between respiratory neurones and CVPN, and is characterised by relatively low levels of vagal tone on the heart (Taylor 1992). In teleosts the bursts seem to be driven reflexly by stimulation of peripheral chemoreceptors and mechanoreceptors when respiratory drive and cardiac vagal tone are high (Taylor et al. 1999, 2009b). These differences seem fundamental. However, reflex control from peripheral receptors is important in determining activity in CVPN, and consequently heart rate, in all fish including elasmobranchs (see Fig. 3a and c) as well as teleosts (see Fig. 4c and d), and evidence from current investigations suggests that there are elements of central feed-forward control of cardiorespiratory interactions in some teleosts as well as elasmobranchs. Consequently, it seems probable that variable combinations (relating to conditions

and possibly to species differences) of feed-forward control via central interactions, plus feed-back control from peripheral receptors, determine activity in CVPN in fish. This in turn can recruit the heart to the respiratory rhythm, though it may subserve different roles in different groups of fish.

3.2 Air-Breathing Fish

Many of the primitive ray-finned fishes are air-breathers and air-breathing also seems to have evolved numerous times within teoleosts (Graham 1997). The anatomy and physiology of the air-breathing organs vary enormously among species, but it is characteristic that heart rate increases during air-breathing. This tachycardia probably contributes to a temporal matching of perfusion and ventilation of the air-breathing organ, although the significance of the putative function of this matching remains to be studied experimentally. Thus, it is noteworthy that complete pharmacological abolition of the heart rate changes in the air-breathing jeju does not affect oxygen uptake (McKenzie et al. 2007), and it is clearly of interest to investigate the functional correlates of cardiorespiratory interactions in air-breathing fishes.

In lungfish (dipnoi), a vagal innervation of the heart is well established, whilst the sympathetic chain seems poorly developed (Axelsson et al. 1989). In the African lungfish *Protopterus sp*, there were very small changes in heart rate during the intermittent ventilation of the lungs, while heart rate increased both in the South American and in the Australian lungfishes, *Lepidosiren paradoxa* and *Neoceratodus forsteri* (Johansen and Hanson 1968; Axelsson et al. 1989; Fritsche et al. 1993; Sanchez et al. 2001). The rather small influence of lung ventilation may, at least in part, reflect the surgical difficulties associated with physiological instrumentation of these animals, and studies on minimally instrumented lungfish would be of great interest. In any event, although heart rate does not change much with breathing, pulmonary ventilation is associated with a marked rise in pulmonary blood flow in all species of lungfish (Johansen et al. 1968a; Fishman et al. 1985; Burggren and Johansen 1986; Axelsson et al. 1989; Fritsche et al. 1993). This rise in pulmonary blood flow is caused by a combination of β-adrenergic and cholinergic mechanism (Johansen and Reite 1968) but the afferent signals leading to the vascomotor responses remains to be studied. Lungfish are endowed with pulmonary stretch receptors as well as both central and peripheral chemoreceptors making it likely that feedback from these receptors is involved (Delaney et al. 1983).

Changes in heart rate during ventilation are also evident in *Arapaima gigas*, an obligatory air-breathing, osteoglossid fish that relies on the heavily vascularised swimbladder for gas exchange (Farrell 1978). In the jeju (*Hoplerythrinus unitaeniatus*), a teleost fish that also uses a modified swimbladder as air-breathing organ, heart rate decreases during expiration, but increases drastically during the subsequent inhalation that inflates the swim bladder (Farrell 1978; McKenzie et al. 2007). These heart rate changes were primarily of cholinergic origin and an in-depth analysis of the heart rate variability in this species indicated that the heart rate

changes were qualitatively similar to RSA in higher vertebrates (McKenzie et al. 2007). Air-breathing also causes heart rate to increase in the climbing perch where the air-breathing organ is located in a suprabranchial cavity (Singh and Hughes 1973), and similar responses have been reported for the electric eel and two related species of synbranchid eels, all of which use the buccopharyngeal cavity for gas exchange (Johansen et al. 1968b; Graham et al. 1995; Skals et al. 2006). An example of the rapid heart rate change upon an air-breath in *Synbranchus marmoratus* is shown in Fig. 5 (Skals et al. 2006). As seen in this example, expansion of the buccopharyngeal cavity leads to a marked rise in heart rate, which is attended by a rise in central venous pressure, which is likely to preserve cardiac filling, keeping stroke volume constant in spite of the decreased filling time (Skals et al. 2006).

While changes in cholinergic tone seem to dominate the afferent regulation of the heart rate changes associated with air-breathing in both primitive and derived fishes, the efferent mechanisms remain to be studied in more detail. However, in the electrical eel as well as the synbranchid eels, manual inflation of the buccopharyngeal cavity elicits heart-rate changes that are similar to those occurring during spontaneous air-breathing (e.g. Graham et al. 1995). This unequivocal evidence for stretch receptor feedback being involved in the regulation of the cardiorespiratory interactions is interesting, given the large variation in respiratory structures among the various air-breathing fish, and it would be interesting to identify the central projections in an array of different air-breathing fishes. The apparent central role of stretch receptor feedback obviously does not exclude the possibility of centrally generated interactions leading to feed-forward control. Also, it is quite likely that inputs from chemoreceptors are involved. Thus, as shown in the example of Synbranchus (Fig. 4), which is typical for other species of air-breathing fish as well, heart rate decreases progressively during the breath hold. During this time, PO_2 of the gas within the air-breathing organ decreases while CO_2 accumulates to a lesser extent, given that it may be excreted to the water over the gills or accumulates in body fluids.

3.3 Amphibians

The larvae of all amphibians are aquatic, but the gills are lost in virtually all species during development, but the adult forms maintain bimodal gas exchange where the structurally simple lungs are supplemented with cutaneous gas exchange. The lungs are ventilated by muscles inserted around the bucco-pharangeal cavity, innervated by efferent motor output from cranial nerves, which generate a positive pressure driving air into the lungs. The respiratory rhythm is generated within the central nervous system (CNS) and is modulated by peripheral chemo- and mechanoreceptors as well as central chemoreceptors in the medulla. The cardiovascular system of amphibians, as well as reptiles, is complex because the ventricle is undivided, allowing for mixture of divided ventricle with blood flow to the lungs, skin and body controlled independently. Thus, cardiorespiratory responses

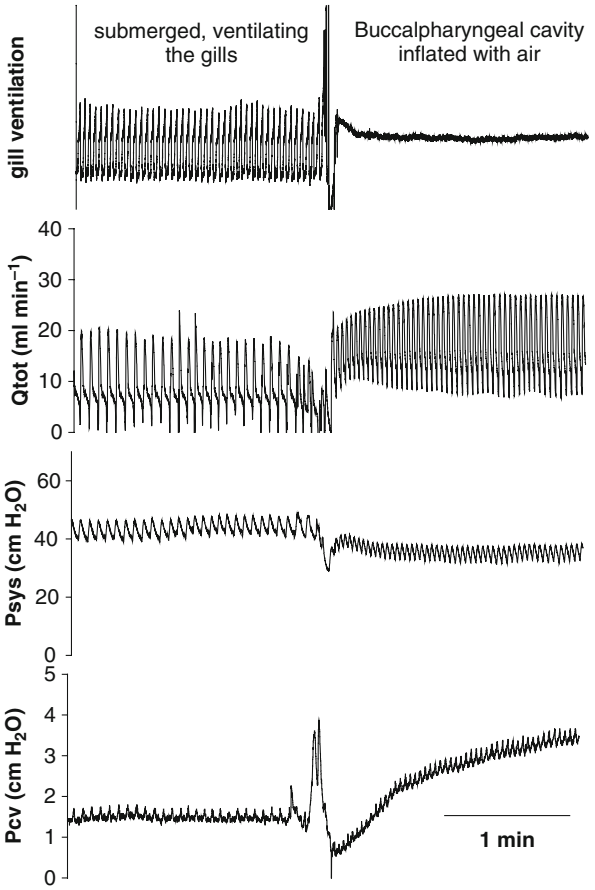

Fig. 5 The changes in heart rate and blood flow in the ventral aorta associated with the transition from aquatic gill ventilation to inflation of the buccopharyngeal cavity with air in the air-breathing fish *Synbranchus marmoratus*. Both heart rate and cardiac output (Qtot) increase rapidly in association with the air-breath because stroke volume is maintained by an increase in central venous pressure (*Pcv*) that probably is caused by α-adrenergic venous constriction (*Psys* is the pressure in the dorsal aorta). (Taken from Skals et al. 2006)

to increased metabolism or hypoxia can consist of increased ventilation, increased heart rate and/or redistributions of blood flows (see Wood 1984; Wang and Hicks 1996b).

In addition to the control of heart rate, the vagus (cranial nerve X) also regulates vascular resistance in the pulmonary artery which, in turn, affect cardiac shunts within the undivided anuran ventricle. Thus, increased vagal tone increases resistance to pulmonary blood flow and induces large right-to-left shunts and leads to lower oxygen concentrations in the arterial blood. Thus, in combination with the lower heart rate, high vagal tone is associated with a reduction in systemic oxygen delivery, and high vagal tone and the predominance of right-to-left cardiac shunts

are indeed most prevalent in resting undisturbed animals where metabolism is low (e.g. Gamperl et al. 1999; Wang et al. 2001b). In active animals, lung ventilation is associated with a relative left-to-right shunt, causing blood flow to be redirected to the lungs (Shelton 1970). However, little is known about the control of central vagal motor outflow to the heart and pulmocutaneous artery. Anatomical evidence indicates a close proximity of the centers responsible for respiratory rhythmogenesis and the vagal motoneurons involved in cardiovascular regulation. Furthermore, anurans in which phasic feedback from chemo- and mechanoreceptors is prevented by artificial ventilation exhibit cardiorespiratory interactions that appear similar to those of conscious animals. These observations indicate interactions between respiratory and cardiovascular centres within the CNS (Wang et al. 1999). Thus, similarly to mammals and other air-breathing vertebrates, the cardiorespiratory interactions in anurans result from both feed-back and feed-forward mechanisms. Because amphibians are positioned centrally in the phylogeny of air-breathing vertebrates and they metamorphose from gill-breathing larvae to lung-breathing adults, an understanding of the mechanisms controlling their cardiorespiratory systems may reveal the fundamental properties that were associated with the evolutionary emergence of air-breathing.

3.4 Reptiles

As discussed previously, reptiles represent an ancient polyphyletic group and generalisations regarding the topography and control of their cardiorespiratory systems must be avoided. Thus, while all extant members of reptiles are ectothermic and committed air-breathers, their mechanisms for pulmonary ventilation, lung structure and morphology of the heart vary considerably among taxa. Cutaneous gas exchange contributes significantly only under special situation such as hibernation at low temperature (Feder and Burggren 1985). Turtles and tortoises (the anapsids) are considered the most primitive extant group of reptiles, and most species of this clade have poorly divided ventricles and a ventilatory mechanism that is highly specialized to take account of their shell (Landberg et al. 2003). In common with the turtles, lizards and snakes (squamates) lack a diaphragm, and lung ventilation is normally generated by intercostal muscles acting on the rib cage. A primitive buccopharyngeal or gular pump, like that described in amphibians, was considered to be used primarily for generating airflows within the oral cavity, presumably for olfaction. As lizards run in a serpentine manner, employing segmental muscles from the body wall, it was asserted by some investigators that they are unable to utilise thoracic, aspirational breathing while running. However, an alternative mode of ventilation, involving a gular pump which alternated with the costal pump, has been described in an agamid lizard (Al-Ghamdi et al. 2001) and during exercise in a varanid (Brainerd and Owerkowicz 1996; Owerkowicz et al. 1999).

Most reptiles breathe intermittently, and the breathing pattern, particularly of aquatic species, is often characterized by long-lasting breath-holds. In many of the

aquatic species, periods of pulmonary ventilation consist of several breaths taken continuously, while many terrestrial species tend to ventilate the lungs with single breaths separated by shorter intervals of apnea. Regardless of habitat, the breathing cycle is terminated by an inspiration, and the lungs therefore remain inflated during apnoea. As in amphibians, it has been suggested that the initiation of discontinuous bouts of breathing is initiated by a complex interplay of stimulation of central and peripheral chemoreceptors as PO_2 in the blood and lungs decreases and CO_2 increases, rather than by centrally generated ventilatory rhythms (Douse and Mitchell 1990; Milsom 1990). This may enable the flexibility of response essential for an ectothermic vertebrate, as the thresholds for stimulation will vary with temperature, in accord with the animal's oxygen. However, unidirectionally ventilated alligators display episodic breathing (Douse and Mitchell 1991), so that centrally generated rhythmicity may have a role in its initiation.

The cardiac ventricle of all squamates is incompletely divided, but varanid lizards and pythons have evolved the capacity for ventricular pressure separation allowing for high systemic blood pressures, while keeping the pressure in the pulmonary circulation low. In crocodilians, which are in fact more closely related to birds than the rest of the reptiles, the ventricle is anatomically divided, and pulmonary ventilation is driven by muscles of the body wall moving the liver, which is attached to a transverse connective tissue sheet resembling a mammalian diaphragm (Farmer and Carrier 2000).

Given the incomplete anatomical separation of cardiac ventricle in reptiles, there is a possibility of admixture between the oxygenated blood that returns to heart from the lungs with the oxygen-poor systemic venous blood. The direction and magnitude of these cardiac shunts vary consistently with ventilation, and all experimental studies agree that periods of apneoea are associated with low pulmonary blood flows and a net right-to-left shunt, while ventilation is associated with a reduction in the right-to-left shunt, or even a development of left-to-right shunts where blood is recirculated with the pulmonary circulation (e.g. Lillywhite and Donald 1989; Wang and Hicks 1996b). It is also clear that large right-to left shunts are characteristic of undisturbed and resting animals, whilst exercise or handling stress caused pulmonary blood flow to increase (Wang et al. 1997, 2001a). The tight correlation between heart rate and the net shunt pattern makes it very likely that the smooth muscle surrounding the pulmonary artery is innervated by the same vagal motoneurons as those innervating the heart, securing a functional coupling.

The efferent control of pulmonary blood flow and the rise in heart rate during ventilation is not well understood. Many studies have noted that heart and pulmonary blood flow tend to increase before lung ventilation is initiated, which would indicate a centrally generated release of vagal tone on the heart and the pulmonary artery. Also, artificial inflation and deflation of the lungs in anaesthetized and fully recovered turtles did not elicit cardiovascular responses in one study on turtles (Herman et al. 1997), while similar manipulation of lung volume in the same species elicited marked changes in heart rate and pulmonary blood flow in an earlier study (Johansen et al. 1977). The reason for such disparate findings is not clear, but points to a complex interaction between stretch receptor feedback from the lungs

and the efferent cardiovascular control. The role of chemoreceptor stimulation is also uncertain and needs to account for the changes in ventilatory patterns, which in itself changes pulmonary blood flow and the direct effects of hypoxia on the pulmonary vasculature (Wang et al. 1997; Skovgaard and Wang 2006)

The functional significance of the tight interaction between heart rate and cardiac shunt patterns remains enigmatic (e.g. Hicks and Wang 1996). This is even the case in turtles, which probably exhibit very pronounced heart rate and blood flow changes. Thus, when the rise in pulmonary ventilation during ventilation was prevented by mechanical occlusion of the pulmonary artery, there was no effect on oxygen uptake or CO_2 excretion in fully recovered turtles (Wang and Hicks 2008).

4 The Neural Basis of Cardiorespiratory Interactions

In mammals, heart rate varies with the respiratory cycle, accelerating during inspiration. It is known to be driven by respiration-related fluctuations in the efferent, inhibitory supply to the heart via the cardiac vagus. This is generated centrally by an inhibitory input to cardiac-vagal preganglionic neurones (CVPN) in the ventro-lateral nucleus ambiguus (NA) from inspiratory neurones in the neighbouring ventral respiratory group. The consequent gating of vagal outflow causes heart rate to rise during inspiration (Jordan and Spyer 1987). Consequently, instantaneous heart rate varies on a beat-to-beat basis with respiration and these variations in the heart rate variability signal (HRV) are termed respiratory sinus arrthymia (RSA).Its functional role in improving pulmonary oxygen uptake was recently discussed (Hayano and Yasuma 2003). Analysis of these beat-by-beat changes, using frequency domain analysis, has been recognized as an important tool for examining the underlying autonomic effectors controlling cardiac output (Campbell et al. 2004). In conscious dogs, Akselrod et al. (1981) demonstrated that random process analysis of the HRV signal provided a sensitive, quantitative measure of rapidly reacting cardiovascular control mechanisms, revealing three distinct components. These were: (1) the high-frequency component (0.3–0.4 Hz) associated with central respiratory drive and solely vagally mediated, (2) a mid-(0.1–0.3 Hz), and (3) a low (0.07–0.1 Hz)-frequency component associated with blood pressure control systems and thermal vasomotor activity respectively. Both of these latter components had a mixture of sympathetic and vagal contributions (Bootsma et al. 1994). The “polyvagal theory” has suggested that the beat-to-beat modulation of heart rate that generates RSA is restricted to mammals, which have evolved myelinated vagal pathways that originate in the NA (Porges 1995). This assertion has been contested by Grossman and Taylor (2006) who pointed out that the beat-to-beat control of cardiac interval has been reported in both resting dogfish (Taylor 1992), and hypoxic trout (Randall and Smith 1967), and that settled rattlesnakes show respiratory modulation of heart rate resembling mammalian RSA (Campbell et al. 2006). These species have CVPN located both in the DVN and in a ventro-lateral

location outside the DVN that may constitute a primitive NA (Taylor 1992). So a dual location for CVPN seems to be a common feature of the vertebrate brainstem with putative links to control of heart rate variability and cardiorespiratory interactions.

5 Fish

Cardiorespiratory interactions (CRI) have been reported in both resting dogfish, *Scyliorhinus canicula* (Taylor 1992) and hypoxic trout, *Oncorhynchus mykiss* (Randall 1966). Cardiac vagotomy or injection of atropine abolished CRS in the dogfish (Taylor 1992), while in the sculpin, *Myoxocephalus scorpius*, injection of atropine raised mean heart rate in normoxia and abolished a hypoxic bradycardia, while cardiac vagotomy abolished heart rate variability (Campbell et al. 2004). These observations confirm the dependence of beat-to-beat variability of heart rate on tonic vagal control. However, the neurological basis for CRI in fishes in still largely unresolved.

6 Elasmobranchs

Recordings from the central cut end of a branchial cardiac branch of the vagus in decerebrate, paralysed dogfish revealed high levels of spontaneous efferent activity that could be attributed to two types of unit (Taylor and Butler 1982; Barrett and Taylor 1985a,c). Some units fired sporadically and increased their firing rate during hypoxia. Injection of capsaicin into the ventilatory stream of the dogfish, which was accompanied by a marked bradycardia, powerfully stimulated activity in these non-bursting units recorded from the central cut end of the cardiac vagus (Jones et al. 1993). Consequently, we suggested they may initiate reflex changes in heart rate, as well as play a role in the determination of the overall level of vagal tone on the heart. Other, typically larger, units fired in rhythmical bursts which were synchronous with ventilatory movements (Taylor and Butler 1982; Barrett and Taylor 1985a). We hypothesised that these units, showing respiration-related activity which was unaffected by hypoxia, may serve to synchronise heart beat with ventilation (Taylor 1992).

The separation of efferent cardiac vagal activity into respiration-related and non-respiration-related units was discovered to have a basis in the distribution of their neuron cell bodies in the brainstem, described above. Direct connections between bursting CVPN and RVM are possible in the dogfish hindbrain, as both are located in the DVN with an overlapping rostro-caudal distribution (Taylor 1992; Taylor et al. 1999). Extracellular recordings from CVPN identified in the hindbrain of decerebrate, paralysed dogfish by antidromic stimulation of a branchial cardiac branch revealed that neurons located in the DVN were spontaneously active, firing in

rhythmical bursts which contributed to the respiration-related bursts recorded from the intact nerve (Barrett and Taylor 1985c and see Fig. 3). Neurons located ventrolaterally outside of the DVN were either spontaneously active, firing regularly or sporadically but never rhythmically, or were silent (Fig. 3). Thus, the two types of efferent activity recorded from the cardiac nerve arise from the separate groups of CVPN, as identified by neuranatomical studies (Taylor 1992). There is clear experimental evidence for central, feed-forward control of CRI in dogfish, as well as reflexcontrol originating from mechanoreceptors on the gills (Barrett and Taylor 1985c; Taylor 1992 and see Fig. 3). Thus, in the intact fish, normal breathing movements that stimulate peripheral mechanoreceptors on the gills may, by a reflex pathway, generate activity in CVPN and consequently in the cardiac vagi, affecting heart rate. This implies that the typical reflex bradycardia in response to hypoxia may arise both directly, following stimulation of peripheral chemoreceptors, and indirectly, via increased ventilation, which by stimulating branchial mechanoreceptors may increase vagal outflow to the heart. This is reminiscent of, but opposite in kind to, the hypoxic response in the mammal, where stimulation of lung stretch receptors causes an increase in heart rate (Daly and Scott 1962). As well as slowing the heart, respiration-related efferent activity in the cardiac vagi may entrain the heart (Taylor et al. 2006, 2009c).

7 Teleosts

Most studies on teleosts have stressed the importance of inputs from peripheral receptors in the genesis of cardiorespiratory synchrony (Randall 1966; Randall and Smith 1967). In pacu, *Piaractus mesopotamicus*, respiration-related, bursting activity was only recorded from the cardiac vagus in normoxic fish when they were hyperventilating or coughing, implying that the bursts arise reflexly, following stimulation of branchial mechanoreceptors. In fish rendered moderately hypoxic by reduction of the flow of water irrigating the gills, a period of spontaneously increased ventilatory amplitude was accompanied by respiration-related bursts of activity in the cardiac vagus, which were not apparent in the inactive, normoxic fish, and appeared to recruit the heart. In both pacu and dogfish, phasic peripheral stimulation of the cardiac vagus or central stimulation of respiratory branches of cranial nerves VII, IX and X entrained the heart over a wide range of frequencies (Fig. 4). However, central stimulation of the mandibular branch of the Vth cranial nerve, innervating the jaw was without effect on heart rate, possibly reflecting the fact that the distribution of motoneurones supplying this nerve does not overlap with CVPN. Nevertheless, activity in the cardiac vagus was synchronous with activity in the Vth cranial nerve, and both anticipated activity in other cranial nerves supplying respiratory muscles, implying that central feed-forward control was determining this relationship (Taylor et al. 2009c).

8 Amphibians

The intermittent ventilatory patterns of anurans are often associated with concomitant cardiovascular changes. Thus, a number of studies have shown that heart rate increases upon inflation of the lungs, and it is also clear that the pulmonary blood flow increases (reviewed by Wang et al. 1999). These cardiovascular responses are primarily caused by release of vagal tone on the heart and pulmonary artery. Thus, vagotomy or atropine injection reduces or abolishes cardiorespiratory coupling; however, the underlying mechanisms are not known. Cardiorespiratory coupling in amphibians may originate from direct influences of activity in the centres responsible for respiratory rhythm generation on the cardiac and pulmonary arterial vagal motoneurons. These interactions occur within the CNS and are independent of afferent feedback. Thus, in decerebrated and paralyzed toads (*Bufo marinus*), where the lungs were unidirectionally ventilated to maintain constant degree of lung inflation, pulmonary blood flow increased during periods of ventilatory activity in the respiratory muscles innervating the buccal cavity (Wang et al. 2004). An example of such a response is shown Fig. 6.

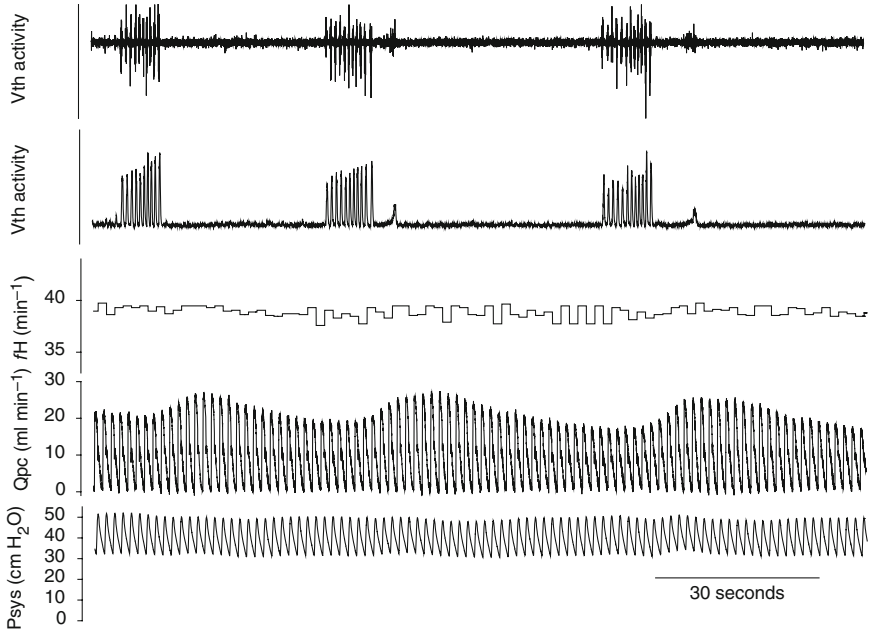

Fig. 6 Cardiovascular changes associated with ventilatory activity in a decerebrated and paralysed toad (*Bufo marinus*). Ventilatory activity was measured as nervous activity in the fifth cranial nerve (*two upper traces*), which innervates the respiratory muscles in the buccal cavity. Because the toad was unidirectionally ventilated and paralysed, there were no changes in afferent input during bouts of fictive ventilation, and the rise in pulmocutaneous blood flow (Qpc) and the attendant changes in systemic blood flow during ventilation seems to be caused by central feed-forward mechanisms. There were no obvious changes in systemic blood pressure (*Psys*) or heart rate (*fH*). (Taken from Wang et al. 2004)

Stretch receptor feedback from the lungs, however, is also important for the cardiovascular responses associated with lung ventilation, and inflation of the lungs of anurans often elicits a rise in pulmonary blood flow and heart rate (e.g. West and Burggren 1984; West and Van Vliet 1992) During breathing, changes in lung volume stimulate pulmonary stretch receptors and it is possible that their afferent activity serves as a feedback mechanism to the cardiovascular centres that, in turn, release cardiac vagal tone on the heart and pulmonary artery. In some experiments on anaesthetized *Bufo, Rana* and *Xenopus*, artificial lung inflation elicited cardiovascular responses that were similar to those observed during spontaneous breathing, and these responses were abolished following atropine injection and during deep anesthesia (West and Burggren 1984; Wang et al. 1999). Changes in thoracic pressure and volume during pulmonary ventilation may also alter the venous return to the heart and change cardiac output through the classical Frank-Starling mechanism (e.g. Segura et al. 1981). Apart from nervous control, resistance to pulmonary blood flow can be altered within the pulmonary circulation per se, where lung gas composition may exert a direct influence on vascular tone (West and Burggren 1984).

9 Reptiles

Reptiles are typically periodic breathers, and during bouts of breathing the degree of shunting of blood flow to the lung increases. Vasomotor control is important in diverting blood between the pulmonary and systemic systems. In turtles and lizards, the net direction and magnitude of shunt flow are affected by resistance in the pulmonary circuit, relative to the systemic circuit, by active vagal, cholinergic regulation of pulmonary arterial resistance. Control of pulmonary blood flow in reptiles is achieved by vagal cholinergic constriction of the pulmonary artery. Peripheral electrical stimulation of the vagus or intravenous injection of acetylcholine each result in bradycardia and an increase in pulmonary vascular resistance, which reduces pulmonary blood flow (Taylor et al. 1999). These cardiovascular changes are abolished by administration of atropine. The rattlesnake, *Crotalus durissus*, has a single lung and functional pulmonary arch, designated as the right arch, though it is innervated by the left vagus. Peripheral electrical stimulation of the left vagus slowed the heart and stopped blood flow to the pulmonary arch (Taylor et al. 2009). Blood flow is also under adrenergic control. In *Boa constrictor* vagal, cholinergic tone predominated over sympathetic, adrenergic tone in inactive animals. The increase in heart rate during enforced activity was due largely to complete withdrawal of inhibitory vagal tone, while the increase following a meal was mediated by small changes in overall autonomic tone with evidence of involvement of non-adrenergic-non-cholinergic (NANC) factors (Wang et al. 2001a).

Reptiles probably exhibit the most pronounced cardiovascular changes associated with ventilation of all vertebrate groups. Thus, a tachycardia during ventilation is characteristic of most species belonging to all major groups of reptiles (e.g.

Johansen 1959; Andersen 1961; Pough 1969; Huggins et al. 1970; Jacob and McDonald 1976; Heatwole 1977; Jacob 1980). The heart rate changes seem most pronounced in aquatic species, and an early description of the tachycardia during ventilation in turtles even suggested that bradycardia during breath-hold represent the normal state (Belkin 1964). In the free-diving turtle *Trachemys scripta*, pulmonary blood flow increased more than threefold at the onset of breathing, during recovery from breath-holds lasting longer than 5 min (Wang and Hicks 1996a). Systemic blood flow also increased during ventilation. These increases were accomplished entirely through changes in heart rate during ventilation, with stroke volume unchanged. Systemic blood flow always exceeded pulmonary flow, so that a net right-to-left cardiac shunt prevailed, regardless of ventilatory state. Nevertheless, because pulmonary flow increased markedly during ventilation, the ratio of pulmonary to systemic flow increased from 0.3 to 0.8. In both the turtle, *Pseudemys scripta*, and the tortoise, *Testudo graeca*, the onset of lung ventilation was closely accompanied by a tachycardia (Burggren 1975). As stimulation of pulmonary stretch receptors, arterial chemoreceptors, and baroreceptors or water receptors was without effect on heart rate, it was concluded that this ventilation tachycardia resulted from central interactions between respiratory and cardiac neurons in the medulla. However, lung stretch receptor afferents have been recorded from the vagus nerve of reptiles (Sundin et al. 2001). All changes in heart rate were mediated by alterations in vagal tone.

The heart rate changes with ventilation disappear upon blockade of the autonomic nervous innervation of the heart and are clearly regulated. Wang et al. (2001a, b) showed that there are slight changes in fH related to lung ventilation in snakes, but it is uncertain whether these components formed distinct oscillations in fH at the frequency of fV, and could therefore be categorised as RSA. On the basis of power spectral analysis of heart rate, Gonzalez and De Vera (1988) argued that there was no spectral component of the heart rate signal with ventilation in a small lizard *Galloti galloti*. However, the use of fH dataloggers for long-term monitoring in undisturbed rattlesnakes (Campbell et al. 2006) enabled us to determine HRV in settled, recovered animals when sympathetic tonus was low and vagal tonus high. These animals showed oscillatory components in the HRV signal at the frequency of ventilation that were abolished by injection of atropine. Results from this study agreed in part with Gonzalez and De Vera (1988), in that two peaks were detected in the fH spectra of the rattlesnake. However, the frequency and amplitude of these peaks were relative to fH, with high fH favouring the lower frequency peaks and low fH the high frequency peaks. The removal of these peaks, independently, by pharmacological blockade was accompanied by the associated changes in fH, suggesting that these peaks represented sympathetic and parasympathetic drive on the heart. Furthermore, the high peaks, that were removed by the cholinergic blocker atropine, indicated an oscillatory component in HRV that occurred at the frequency of the respiratory cycle. The respiratory cycle of rattlesnakes consists of a prolonged inspiration followed by a relatively short expiration. Heart rate slowed upon expiration and increased during inspiration, which is similar to the changes in heart rate observed in conscious unrestrained mammals and characterized as RSA (Hayano

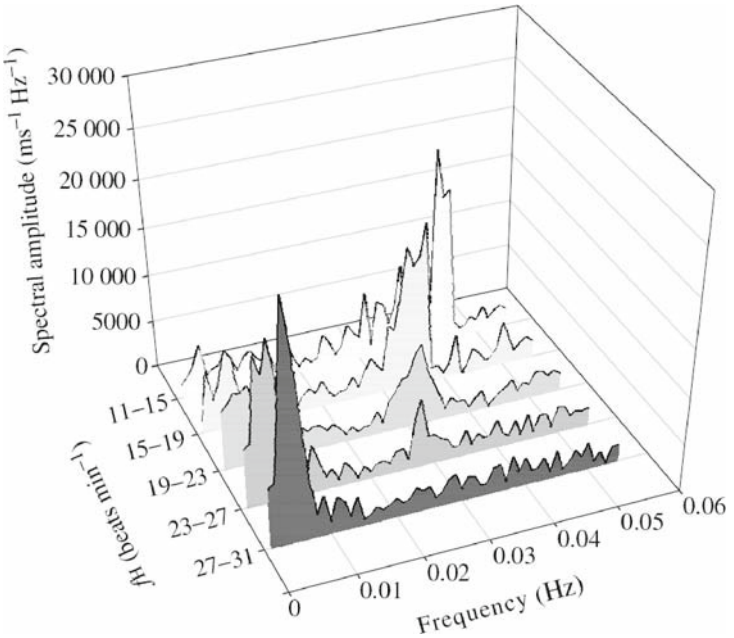

Fig. 7 Power spectra generated from heart beat interval data recorded for 110 h from four rattlesnakes, *Crotalus durissus*, fitted with external miniature dataloggers. Heart rate varied with recovery from operative procedures and over a diurnal cycle. For power spectral analysis, ten individual data sets, each consisting of 512 consecutive R-R intervals, were chosen from each animal, within each of 5 fH categories. The resultant power spectra within 0.001 Hz frequency bins were pooled to produce the plots. (Taken from Campbell et al. 2006)

and Yasuma 2003). These peaks showed up clearly following power spectral analysis of heart rate variability (Fig. 7). Thus, this study contrasts with that of Gonzalez and De Vera (1988), as we were able to present clear evidence for respiratory modulation of heart rate which closely resembled that recorded from mammals, and accordingly may be classed as RSA. These data refute the proposition that centrally controlled cardiorespiratory coupling is restricted to mammals, as propounded by the polyvagal theory of Porges (1995).

Changes in heart rate during bouts of ventilation that consist of many consecutive breaths are less easy to characterise. Thus, as shown in Fig. 8, which depicts an example of heart rate changes during intermittent ventilation of a turtle, it is clear that heart rate often remains high and invariable during the entire breathing episode. Thus, while heart rate clearly increases in connection with pulmonary ventilation, there is no variation associated with inspiration and expiration during the individual breaths. It may therefore be appropriate to label these interactions as cardiorespiratory coupling.

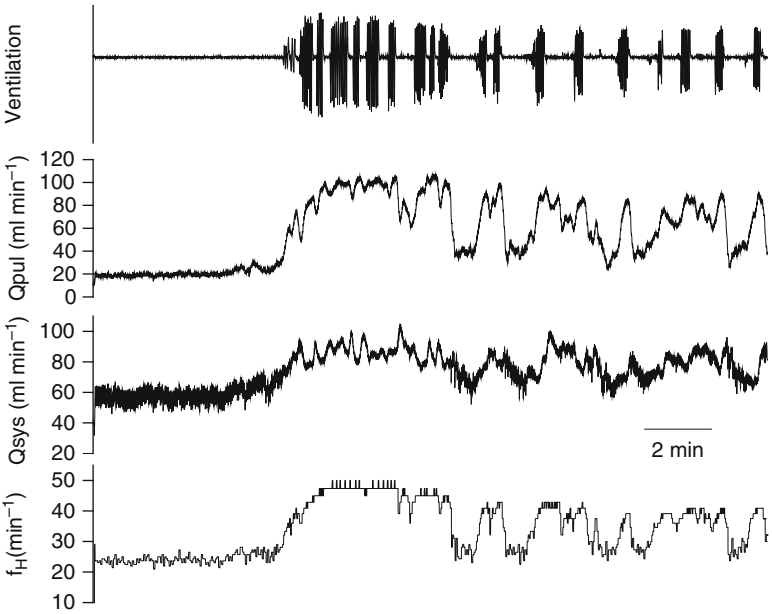

Fig. 8 Heart and central vascular blood flow in an aquatic turtle, *Trachemys scripta*, during spontaneous apnoea and ventilation. The ventilatory bouts, which consist of several consecutive breaths, are accompanied by a large rise in heart rate (fH) and pulmonary blood flow (*Qpul*), while systemic blood flow (*Qsys*) remains unchanged. As a consequence of a strong vagal tone on the smooth muscle that surrounds the pulmonary, large right-to-left cardiac shunts prevail during apnoea, while relaxation of this sphincter during ventilation reverses the cardiac hunt pattern. (Taken from Wang and Hicks 1996a)

10 Mechanisms of Phasic Vagal Control of the Heart

There are at present no definitive studies of the mechanisms by which respiration-related efferent activity in the cardiac vagus recruits the heart in fish, amphibians or reptiles. In the dogfish, injection of the muscarinic cholinoceptor antagonist, atropine, abolished both the cardiac arrest due to tonic efferent electrical stimulation of the cardiac vagus and the recruitment of the heart by phasic stimulation, so that both are due to stimulation of muscarinic receptors by acetylcholine (Taylor et al. 2006). In dogfish, these separate effects may relate to the topographical and physiological separation between CVPN in the DVN and putative NA (Fig. 3). The denervated heart in dogfish was entrained by bursts of activity delivered down the peripheral cut end of the cardiac vagus at frequencies lower and somewhat higher than its intrinsic rate. However, when these phasic stimuli were combined with lower amplitude continuous stimulation that simulated the activity recorded from the central cut end of the cardiac vagus, the heart was only entrained at frequencies lower than intrinsic heart rate (Taylor et al. 2006). A similar pattern of entrainment was observed when respiratory nerves were stimulated centrally in pacu (see Fig. 4).

Thus, as stated above, the non-bursting units recorded from the cardiac vagus may be responsible for slowing the heart while the respiration-related activity modulates heart rate on a beat-to-beat basis when overall vagal tone on the heart is low, possibly signifying that the non-bursting units are silent. Whether these separate neural units innervate different parts of the heart is unknown.

Finally, we are left with a leading question: How can activity in the cardiac vagus which is normally inhibitory, when delivered in pulses, recruit the heart at rates above its intrinsic rate? By its nature, this is likely to be a direct induction of the heart beat rather than an effect on cardiac intervals. When bursts of electrical stimuli were delivered peripherally down the cardiac vagus of the dogfish at 43 bursts min^{-1} the heart slowed, but it was observed to beat at exactly half the bursting rate, implying that it was induced to beat by alternate bursts (Taylor et al. 2006). This question has long been of interest to mammalian physiologists. In mammals the cardiac vagi show bursting activity of variable frequencies (Jewett 1964; Katona et al. 1970; Kunze 1972; Taylor et al. 1999), and the heart can be entrained by bursts of electrical activity delivered down the cardiac vagi over a range of stimulation frequencies both lower and higher than the intrinsic heart rate (e.g. Levy et al. 1969, 1972, 1981; Pokrovskii 1984, 2003). In a classic study of the chronotropic effects of brief cardiac vagal stimulation in cats, Brown and Eccles (1934) identified a relationship between the phase of the cardiac cycle during which the efferent stimulus was delivered and its chronotropic effect, with two inhibitory phases and a brief phase of relative or actual cardioacceleration. Martin (1977) noted that atrioventricular conduction time in the heart of the dog was shorter in the presence of vagal stimulation. Similar complex relationships were reviewed by Levy et al. (1981). Pace et al. (1984) showed that maximum R–R intervals were triggered when a brief efferent stimulus was delivered to the cardiac vagus during the phase of slow depolarization of the cardiac pacemaker cells. The minimum R–R intervals occurred when the stimulus was delivered before this phase. These observations imply that the effect of each burst depends on the phase of the cardiac cycle at which it is applied. Thus, the vagal effect on the heart cannot be measured merely in terms of the amount of acetylcholine delivered per unit time. Similar studies are long overdue on amphibians and reptiles.

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The Endocrine–Paracrine Control of the Cardiovascular System

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Abstract Over the last 50 years, a large number of cardiovascular studies have identified in vertebrates the ability of cardiac non-neuronal cells to synthesize and release catecholamines (CAs) and the natriuretic peptides (NPs). Thanks to compartmentalized cardiac and vascular receptors, these substances, through activation of local autocrine and paracrine circuits, regulate cardiovascular homeostasis in health and disease. In particular, biomedically oriented research has extensively analysed CAs and NPs in mammals, since these substances are regarded with interest in view of their potent diagnostic and therapeutic implications. This knowledge has firmly established the concept of the heart as an endocrine organ. Such a scenario was dramatically enriched by the identification of a growing number of molecules (i.e., angiotensin II, adrenomedullin, ghrelin, neuropeptide Y, etc.) which, produced by the heart, exert endocrine/paracrine/autocrine cardiac actions. More recently, chromogranin-A (CgA) and its derived cardio-suppressive and anti-adrenergic peptides (vasostatin-1 and catestatin) have revealed themselves as new players in this framework, functioning as cardiac stabilizers, particularly in the presence of intense excitatory stimuli such as those acting under stress, including CA responses. The intracardiac nitric oxide synthase (NOS)/nitric oxide (NO) system works as a very sensitive autocrine/paracrine spatio-temporal organizer through connection–integration processes, playing a role in network configuration. This chapter comparatively summarizes the information available on the hearts of cold-blooded vertebrates with regard to these major endocrine and paracrine agents, although many serious gaps are particularly evident in amphibians and reptiles due to discontinuous information being available. Some paradigmatic examples will help the reader to grasp, with a historical approach, the ways in which incipient endocrine agents, with their molecular loops, have evolved as important cardiac modulators, and how they have become critical intermediates during evolutionary transitions or in a distinct phylogenetic lineage. At the same time, a better

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understanding of the old evolutionary roots of these networks, and how they have evolved from relatively less complicated designs, can help to disentangle the experimental complexity which characterizes the endocrine heart at higher organization levels.

1 Introduction

Due to restrictions on space, we could not attempt to trace back to the fascinating history of the conceptual development of the endocrine heart. However, whenever possible we have included some insights over the last century on seminal studies performed on cold-blooded vertebrates that paved the way to the emergence of the endocrine heart concept.

The heart has played a central role in the development of the knowledge concerning neurosecretion. About 100 years ago, by using the frog heart as a tool, Loewi (Loewi 1921) demonstrated that the mechanical effects of electrical stimulation of the innervation were mediated by "*Vagus-Stoff*," acetylcholine, or by "*Sympatikus-Stoff*," adrenaline, providing the basis to functionally characterize how the sympathetic and parasympathetic systems were able to influence effector tissues.

The late 1950s represented the beginning of the endocrine story (*sensu strictu*) of the vertebrate heart. Pioneer works revealed, within the mammalian atrial myocardium, the presence of dense core granules resembling, both morphologically and functionally, those of the endocrine cells (Kisch 1956; Bompiani et al. 1959; Palade 1961; Jamieson and Palade 1964; de Bold and Bencosme 1973; Marie et al. 1976; de Bold 1979). Soon after the first reports on mammals, comparative scientists showed that these granules were also present in myocardial cells of lower vertebrates, not only at the atrial, but also at the ventricular and endocardial level (Santer and Cobb 1972; Helle et al. 1972; Helle and Lönning 1973; Cantin et al. 1979; Helle et al. 1983).

It was only in 1981 that de Bold and coworkers published the first indication concerning the putative function of the constituents of these granules (de Bold et al. 1981). By using a hundred rat atria, they produced an extract that, infused in rats, caused diuresis and natriuresis. Accordingly, the unknown granular substance was called atrial natriuretic factor (ANF). It was then isolated and characterized as a 28 aa peptide which was called atrial natriuretic peptide (ANP) (Flynn et al. 1983; De Bold and Flynn 1983). Soon afterwards, another two natriuretic peptides (NPs) were identified; brain natriuretic peptide, now called B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (Sudoh et al. 1988, 1990). So far, other two NPs have been identified and sequenced. One was found in the venom of the green mamba snake (*Dendroaspis angusticeps)*, thus it was called DNP (*Dendroaspis* natriuretic peptide) (Schweitz et al. 1992). The second, identified in the eel ventricle, was called VNP (ventricular natriuretic peptide) (Takei et al. 1991, Takei et al. 1994c). NPs were also found in the heart of a large number of non-mammalian vertebrates (Netchitailo et al. 1987; Takei et al. 1990; Bjenning et al.

1992; Larsen et al. 1994; Kawakoshi et al. 2003), invertebrates and plants. In all living organisms, NPs act by orchestrating ion fluid homeostasis, which in vertebrates includes direct regulation of cardiovascular performance.

To date, the word "NPs" is synonymous of cardiac hormones. However, it is now clear that the picture is not so simple. In fact, cardiac cells are able to produce a large number of endocrine and/or paracrine and/or autocrine substances (i.e., catecholamines, angiotensin II, ghrelin, adrenomedullin, neuropeptide Y, etc.) which, by acting in concert with the neurotroansmitters released by autonomic nervous terminals, increase the variety of chemical signals to be utilized for a finely tuned heart responsiveness to local and/or systemic challenges

The established knowledge on the endocrine heart stimulated an impressive proliferation of exciting research on the molecular biology of cardic hormones, their receptors and regulation. Since many studies, employing sophisticated molecular biology techniques, were biomedically oriented and thus performed on mammals, this advanced mammalian knowledge necessarily represents a fundamental theoretical point of reference. Consequently, we felt the need to provide the reader with this basic synthetic information so he can have a better critical perception conerning what has been accomplished in terms of evidence related to cold-blooded vertebrate hearts.

A large number of books and reviews have been written on the cardiac functional morphology of cold-blooded vertebrates, and its rearrangement related to major evolutionary events (single and double circulation patterns, the addition of a pulmonary circuit, heart-chamber septation, etc). For the reader who is not familiar with this knowledge, we will summarize the basic morpho-functional traits pertinent to the issues of this chapter.

2 Basic Cold-Blooded Vertebrate Heart

From a phylogenetic point of view, the fish heart is the prototype of the higher vertebrate hearts, as elucidated by zebrafish embryology (Stainier and Fishman 1994). The fish heart consists of four chambers in series: the sinus venosus, the atrium, the ventricle, and the outflow tract (*bulbus cordis*), i.e., a proximal conus arteriosus and a distal bulbus arteriosus. In a typical water-breathing fish, the peripheral venous blood flows in sequence from the sinus venosus, to the atrium, to the ventricle and to the bulbus cordis, from where it is pumped to the gills to be oxygenated; thence, it is distributed to the body and back to the heart.

Most teleosts and the lungfish (belonging to the sarcopterygians, which gave rise to the very first terrestrial vertebrates, the amphibians) have an entirely *trabeculated* heart, in which the ventricle in particular is made up by *trabeculae* (projecting cones of myocardial muscle) and is named *spongiosa*, because of its spongy texture when viewed from the lumen. Covered by the endocardium, the *spongiosa* is supplied by the intertrabecular lacunary system. Other fish (elasmobranchs, many teleosts) have a mixed type of ventricle, i.e., an external *compacta* (densely arranged

myocardial bundles) and an inner *spongiosa* (Tota 1983; Tota et al. 1983; Icardo et al. 2005). In this chapter, the hearts of the Antarctic teleosts and the African lungfish *Protopterus dolloi* exemplify the entirely trabeculated ventricle, while eel and tuna hearts exemplify the mixed type of ventricular myoarchitecture. The entirely trabeculated ventricle can only be supplied by the lacunary system (avascular heart) or, in addition, can have a vascular supply (usually coronary arteries); the compacta is always vascularized (Tota et al. 1983; Tota 1989). The fish heart is a venous heart, being only, or mostly, supplied by venous blood. In comparison with the warm-blooded vertebrates, it generally functions as a low-pressure region facing relatively low and variable p_{02} levels (Farrell and Jones 1992; Olson 1998). However, among the vertebrates, fish exhibit the highest interspecific variation not only in myo-angio-architecture, as illustrated by the different types of ventricle structure (Farrell and Jones 1992; Tota et al. 1983), but also in patterns of morphodynamic mechanical performance (Tota and Gattuso 1996).

The amphibian heart is made up of a sinus venosus, right and left atria divided by an anatomically complete internal septum, a ventricle lacking any internal subdivision (with the exception of the salamander species Siren), and a conus arteriosus with a spiral valve. Due to the remarkable variety of respiratory and circulatory patterns shown by the amphibians, the mode of action of the "amphibian heart" cannot be generalized (Foxon 1964). Since most of the endocrine evidence which is available concerns the frog heart, here we will only consider the heart of *Rana* as a paradigm. While "venous" blood flows in the right atrium, more oxygenated "arterial" blood is drained in the left atrium. According to the so-called "classical hypothesis," the blood from the two atria remains to a large extent unmixed in the single ventricle, with a selective-like distribution during ventricular systole. In fact, the "venous" blood during early systole, under the influence of the spiral valve of the conus, leaves first, being distributed to the low-pressure pulmo-cutaneous arch, hence perfusing the lungs and the skin; in the middle phase of ventricular systole, the more mixed blood from the centre of the ventricle is ejected on the other side of the spiraI valve of the conus, flowing in the systemic arches. The last to leave the ventricle is the "oxygenated" blood, which under the greater blood pressure is canalized to the brain through the high-resistance carotid arches (Foxon 1964).

In relation to the endocrine heart, it is relevant that the large surface area of the EE lining the lumenal cavity of the cold-blooded trabeculate hearts may act as a sensor–integrator device, through its large numbers of receptors for endocrine and growth factors (Brutsaert 2003, and references therein) and its autocrine–paracrine ability (e.g., via EE – NOS/NO-mediated signaling: see below). Accordingly, the trabeculate hearts represent suitable natural models for analysing the humoral cross-talk between the EE and the subjacent myocardium, providing new opportunities for understanding the early ontogenetic and phylogenetic roots of the autocrine–paracrine control of the vertebrate heart. For example, it was shown that during embryogenesis the EE cells are able to regulate cardiac morphogenesis by sensing and transducing biomechanical stimuli caused by pulsatile blood flow (Hove et al. 2003, and references therein).

3 Catecholamines

Metabolic, contractile and conduction properties of the vertebrate heart are influenced by catecholamines (CAs) through their binding to cardiac α- and β-adrenoceptors. CAs, typically adrenaline and noradrenaline, reach cardiac adrenoceptors via both the circulation and the sympathetic nerve terminals. However, in several poikilotherm vertebrates, such as cyclostomes, elasmobranchs and, to a lesser extent, teleosts, the sympathetic nervous system (SNS) differs from the mammalian type, because it lacks the longitudinally connected sympathetic chains. Moreover, in many cases, as recognized in the late 1960s, the peripheral nerves are replaced by aggregates of chromaffin cells (Burnstock 1969) that in many species become components of the diffuse neuroendocrine tissue. As later detailed, in the cardiovascular system they appear located in hemodynamically strategic regions, such as the wall of the large arteries (cyclostomes, teleosts, lungfish), the axillary bodies (elasmobranchs), or embedded in the cardiac muscle (teleosts, elasmobranch, lungfish, amphibia). They often associate with sympathetic nerves and/or, autonomic-like ganglionic cells, receive cholinergic stimulation, thereby providing a regionally defined CAs production which contributes to the humoral cardiovascular control (for ref., see Gannog and Burnstock 1969; Abrahamsson et al. 1979; Tota 1999). The identification of these intracardiac sites for CA production and release in poikilotherm vertebrates has remarkably contributed to pave the way to the modern concept of the endocrine heart.

3.1 Cardiac Catecholamines

From fish to reptiles, the heart undergoes an increasing and diversified innervation, showing a pattern which, moving from the absence of any type of innervation in the hagfish or the presence of only cholinergic nicotinic nerves in lampreys and elasmobranchs, reaches the establishment of both sympathetic and parasympathesic nerves in teleosts, amphibians and reptiles. In elasmobranchs and Dipnoi there is no direct cardiac sympathetic control, and therefore the modulation of inotropism and chronotropism involves the CAs produced by cardiac chromaffin cells. In contrast, in teleosts, amphibians and reptiles, the heart receives adrenergic stimulation both neurally and via the circulation (for references, see Taylor 1992). By comparison, in birds and mammals the adrenergic control of the heart reaches higher complexity. In fact, in addition to the rich extrinsic innervation, an intrinsic heterogeneous population of afferent, efferent, and interconnecting short neurons and intracardiac ganglia provides the basis for a local cardiac neural integration through a number of neurotransmitters, including adrenaline and noradrenaline (Slavíková et al. 2003). In addition, intracardiac chromaffin cells are present from the initial developmental stages until adult life (Kohn 1903; Papka 1976; Ehinger et al. 1968; Ellison and Hibbs 1974). Together with these cells, the heart of both fetal and adult mammals, e.g., rat and human, shows specialized nonneuronal cardiocytes which possess

CAs biosynthetic enzymes, and are called *intrinsic cardiac adrenergic* (ICA) cells (Huang et al. 1996). Because of the presence of numerous clear cytoplasmatic exocytotic vesicles, ICA cells differ from cardiac chromaffin cells, which contain electron-dense core granules and are largely confined to atrial parasympathetic ganglia. ICA form clusters which are distributed in atria and ventricles, often closely associated with the coronary microvasculature and in intimate contact with atrial and ventricular myocytes in adult rat heart (Huang et al. 1996). They are capable of paracrine adrenergic signaling, are linked to fetal development, cardiac pacemaking and conduction, blood pressure regulation (Huang et al. 1996; Ebert and Thompson 2001; Rumantir et al. 2000), and are also of central importance in the survival of transplanted human hearts (Huang and Ewy 2001). From the perspective of this recent mammalian knowledge, studies addressed at exploring ICA cells in poikilotherm hearts appear timely and rewarding.

3.2 Autocrine/Paracrine Cardiac Effects of Catecholamines

The different source and topologic arrangements which are at the base of the catecholaminergic control of the heart are often mirrored by the different cardiac sensitivities to CAs, going from the insusceptibility of the Myxinoid heart to the complex and often equivocal responses displayed by teleost and amphibian hearts. Therefore, while a general evolutionary trend can be reasonably traced, the importance of inter- and intraspecific differences, even when present in closely-related species, must be taken by the reader into serious consideration. Furthermore, both theoretical and methodological aspects may have hampered the clarity and interpretation of a number of results, particularly in the case of the less recent ones. In fact, prior to the recent recognition of the impact of stress and stress responses in lower vertebrate physiology and adaptation (Wendelaar Bonga 1997; Cossins et al. 2006), many studies were performed without any serious quantitative evaluation of the stress-induced changes imposed by the experimental conditions upon either the organism or the organ preparation. This issue appears particularly crucial when the major stress effector molecules, i.e., the CAs, are under study. Another limitation has been the use of pharmacological but not physiological concentrations of CAs. This has precluded the identification of the fine modulation (e.g., the biphasic inotropic response) exerted by CAs as components of the complex adrenoceptor-G-protein coupled signal-transduction pathways, including nitric oxide (NO)-cGMP and cAMP, which, on the basis of the mammalian studies have revealed themselves as major regulators of almost each aspect of cardiac performance.

An early evolutionary response to CAs stimulation is highlighted by the lampetroid heart (Nilsson 1983). Unique among vertebrates, in cyclostomes acetylcholine (ACh) accelerates heart rate, while decreasing inotropism. Since the pioneering study of Ostlund et al. (1960), the existence of an intracardiac CAs production has been suggested by the identification of atrial and ventricular CAs containing fluorescent granules (Otsuka et al. 1977). This cardiac chromaffin

tissue forms an intrinsic control system which responds to intracavitary stimuli by releasing adrenaline. In turn, adrenaline stimulates the release of noradrenaline, and probably also dopamine, from other cardiovascular chromaffin cells (Dashow and Epple 1985). Noradrenaline targets the myocardium via β-adrenergic receptors (ARs), providing CAs stimulatory actions which, however, appear less powerful than those induced by ACh (for references see Taylor 1992).

In elasmobranchs, heart performance is influenced either directly or indirectly by a basal CA release from the intracardiac stores, as well as from suprarenal and axillary bodies (Saetersdal et al. 1975). Physical disturbance, exercise, and hypoxia are known stimuli for elevating plasma CAs levels in dogfish species (Butler et al. 1986; Opdyke et al. 1982a; Metcalfe and Butler 1989). However, in *Squalus acanthias* CAs do not increase during hypercapnic and hypoxic stress. Such an apparent contradiction (Perry and Gilmour 1996) may be related to the experimental conditions, i.e., short- vs long-term exposure to hypoxic medium. The predominant trigger for CAs secretion is ACh released by preganglionic cholinergic fibres of the SNS (Nilsson et al. 1976; Abrahamsson 1979; Opdyke et al. 1983; reviewed by Randall and Perry 1992). Plasma CAs indirectly regulate cardiac filling pressure via an α-adrenergic mediated increase of the venous pressure (*S. acanthias*: Sandblom et al. 2006), and by a presynaptic modulation of the vagal inhibition (*S. acanthias*: Agnisola et al. 2003). The direct modulation is exerted either by CAs released by the axillary bodies and immediately sucked into the heart during each cardiac cycle, or by those released by the sinus venosus, atrial, and ventricular stores. This results in positive chronotropic and inotropic effects (Capra and Satchel 1977) that occur via β-ARs, resembling the mammalian β2-type (Ask 1983). Moreover, CAs modify the motility of the well-developed coronary system, thus contributing to adapt coronary performance to the requirements of the myocardium (see Axelsson 1995). An elegant electrophysiological study in shark described the intracellular cascades involved in the β-adrenergic-dependent modulation of myocardial contractility. In the presence of very limited intracellular Ca^{2+} stores (e.g., a less-developed sarcoplasmic reticulum), the myocardial contraction evoked by β-adrenergic stimulation requires a time-dependent modulation of the Ca^{2+} transients via the $Na^{+} - Ca^{2+}$ exchanger. This initially contributes to increase Ca^{2+} entry and subsequently facilitates Ca^{2+} efflux, thus accelerating CA-dependent relaxation (Woo and Morad 2001).

The air-breathing lungfish (Dipnoi) represent ideal models to study the evolution of cardiac adrenergic control in vertebrates. Their autonomic nervous system, which is less differentiated than in teleosts, appears progressively adapted for terrestrial existence. So far, preliminary, but encouraging morpho-functional observations have started to sketch out the morphological endocrine–paracrine requisites of their cardiovascular CAs-mediated regulation. In several lungfish species, e.g., the African *Protopterus aethiopicus* and *P. annectens*, as well as the genus Lepidosiren, chromaffin cells, producing a primary CA identified as dopamine, are located singly and/or in cluster in the wall of the sinus venosus and in the auricle (for ref., see Abrahamsson et al. 1979; Larsen et al. 1994). These cells, identical to those found in several autonomic ganglia of a variety of vertebrates, represent an intermediate

phenotype between mature chromaffin cells and primitive sympathetic cells, and are situated in the position where chromaffin cells or their precursors locate in mammals during ontogenetic development (Scheuermann 1993). As in other vertebrates, CA-producing cells locate in subendocardial areas of the atrium, suggesting either a direct release of CAs into the intraluminal blood, or a paracrine control of the subjacent myocardium (Fritsche et al. 1993; Larsen et al. 1994). So far, few functional studies have analysed the responses of the lungfish heart to CAs. In teleosts, CAs are believed to activate several cardiovascular homeostatic responses in order to face the detrimental effects associated with acute stressors, such as those associated with variable O_2 availability, temperature acclimation, and exercise (see Perry et al. 2004 and references therein). These stimuli may be even more important in lungfish, which, during the dry tropical season, tolerate drought periods by aestivating in subterranean mud cocoons (Smith 1935; Janssens and Cohen 1968). Lungfish aestivation requires a number of cardiorespiratory and metabolic changes, including decreased oxygen consumption, complete reliance on air breathing with a consequent reorganization of the branchial/lung vascular perfusion, slowing of heart rate, and drop in blood pressure (Amelio et al. 2008). In *P. dolloi*, during terrestrial aestivation plasma CAs level does not change (Perry et al. 2008). However, during exposure to aerial hypoxia the fish is able to mobilize stored CAs (Perry et al. 2005). It is unknown whether and to what extent these mobilized CAs are active on the heart or on the blood vessels. During aerial hypoxia, the increase in plasma CAs is not accompanied by heart rate variations (Perry et al. 2005). It cannot be excluded that either circulating or locally produced CAs may exert long-term actions on the heart, for example contributing to the ultrastructural changes which occur during aestivation (Icardo et al. 2008). However, this challenging question remains to be investigated.

Phylogenetically, teleosts are the first vertebrates to receive cardiac sympathetic innervation via a vagosympathetic trunk made up of cholinergic and adrenergic fibers (Laurent et al. 1983). Such a double nervous control is associated with a notable intrinsic heterometric ability (i.e., Frank–Starling response) which makes the heart able to adapt its inotropic performance to the peripheral perfusion requirements. Accordingly, the teleost heart shows an elevated adaptive and acclimatory flexibility, which allows many species to face changes in environmental salinity, extreme temperatures, variable oxygen availability, sustained enforced activity, etc. The variable degree of intracardiac blood supply, which goes from the avascular condition of the fully trabeculate (*spongy*) ventricle to the vascularized outer compact myocardium of several very active species (see above), provides remarkable patterns of cardiac nonuniformity. Therefore, the CA-mediated cardiac responses should be also analysed in relation to this morpho-functional heterogeneity. However, despite the initial momentum (see for example, Satchell 1991; Randall and Perry 1992), there has been a rapid decline of interest in exploring whether and to which extent local adrenergic circuits may contribute to modulate heart performance of both stressed and unstressed teleosts.

Under resting conditions, CAs circulate in low concentrations, and thus the major control of the teleost heart is exerted by the nervous activity (Axelsson 1988). CAs

exert a basal adrenergic excitatory tone which predominates over the cholinergic one. Adrenergic tone is mediated by α- and β-ARs associated with both the pacemaker and the working myocardium (Axelsson et al. 1987; Gamperl et al. 1994). Adrenergic stimulation, mainly described in isolated heart preparations, evokes an increase in heart rate (Graham and Farrell 1989) and a slight improvement of the Starling response (Farrell et al. 1986). Under stress challenges, such as hypoxia, air exposure, anemia, acidosis, hypercapnia, exhaustive exercise, and physical disturbances, there is a sudden release of CAs from the chromaffin cells, which abundantly populate the walls of the posterior cardinal vein close to the head kidney (Nandi 1961). These CAs reach the heart, adding their action to that of sympathetic terminals and cardiac chromaffin cells (Nilsson and Holmgren 1992; Farrell and Jones 1992). CAs also activate vascular and respiratory responses, thus contributing to alleviate the stress-associated detrimental effects (Farrell et al. 1986).

Although in teleosts the prevalent chromaffin CA is adrenaline (86% adrenaline to 14% noradrenaline in the head kidney of the Atlantic cod: Abrahamsson and Nilsson 1976), the ratio of circulating adrenaline/noradrenaline varies depending on the species (for ref., see Randall and Perry 1992). This variability is accompanied by a species-specificity of the ratio α/β ARs, although a prevalent β2-ARs component is believed to characterize the cardiac response to CAs (Gamperl et al. 1994). For example, the seasonal cardiac responsiveness to CAs (Peyraud-Waitzenegger et al. 1980) may be a consequence of a variable adrenoceptor expression. This may also explain the negative inotropism elicited by isoproterenol (ISO)-exposure in 30% of eel heart preparations (Imbrogno et al. 2006), which is observed in addition to the classic positive inotropic effect (Tota et al. 2004). However, the recent identification of a novel type of cardiac β-receptor in two teleost species requires this view to be reappraised. In the trout *O. mykiss*, these novel adrenoceptors are richly expressed in the heart and are homologous to the mammalian β3-ARs (Nickerson et al. 2003). In mammals, activation of cardiac β3-ARs by both endogenous and synthetic agonists elicits negative inotropic (for references see Gauthier et al. 2000) and negative lusitropic actions (Angelone et al. 2008), both involving the NO – cGMP pathway. In teleosts, the physio-pharmacological characterization of cardiac β3-adrenoceptors has extended to these fishes the possibility of an intrinsic β3-adrenergic inhibitory tone, which may adjust the cardiac responses to excessive excitatory stimulations (exposure to systemic and/or intracardiac CAs, angiotensin, endothelin), thus protecting the heart against stress. As in mammals, in *A. anguilla* β3-ARs activation decreases cardiac mechanical performance through a PTx-sensitive Gi protein mechanism. This is consistent with a major β3-ARs myocardial localization. However, the β3-ARs-mediated inotropic action may also include a paracrine EE-dependent modulation, as indicated by the obligatory role played by the NO – cGMP – cGMP-activated protein kinase (PKG) cascade in mediating the effects of β3 activation (Imbrogno et al. 2006). This recent evidence shows that, as epitomized by the more extensive and sophisticated mammalian studies, CAs regulate fish cardiac performance in a *ying/yang* fashion much more complex than hitherto perceived.

As in many fields of biological research, amphibians have also provided suitable natural models for incrementing basic knowledge in the context of the neuroendocrine heart. During the early 1920s, Otto Loewi, in his seminal study rewarded with the Nobel Prize in Physiology an Medicine (Loewi 1921), initially described adrenergic regulation of cardiac contractility in the denervated frog heart using *Accellerans-Stoff* or *Sympathin*, which was later identified as adrenaline (Loewi 1936).

In amphibians, CAs are synthesized and stored in the heart starting from the early developmental stages, and may therefore serve as paracrine modulators. In *Xenopus laevis* larvae, the non innervated heart shows a progressive developmental increment of adrenaline, noradrenaline and dopamine content (Kloberg and Fritsche 2002). This is paralleled by a growth-dependent increase of the adrenergic tone which is responsible for the high heart rate observed during late development (Jacobsson and Fritsche 1999). The cardiostimulatory effect of CAs is maintained during the adult life. In the cane toad (*Bufo marinus*), β-adrenergic stimulation of pacemaker cells increases intracellular calcium concentration and firing rate (Ju and Allen 1999). Nonneural CAs (including those produced by intracardiac stores) stimulate heart rate by activating extrajunctional β-ARs linked to a cAMP-dependent metabotropic pathway. In contrast, CAs produced by sympathetic nerve activity possibly act via junctional non α- non β-adrenoceptors linked to Ins(1,4,5)P3-dependent signaling (Bramich et al. 2001). Similar mechanisms may operate also in the case of the adrenergic chronotropic control described in mammals.

Although in amphibians adrenaline is considered to be the cardiac CA, β-adrenergic activation by ISO is routinely used to describe the effects of adrenergic stimulation on myocardial contractile performance. In frog, the myocardium exhibits some of the features associated with the ISO response, including potentiation of phasic contraction (twitch) followed by enhanced inhibition of maintained (tonic) tension (see Fan et al. 1996 and references therein). This is known as the "paradoxical" cardiac action of adrenaline (Graham and Lamb 1968). In addition, in toad isolated ventricular strips, ISO induces a positive lusitropic effect. Both inotropism and lusitropism are mediated by the β2-adrenergic-dependent decrease of myofibrillar sensitivity to Ca^{2+} (Petroff et al. 1994; Fan et al. 1996). As in elasmobranchs and teleosts, in amphibians cardiomyocytes exhibit a scarce sarcoplasmic reticulum, a poor Ca^{2+}-ATPase/phospholamban complex and an absence of significant intracellular Ca^{2+} pools. It has been proposed that an ISO-dependent downregulation of the Na^{+}–Ca^{2+} exchanger, and thus of the transmembrane Ca^{2+} transport, provides the basis for the β-adrenergic inhibition of tonic tension and positive lusitropism (Fan et al. 1996). Interestingly, amphibian cardiac cells have provided, also for mammals, a key paradigm for dissecting the mechanisms of β-dependent modulation of contractility. In fact, frog ventricular myocytes were largely utilized to elucidate the functional coupling between β2-ARs and Ca^{2+}. It was found that, thanks to a highly compartmentalized pool of phosphodiesterases (PDEs) (i.e., PDE3 within the cytosol, and PDE4 in proximity of the membrane), activation of β2-ARs modifies the intracellular spatial profile of cAMP, the activity of cAMP-dependent protein kinase (PKA), and thus of its closely localized

substrates, including L-type Ca^{2+} channels (Jurevicius et al. 2003). This in turn determines the rate of $I_{Ca2,L}$, influencing contractility. Indeed, the identification of this fine-tuned mechanism has significantly contributed to the clarification of the routes by which cardiac cells respond to different external stimuli, modulating the amount of a single intracellular messenger according to a restricted spatio-temporal pattern.

The reptilian heart represents a very interesting model of architectural organization, being characterized by an almost complete separation of the ventricles and the presence of a well-developed coronary supply. However, for years it has been more or less excluded by the analytical investigations focused on local CAs control. Because of the intensive challenges experienced during their life (diving and anoxia in turtles and water snakes, gravity adaptation in semiarboreal species, high temperature and water deprivation exposure in desert species, elevated and prolonged metabolic rate associated with digestion, etc.), major interest has been dedicated to describing the influence of both circulating CAs and sympathetic nervous activity on the cardiac and vascular adaptations necessary to face immediate and/or long lasting requirements of the organism.

To date, no conclusive data are available regarding non nervous CAs stores in atrial and ventricular myocardial walls of reptiles. Chromaffin cells, morphologically linked to nearby nerve bundles, are present in the common wall of both pulmonary and left aortic arteries of the lizard *Trachydosaurus rugosus* (Berger et al. 1982). This calls for a paracrine regulation of the motility of these vessels. However, since these chromaffin cells are located in outflow regions of the heart, it is unlikely that they may provide CAs for either atrial or ventricular receptors (Hicks and Farrell 2000). Further investigations are needed to establish the catecholaminergic properties, as well as the presence of paracrine adrenergic circuits in the reptilian heart. Indeed, there is no reason to believe that it differs from the heart of all the other vertebrates in terms of intrinsic non nervous CAs production.

Under resting conditions, the reptilian heart is under a major cardiodepressant cholinergic tone and a much weaker stimulatory adrenergic influence (Burggren 1987; Hedberg and Nilsson 1975). α- and β-adrenergic activities modulate heart rate, and through venous return regulation, affect cardiac filling and thus contractility (Skals et al. 2005). During stress (i.e., enforced activity, aggressive behavior, warm acclimation, anoxia, etc.), this cholinergic tone is depressed, while the consequent increased adrenergic tone exerts important effects on the performance of both the heart and critical vascular districts, including peripheral resistance vessels (Wasser and Jackson 1991; Wang et al. 2001; Hicks and Farrell 2000; Skals et al. 2005). An example is the temperature-dependent adrenergic influence which is exerted on the peripheral resistances, as well as on both cardiac rate and output of the turtle *Trachemys scripta* acclimated to anoxia (Hicks and Farrell 2000; Stecyk and Farrell 2007). During forced activity, a many-fold increase in circulating CAs strongly correlates with a positive chronotropic effect and an increase in blood pressure (*Coluber constrictor*: Stinner and Ely 1993; *Boa constrictor*: Wang et al. 2001). In isolated atria preparation of *Naja naja* and *Ptyas korros*, α- and β-agonists elicit dose-dependent increases in atrial beating rate and tension. The pharmacological

characterization of these responses has suggested the presence of a prevalent population of postsynaptic β-ARs (Chiu and Sham 1985), whose activation may occur via circulating CAs which may reach the sino-atrial region through the coronary circulation, and/or through presynaptic sympathetic activity. However, conflicting data are reported concerning the chronotropic response of the reptilian heart to CAs. In fact, in *B. constrictor*, the physiological increase in heart rate which occurs during the prolonged digestion of a big meal was found to be abolished by β-adrenergic stimulation (Wang et al. 2001).

3.3 Cardiotoxic Effects of Catecholamines

In addition to their action on heart rate and contractility, in both poikilotherm and homeotherms CAs are known as cardiotoxic agents. Exposure to excessive CAs amounts induces serious structural and functional lesions at the level of the myocardiocytes, thus contributing to the cardiac injuries related to the so-called "sympathetic storm" (Samuels 2007).

The cardiac architectural heterogeneity of many poikilotherm species (see *Basic cold-blooded vertebrate heart*) has provided for years appropriate experimental conditions to discriminate between vascular (coronary)- and myocardial-dependent reactions induced by CAs toxicity. Because of its elevated resistance to the cardiotoxic action of CAs, the cold-blooded vertebrate heart is well suited to uncovering tissue, cellular, and molecular mechanisms which may contribute to cardioprotection. One aspect of such natural cardioprotection may be related to the lower levels of temperature, heart rates, contraction velocity and oxygen consumption, causing the work of the poikilotherm heart to be less intense than in homeotherms. This may represent a beneficial effect against those unfavorable actions induced by intracardiac and/or circulating CAs. It suggests the hypothesis that species-specific differences in either the amount/activity of the cardioinhibitory β3-ARs or the presence of "antiadrenergic" counter-regulatory peptides (see below) may contribute to determine the susceptibility of the heart to CAs injuries. Indeed, locally released protective factors may orchestrate compensatory mechanisms to prevent and/or counteract the effects of excessive CAs exposure. In the light of the emerging new adrenergic mechanisms which control the mammalian heart (e.g., ICA cell-derived CAs: Huang et al. 2005), a reappraisal of the role of CAs in the poikilotherm heart is opportune.

Turtles were the first poikilotherms to be analyzed in terms of CAs cardiotoxicity (*Testudo horsfieldi*: Ostadal et al. 1968). It was found that 48-h ISO administration induced necrosis only in the avascular spongy myocardium, leaving the outer compact and vascularized layer intact. This is of particular interest since the reptilian myocardium is usually anoxia-resistant (Gesser and Poupa 1978). Fishes (e.g., trout and tuna) showed a lower susceptibility to ISO-induced lesions (Poupa and Ostadal 1969; Ostadal and Rychterova 1971). Cardiac muscle becomes even less susceptible in amphibians, in which, however, the resistance to CAs toxicity is strongly broken

down by increasing environmental temperature, thus showing a seasonal variation (Poupa and Carlsten 1970). In frogs, CAs-induced damage to the myocardium is mainly localized in the ventricle at the level of the base and the apex. This damage is associated with paradoxical systolic movements of the aneurysmatic ventricular wall during the cardiac cycle. Functionally, the damaged ventricle shows alterations in the force development, and resistance to anoxia. The injured regions of the myocardium show degeneration of the trabecular arrangement. Myocytes appear edematous, showing enlarged mitochondria, disarranged myofibrils and contraction bands (see for references Carlsten et al. 1983a).

4 Natriuretic Peptides

The cardiac natriuretic peptides (NPs) are the best characterized cardiac hormones throughout vertebrates. Figure 1 illustrates the structure of the five NPs so far identified. They were firstly identified by de Bold and coworkers as the major constituents of rat atrial granules, being capable of inducing diuresis and natriuresis when infused into rats (de Bold et al. 1981). Impressive research has now extensively described the nature and the functional significance of the NPs system in almost all living organisms.

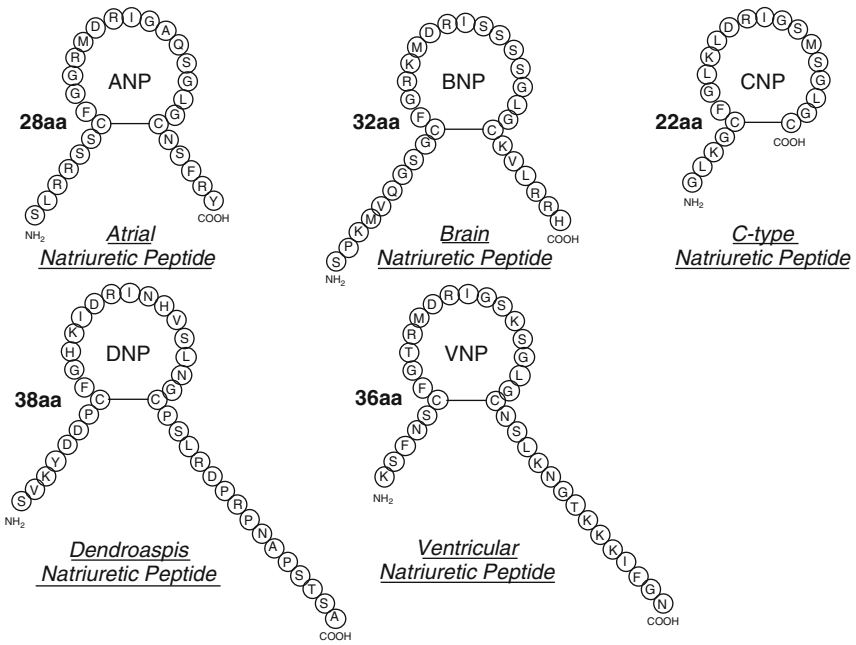

Fig. 1 Amino acid sequence and structure of biologically active natriuretic peptides in vertebrates. See Introduction for references

Universal NPs distribution is confirmed by their presence in single-cell organisms (e.g., *Paramecium multimicronucleatum*; Vesely and Giordano 1992), in plant (e.g., *Dracena godseffiana*: Veseley and Giordano 1991; *Metasequoia*: Yang et al. 1999; *Hedera helix*: Billington et al. 1997; *Zea mays*: Pharmawati et al. 1998) and in invertebrates (e.g., the roundworm, *Ascaris suum*: Brownlee et al. 1993; the silkworm *Bombyx mori*: Kim et al. 1994; the blue crab, *Callinectes sapidus*, the oyster, *Crassostrea virginica*: Poulos et al. 1995) (Table 1). Based on this almost ubiquitous presence and the sequencing of comparison data, it is now clear that NPs have a long evolutionary history. They appeared 565 million years ago as an ancestral peptide, presumably CNP, and diverged to form four groups composed of structurally similar peptides. 360 million years ago, there was the divergence of the sequences present also in man (Inoue et al. 2003a). It is still unclear to which group DNP belongs.

The major role of NPs is the orchestration of ion/fluid balance. In both mammalian and nonmammalian vertebrates, NPs are components of a homeostatic loop which links blood volume expansion and myocardial stretch, with consequent cardiovascular regulation via concerted effects on the heart and the vasculature.

The NPs pool varies among vertebrates. The hagfish *Eptatretus burgeri* expresses a single NP named EbuNP (Kawakoshi et al. 2003). CNP is the only NP present in elasmobranchs (Suzuki et al. 1994; Takei 2000). Instead, in the Chondrostean *Acipenser transmontanum*, all ANP, BNP, CNP and VNP genes are present (Kawakoshi et al. 2004). In teleosts, both ANP and BNP are present in pufferfish and tilapia, but ANP was undetectable in medaka. In contrast, eel and rainbow trout do not possess BNP, while showing VNP (Takei et al. 1991, 1994a, b). Tetrapods typically have ANP, BNP and CNP. ANP and BNP are present in amphibians and mammals, while birds apparently lack ANP (see Takei 2000), and show the presence of a so far unknown NP, named renal NP (RNP) because of its high expression in the kidney (Trajanovska et al. 2007). To date, DNP has been identified only in the venom of the green mamba snake (*Dendroaspis angusticeps)* (Schweitz et al. 1992).

4.1 Production and Release of Natriuretic Peptides

Since in vertebrates the heart is the major site for NPs production and release, the term “natriuretic peptides” is considered to be synonymous of “cardiac hormones.” This is true for ANP and BNP, and, if present, for VNP. In contrast, CNP is mainly released by the vascular endothelium. However, in the two teleosts eel and trout, and in elasmobranchs (e.g., *S. canicula*, *T. shyllia*), in which CNP is the primary, if not exclusive NP, the peptide is also produced and released by the heart (see Loretz and Pollina 2000; Inoue et al. 2003b). It is possible that, in the elasmobranch heart (see Tota 1983 for references and comments), the large endothelial surface of the well-developed arterial and venous coronary supply represents a relevant source for the autocrine/paracrine CNP.

In mammals, the adult heart mainly synthesizes and releases NPs at the level of the atrial myocardial secretory granules. In particular, as compared to

Table 1 Natriuretic Peptides distribution in living organisms. For references see the text

Living organism		*Species*	*Peptide*
VERTEBRATES			
Mammalia		Several species (i.e. human, dog, mouse, rat, sheep, cow, pig, dromedary, dolphin)	ANP, BNP, CNP
Aves		Chicken (*Gallus gallus*)	BNP, CNP, RNP
Reptilia			
	Squamata	*Dendroaspis angusticeps* (venom)	DNP
	Chelodinia	Long-necked tortoise *(Chelodina longicollis)*	ANP, BNP
Amphibia			
	Anura	Bullfrog (*Rana catesbeiana*), Toad (*Bufo marinus*), African clawed frog (*Xenopus laevis*)	ANP, BNP, CNP
	Caudata	Newt (*Cynops pyrrhogaster*)	ANP, BNP
Osteichthyes			
	Teleostei	Tilapia *(Oreochromis mossambicus)*	ANP, BNP
		Pufferfish (*Takifugu rubripes*)	ANP, BNP, CNP
		Medaka (*Oryzias latipes*)	BNP, CNP
		Eel *(Anguilla anguilla japonica)*, Rainbow trout *(Oncorhynchus mykiss)*, Coho salmon *(Oncorhynchus keta)*	ANP, VNP
	Chondrostei	Sturgeon *(Acipenser transmontanum)*	ANP, BNP, CNP, VNP
Chondrichthyes			
	Elasmobranchii	Japanese dogfish *(Triakis shyllia)*, Shark (*Squalus acanthias*), Dogfish (*Scyliorhinus canicula*)	CNP
Cyclostomata		Hagfish (*Eptatretus burgeri*)	EbuNP
INVERTEBRATES		Roundworm (*Ascaris suum*), Silkworm (*Bombyx mori*), Blue crub (*Callinectes sapidus*), Oyster (*Crassostrea virginica*)	ANP-like
PLANTS		*Dracena godseffiana, Metasequoia, Hedera helix, Zea mays*	ANP-like
UNICELLULAR ORGANISMS		*Paramecium multimicronucleatum*	ANP-like

nonmammalian species (amphibians), the right atrium contains the highest peptide concentration, with respect to the left atrium. Cardiac NPs production is developmentally and spatially regulated. During ontogenesis, both atria and ventricle produce ANP and BNP. Ventricular production declines after birth, and in adult life it is restricted to the atrium. However, in the presence of pressure and volume overloads, cardiac hypertrophy, hypoxia, or chemical stimuli such as α- and β-adrenergic agonists, the ventricular BNP expression increases. Accordingly, it is considered an immediate–early gene. Myocardiocytes produce very little CNP,

which is synthesized and released by cardiac endothelial cells and fibroblasts after growth factor-induced stimulation (see for references Cerra and Pellegrino 2007).

The morpho-functional heterogeneity of the vertebrate heart has its endocrine hallmark in the different regional organization of the cardiovascular NPs system, which is clearly detectable moving from poikilotherms to homeotherms (see Aardal and Helle 1991). In adult nonmammalian vertebrates, the heart is capable of producing NPs more diffusely than in mammals. In fact, in birds, reptiles (Mifune et al. 1996; Cho et al. 1988), amphibians (Netchitailo et al. 1986, 1988; Fukuzawa et al. 1996), teleosts (Reinecke et al. 1985; Uemura et al. 1991; Donald et al. 1992; Loretz et al. 1997), and lungfish (Masini et al. 1996), NPs are largely produced by both atrial and ventricular cells. The presence of ANP immunoreactivity in both atrial and ventricular myocytes of the African lungfishes *P. aethiopicus* (Larsen et al. 1994) and *Protopterus annectens* (Masini et al. 1996) confirms that this is a very old acquisition of the endocrine heart. In general, atrial myocardial NPs production exceeds that of the ventricle, indicating that most of the secretory activity of the heart is concentrated in the atria. This occurs not only in the two-chambered teleost heart, but also in the three-chambered heart of amphibians. However, in the latter, a regional zonation is detectable at atrial level, more NPs being present in the left than in the right atrium (*Rana ridibunda*: Netchitailo et al. 1986, 1988; *Hyla japonica*: Feuilloley et al. 1993). If ANP and BNP are separately analysed, this heterogeneity appears even more evident. In fact, in the newt *Cynops pyrrhogaster* the atrial regions contain more ANP than BNP. This pattern is inverted in the ventricle, which shows a prevalent BNP expression (Kasuya et al. 1992). Interestingly, the abundance of secretory granulation, and thus of NPs production, seems related to the regional hemodynamic gradients and the sensor ability of the different cardiac chambers. In fact, the low-pressure venous regions of the mammalian heart, e.g., atria, as well as the entirely venous heart of poikilotherm vertebrates, are rich in NPs (Aardal and Helle 1991 and references therein). An example is in the newt. In this amphibian, the postcaval vein and the sinus venosus, as well as the trabeculated atrial and septal regions, contain an elevated granular content and a pronounced NP expression (Kasuya et al. 1992). Such a topographic strategy is encountered also in other species. In the hagfish, NP granulation is present in the portal vein heart. In rodents, it is present at the level of the superior and inferior vena cava, and in extrapulmonary veins (Aardal and Helle 1991 and references therein).

In all species examined so far, the primary acute stimulus for atrial NPs release is myocardial stretch induced by blood volume expansion (Ruskoaho 1992). In vertebrates, the atrium receives the blood returning from the circulatory system. Thanks to a rich pool of stretch receptors, this makes this chamber a strategically located sensor for monitoring changes in blood volume and pressure. Stretch-induced NPs release has been confirmed from teleosts to mammals. In trout heart, the increased filling pressure (but not afterload pressure) elevates both cardiac output and cardiac NPs secretion (Cousins and Farrell 1996). An osmotic mechanism also appears to regulate NPs release from the teleost heart. In fact, transfer from freshwater to seawater transiently increases both ANP and VNP in the eel (Kaiya and Takei 1996a). It has been suggested that hyperosmolality is more potent than

hypervolemia (and then atrial stretch) in stimulating atrial ANP release (e.g., eel and rainbow trout; Kaiya and Takei 1996b; Cousins and Farrell 1996), although volemic stimuli may still play a modulatory role (Kaiya and Takei 1996b). However, taking into account the very different mechanisms which characterize piscine osmoregulation (from osmoconformity in hagfishes, to urea, organic and inorganic osmolyte regulation in Chondrichthyans, to inorganic ion regulation in lampreys and Osteichthyans), it is noteworthy that species-specific strategies for regulating NPs release have not yet been established. Since it is often difficult to separate hyperosmolality from the consequent hypervolemia, data from mammals can provide insights with regard to this aspect. Hopefully, investigations concerning NPs expression and release in water-deprivation-tolerant mammals (e.g., *Camelus dromedarius*, Osman et al. 2004) will help the understanding of the major systemic and/or local (at organ level) switches which trigger the NPs system under these circumstances.

4.2 Natriuretic Peptide Receptor System

NPs act via specific receptors (NPRs), which exist throughout vertebrates and are heterogeneously distributed in all target tissues and organs such as the brain, the kidney, the intestine, blood vessels, and the heart itself. The presence of cardiac NPRs is consistent with a paracrine function exerted by these hormones. In all species studied so far, NPRs show at the heart level a regional zonation that might reflect the different hemodynamics of the cardiac chambers.

In nonmammalian species, most information regarding NPRs has been obtained in teleosts and elasmobranchs, and, to a lesser extent, in amphibians, while pertinent knowledge in reptiles and birds is missing. Two NP receptors, NPRA and NPRB, are single spanning transmembrane molecules, which show different affinities for ANP, BNP and CNP. They belong to the family of particulate guanylate cyclases (pGC) which convert GTP into cGMP (see for references Cerra and Pellegrino 2007). NPRA is evolutionarily old. In fact, its cDNA and gene have been identified not only in mammals, e.g., human, rat, mouse, but also in the bullfrog *Rana catesbeiana*, the eel *Anguilla japonica*, and in medaka (Garg and Pandey 2005). In the eel, a NPRA type of receptor, able to bind ANP and VNP with almost equal affinity, was found to be regionally distributed in the heart (Cerra et al. 1996).

NPRB represents the natural receptor for CNP, since it binds the peptide with a 50–500-fold higher affinity than ANP and BNP (Koller et al. 1991). It is present in nonmammalian vertebrates such as the eel and the spiny dogfish *S. acanthias*, the latter only expressing CNP (Aller et al. 1999). In amphibians, current information on this receptor suggests a tissue-specific expression, since its presence has been postulated in vascular smooth muscle (Chiu et al. 1990; DeBruno and Coviello 1992; Uchiyama et al. 1997; Minerds and Donald 2001) but not in the skin, which was unresponsive to CNP (Uchiyama et al. 1998).

The third receptor, NPRC, binds ANP, BNP and CNP with almost the same affinity, but it differs from the other two tetrameric receptors since it is active in a dimeric form and lacks the guanylate cyclase (GC) domain. It is mainly responsible for peptide clearance via rapid internalization and lysosomal degradation. This process regulates NP availability, thus indirectly controlling activation of the other NPRs. However, interaction of CNP with a subpopulation of NPRC can lead to either G-protein-coupled inhibition of adenylate cyclase (AC) or activation of phospholipase C (PLC) and G-protein-gated inwardly rectifying KC (GIRK) channels (see for references Cerra and Pellegrino 2007). NPRC also functions in nonmammalian vertebrates such as elasmobranchs, bony fish, amphibians and the turtle *Amyda japonica* (Toop and Donald 2004). Interestingly, in the eel, NPRC binds also VNP with a high affinity (see for references Takei and Hirose 2002).

Recently, a fourth NPR, detected only in the eel, has been called NPRD. It is GC-free, but, contrarily to NPRC, shows a tetrameric structure like NPRA and NPRB (Takei and Hirose 2002).

4.3 Cardiovascular Actions of Natriuretic Peptides

The NP-induced endocrine actions implicated in the regulation of blood pressure and volume include a large spectrum of distal targets. Cardiac ANP, BNP, and VNP generate an intricate multilevel network, which controls heart-vessel performance and volume–ion blood homeostasis. In contrast, CNP, which circulates at low concentrations in the blood, is considered as a paracrine endothelium-derived mediator (Ahluwalia et al. 2004). However, depending on the species and its environmental challenges, the various NPs may achieve different homeostatic roles, acting prevalently either as hypotensive and cardioactive factors, or as sodium–water extruders. DNP has potent diuretic, natriuretic and hypothensive effects comparable to those of the other members of the family (Schweitz et al. 1992; Collins et al. 2000; Lisy et al. 1999).

Here, we will mainly focus on NPs-induced cardiovascular actions, to exemplify the endocrine/paracrine circuits activated by these cardiac hormones.

4.3.1 Cardiac Actions

Once released, atrial and ventricular NPs may directly act on the heart, contributing to the multiple cardiac inhibitory mechanisms which protect it against overstimulation (hypervolemia, heightened adrenergic tone, stress, etc.). Accordingly, this NPs-dependent cardioinhibition, present in both mammalian and nonmammalian vertebrates, appears as an evolutionarily old component of the homeostatic loop which controls vertebrate cardiac performance.

In teleost and elasmobranch fish, functional investigations and receptor studies showed that NPs induce a direct negative inotropic influence. In both eel and trout

(McKendry et al. 1999), as well as in the dogfish (Montpetit et al. 2001), NPs exert a biphasic pressor–depressor response associated with heart rate modifications. The presence of biologically active (NPRA/NPRB) and NPRC-like receptors in the different chambers of the eel heart (Cerra et al. 1996) indicates that, at least in teleosts, the heart is a target for paracrine/autocrine NP-mediated modulation. NPs influence the performance of the branchial vasculature as well as that of the heart, since both ANP and CNP relax arterial and venous branchial vessels in a dose-dependent manner. This gill regulation represents one of the pieces of functional evidence which support the so-called NP cardio-protective hypothesis (Farrell and Olson 2000). In fact, NP-induced branchial vasodilation participates to the downstream cascade which gradually prevents the excessive hemodynamic load imposed on the heart by elevated afterloads (high branchial perfusion pressures). This event, which occurs beyond the *windekessel* control exerted by the bulbus arteriosus, contributes to a fine-tuned regulation of the piscine heart performance, being additional and/or alternative to the action exerted by the heterometric response (Frank–Starling). Moreover, the possibility exists that if changes in gill perfusion affect the blood supply of the branchial ionoregulatory cells, they may respond by modifying ion transport, thus contributing to regulate blood ion composition (i.e., Na^+, Cl^-) and osmolality. In view of the strong relationship between osmotic state and NPs heart release (see above), these processes may be coupled in an intriguing regulatory cardiovascular loop.

Amphibian heart is sensitive to NPs. On the isolated atria of *R. tigrina*, exposure to ANP induces negative chronotropic and inotropic effects (Chiu and Lee 1992). These actions are also documented for the whole isolated and perfused working heart of *Rana esculenta*. In fact, in the spontaneously beating heart, ANP reduces heart rate, while in electrically paced preparations it also decreases cardiac output and stroke volume (Cerra et al. 2003a). These effects, presumably mediated by anantin-specific ANP-binding sites (NPRA/NPRB?) detected in both the ventricular endocardium and myocardium, are activated by the same concentrations required for negative inotropism (Cerra et al. 2003a). Since in amphibians the heart is more sensitive than in mammals to the stretch-mediated mechanisms (e.g., the Starling response of the heart), it is of relevance that the heart releases hormones, e.g., NPs, that, in turn, prevent the heart from overloading itself. Moreover, in many amphibians the cardiocirculatory system plays a crucial role in preventing dehydration as well as excessive water accumulation, which depend on their aquatic life style (Toews and Wentzell 1995). Conceivably, a NPs-mediated cardiac control may contribute to an improvement in the functional plasticity of the organ, adjusting it against hydration/dehydration stresses. However, notable species-specific variability concerning the cardiac effect of NPs has been documented in amphibians. In fact, studies on the toad *B. marinus* were unable to reveal either cardiac ANP-binding sites or any effect on heart rate and contraction (Minerds and Donald 1997). Similarly, in *R. catesbeiana*, frog ANP and frog BNP were ineffective on inotropism, while frog CNP revealed a negative inotropic capacity (Uchiyama et al. 1997).

As in the case of NPs-elicited vascular actions, data on the direct NPs cardiac modulation from reptiles and birds are scarce. Direct cardiac effects were

only reported by an early study, which demonstrated in chicken that infusion of mammalian ANP 5–28, and/or chicken heart extract caused a marked hypotension (Gregg and Wideman 1986). However, subsequent investigations failed to demonstrate any effect on mean arterial blood pressure and heart rate (Schütz et al. 1992). The identification of an emodynamically strategic heterogeneous zonation of NP-binding sites in sinus venosus, aortic bulb, ventricular wall, and posterior vena cava of the quail *Coturnix coturnix japonica* (Cerra et al. 1993) supports the hypothesis of a regional sensitivity of the bird heart to NPs.

It is well-acknowledged in mammals that NPs exert long-term actions on the heart. The extensive mammalian literature in this field has revealed the importance of the NPs–NPRs system in processes linked to physiological and pathological cardiac remodeling, and, at the same time, the interplay with other homeostatic systems (e.g., NO–cGMP), pointing to the second messenger cGMP as a converging downstream signal. For example, in the presence of pressure and/or volume overload, the mammalian heart reactivates its fetal gene programme, thus reexpressing ANP and BNP in the ventricular cardiocytes (Cavallero et al. 2007). This elevated expression is maintained as the ventricle remodels, and increases with the progression of heart failure. This response appears protective, because of the antihypertrophic effect of the NP–pGC-dependent cGMP formation, helping to counterbalance maladaptive cardiac hypertrophy (de Bold et al. 2001; Tremblay et al. 2002; Molkentin 2003; Holtwick et al. 2003)

Unfortunately, no data are currently available concerning the long-term cardiac effects of NPs (growth and remodeling) in nonmammalian species. Since the nonmammalian (fish and amphibian) heart is able to undergo a dramatic morpho-functional rearrangement in response to the variable hemodynamic demands related to developmental and eco-physiological challenges (e.g., changes in body size and shifts in lifestyle patterns), it represents an impressive example of adaptive plasticity. This is paradigmatically shown by the zebrafish heart, which is an accepted model for studying cardiovascular development (Fishman and Stainier 1994), epigenetic remodeling (Hove et al. 2003), and adult heart regeneration (Poss et al. 2002). Accordingly, fish and amphibian hearts appear as natural models ideally suited for exploring the biological roles of the NP hormones and their long-terms effects on heart growth and myocardial regeneration.

4.3.2 Vascular Actions

In all species studied so far, cardiac ANP and BNP decrease vascular smooth muscle tone, and thus peripheral resistance, while increasing endothelial permeability (see for references Kiemer et al. 2005). CNP, being a selective venodilator, controls venous return and reduces cardiac filling pressure and output. It also induces coronary relaxation, thus affecting local myocardial supply (Hobbs et al. 2004). ANP and BNP decrease sodium and water content via central inhibition of thirst and sodium appetite, inhibition of intestinal water and sodium absorption, and direct stimulation of renal water and sodium excretion. The peptides also inhibit both aldosterone

secretion from the adrenal glomerulosa and vasopressin from the posterior pituitary, thus further promoting renal water and sodium excretion. As the end point, hypervolemia is faced and blood volume is rapidly restored to normal (Takei and Hirose 2002).

NPs vasodilatory actions are present in adult nonmammalian vertebrates from Agnatha to reptiles. In the lamprey *Petromyzon marinus* and in the Atlantic hagfish *Myxine glutinosa*, ANP and CNP dilate ventral aorta (Evans and Harrie 2001). Similar effects are also present in the branchial vasculature at the level of both arterial and venous routes, indicating an ANP- and CNP-dependent regulation of gill perfusion flow which may be of relevance to protect the heart from elevated afterload pressures (Farrell and Olson 2000).

In elasmobranchs, studies on the significance of their simple NP system have been almost exclusively carried out in organs involved in osmoregulation, e.g., the rectal gland. Few cardiovascular investigations have suggested that CNP is more active than ANP in vascular relaxation (Evans et al. 1993). Bolus administration of the homologous shark CNP exerts a biphasic pressor–depressor response in *S. acanthias* (McKendry et al. 1999; Montpetit et al. 2001) associated with a reduction of cardiac output (Montpetit et al. 2001).

In contrast to elasmobranchs, in teleosts NPs cardiovascular actions have been extensively described, showing that ANP is more a sodium and water extruder than a vasodepressant factor. However, *in vitro* and/or *in situ* studies carried out in many teleost species, including the toadfish (Lee and Malvin 1987; Evans et al. 1989), the trout (Olson and Meisheri 1989; Eddy et al. 1990; Conklin and Olson 1994a), the Japanese eel (Takei et al. 1990) and the cod (*Gadus morhua*) (Acierno et al. 1991) have well established that NPs unequivocally elicit vasorelaxation by directly acting on smooth muscle (Olson and Villa 1991). These effects are even stronger in the presence of preconstriction elicited by acetylcholine, epinephrine, carbachol, bradykinin, and thromboxane A2 (Evans et al. 1989; Evans 1991; Conklin and Olson 1994a). Interestingly, in trout, several in situ investigations have reported an initial increase in blood pressure following ANP (and VNP) administration, possibly associated with α-adrenergic stimulation (Olson and Duff 1992; Takei et al. 1994b; McKendry et al. 1999).

In amphibians, a clear vasodilation of both unstimulated and preconstricted vessels has been observed in many species. This effect, being elicited by ANP and CNP with similar potency, appears mediated by both NPRA and NPRB (Chiu et al. 1990; DeBruno and Coviello 1992; Uchiyama et al. 1997; Minerds and Donald 2001). The involvement of these GC-associated receptors is strengthened by the increased cGMP production observed in central arteries of *B. marinus* (Minerds and Donald 2001).

4.3.3 Interactions with CAs

Far from being an isolated hormonal system, NPs interplay with CAs in controlling heart performance. In the heart of mammals (Currie and Newman 1986; Garcia

et al. 1986), as well as in that of poikilotherm vertebrates, the cardiac release of ANP is stimulated by α- and β-ARs. In the amphibian *Rana ridibunda*, ISO and adrenaline stimulations increase ventricular expression of ANP and p38-MAPK, suggesting that the CA-induced p38-MAPK activation contributes to ANP accumulation (Aggeli et al. 2002). This enhanced ANP in both atrial and ventricular cells may reflect the ability of the heart to activate counter-regulatory loops aimed to counterbalance the hemodynamic challenges induced by adrenergic stimulation (e.g., positive inotropic effect). This hypothesis is supported by the antagonistic behavior shown by ANP against β-adrenergic stimulation in the isolated atria of *R. tigrina* (Chiu and Lee 1992). The cardioprotection may be of importance also in the medium- and long-term. In fact, as previously mentioned, exposure to high CAs may induce necrotic damage to the amphibian myocardium, as occurring in relation to temperature changes (Carlsten et al. 1983b; Volkmann 1985; Herman et al. 1986). Consistent with this possibility, in the heart of *R. ridibunda* the pattern of ISO-stimulated p38-MAPK activation and ANP production parallels the variations of experimental temperatures (Aggeli et al. 2002).

In *S. acanthias*, the pressor–depressor response induced by CNP administration associates with increased CAs levels. This is of note since the dogfish represents the only vertebrate that responds to NPs with increased CAs production (McKendry et al. 1999). The mechanism responsible for this CNP-associated CAs increase is still undetermined. In fact, *in situ* CNP perfusion of unstimulated posterior cardinal sinus was unable to increase both adrenaline and noradrenaline secretion. An enhancement in noradrenaline release was observed after carbachol treatment, suggesting the involvement of nicotinc mechanisms (Montpetit et al. 2001). From a functional point of view, elevation of circulating CAs after CNP administration may induce an early increase in arterial pressure. Since the dogfish heart and vasculature lack sympathetic control (Nilsson et al. 1975), in the presence of NPs-induced hypotension, elasmobranchs may recruit humoral vasoconstrictors, including CAs, to preserve cardiovascular function.

In teleosts, the response to bolus administration of ANP and VNP is associated with increased release of CAs from chromaffin cells and/or activation of the sympathetic nervous system (Olson and Duff 1992; McKendry et al. 1999). Although this agrees with the presence of ANP-binding sites in teleost chromaffin tissue (Kloas et al. 1994) and with the inhibition exerted by α-adrenergic antagonists (Olson and Duff 1992), a direct connection between NP stimulation and CA release, as well as their impact on cardiac performance, is still under examination (see Montpetit et al. 2001 for references and comments).

5 Chromogranin-A

During the last 5 years, chromogranin A (CgA) and its derived peptidic fragments have emerged as new players in the scenario of the endocrine heart. The first evidence regarding this protein came from its identification as the major soluble

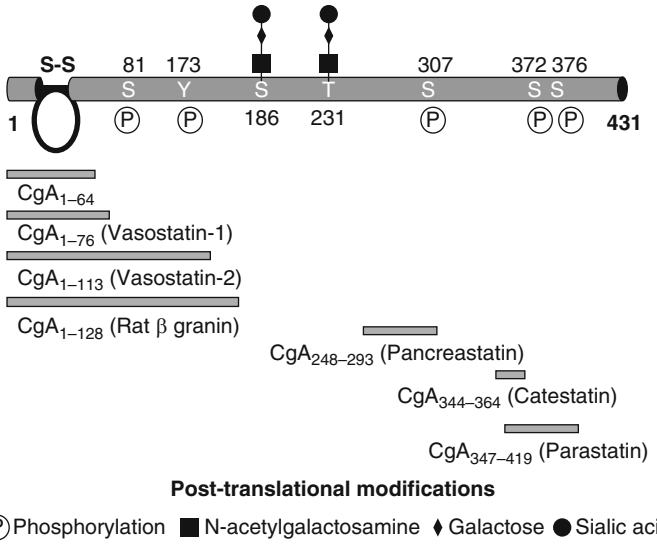

Fig. 2 Schematic illustration of several Chromogranin A-derived peptides. The N-terminal disulfide bridges (S–S) and the post-translational modifications of the bovine sequence are indicated (modified from Tota et al. 2007)

secreted constituent of the secretory granules of the chromaffin cells of the bovine adrenal medulla (Banks and Helle 1965; Blaschko et al. 1967) in which CgA is costored and coreleased with CAs hormones. However, it was only discovered later that CgA is an index member of the granin family of genetically distinct and uniquely acidic proteins that are ubiquitous in the nervous and immune systems as well as in the secretory cells of different tissues such as the adrenal medulla, the parathyroid gland, the gastro–entero–pancreatic system and the heart itself. This recognition paralleled its identification as a prohormone precursor of many fragments generated by the tissue-specific proteolytic cleavage operated by several proteases and prohormone convertases (e.g., PC1/3 and PC2 costored in the neurosecretory granules) on multiple pairs of dibasic sites which lie along the CgA sequence (Fig. 2; see Helle et al. 2007 for extensive references). On the basis of immunoreactive studies, CgA appears to be highly conserved protein throughout vertebrates, from mammals to birds, amphibians, fish and invertebrate classes down to the protozoans (Tota et al. 2007b). CgA is present in man, pig, rat, birds (the quail *C. coturnix japonica*; Reinecke et al. 1991), reptiles (the lizard *Lacerta viridis*; Trandaburu et al. 1999), amphibians (the frog *R. ridibunda*; Reinecke et al. 1991), and teleost fish (the coho salmon, *Oncorhynchus kisutch*; Deftos et al. 1987). CgA-like proteins have also been demonstrated in the nervous system of many invertebrates, including coelenterates (CgA-derived fragment WE-14; Barkatullah et al. 1997), the nematode parasite *Ascaris suum* (the hexapeptide KGQELE, flanking the C-terminus of rat CgA250–301; Smart et al. 1992), and within the secretory granules (trichocystis) of the protozoan *Paramecium tetraurelia* (Peterson et al. 1987). The very

high interspecies homology indicates the long evolutionary history of CgA. Among mammals, only minor differences in primary sequence and posttranslational modifications have been documented, as exemplified by the bovine protein ($bCgA_{1–431}$), which is shorter than the human ($hCgA_{1–439}$) and rat proteins ($rCgA_{1–448}$) (Tota et al. 2007b; Helle et al. 2007). Notably, the finding that the N- and C-terminal domains, $CgA_{1–76}$ and $CgA_{316–431}$, are highly conserved sequences in mammals is consistent with the role of CgA serving as a prohormone for shorter peptides acting as regulatory principles.

Apart from other important functions, e.g., biogenesis and exocitosis of chromaffin granules as well as being a diagnostic marker for neuroendocrine tumours, CgA is endoproteolytically processed to give rise to several peptides of biological importance, including the dysglycemic hormone pancreastatin (Tatemoto et al. 1986), the vasodilator vasostatin (Aardal et al. 1993), and the CAs release inhibitory peptide catestatin (Cts) (human $CgA_{352–372}$, bovine $CgA_{344–364}$; Mahata et al. 1997, 2000, 2003, 2004; Mahata 2004). Cts and pancreastatin have been postulated as important counter-regulatory hormones in "zero steady-state error" homeostasis (i.e., the finely regulated equilibrium generated by the balance between two counter regulatory hormones; Koeslag et al. 1999), a role now extended also to vasostatin 1 (VS-1) in cardiac biology (Helle et al. 2007, and references therein).

The importance of CgA in cardiovascular homeostasis in man is documented by its increased plasma levels in various diseases, such as neuroendocrine tumors (Nobels et al. 1994; Stridsberg et al. 1995) and chronic heart failure (Ceconi et al. 2002), as well as its colocalization with BNP and over-expression in human dilated and hypertrophic cardiomyopathy (Pieroni et al. 2007). Basal plasma levels of CgA correlate with sympathetic tone (Takiyyuddin et al. 1991) showing high heritability (Takiyyuddin et al. 1995). Plasma level of Cts peptide ($\sim$1.5 nM) decreases in patients with essential hypertension, the complex chronic disorder with a poorly understood pathogenesis, and also in normotensive subjects with a family history of hypertension and increased epinephrine secretion (O'Connor et al. 2002); this implies that Cts is an inhibitor of chromaffin cell CAs secretion *in vivo*. Genetic ablation of the chromogranin A (*Chga*) gene results in high blood pressure in mice, which can be rescued by the introduction of the human *CHGA* gene in the $Chga^{-/-}$ background (Mahapatra et al. 2005).

Like CgA, interestingly, also NPs show similar proteolytic processing of the precursor, which gives rise to several derived cardiovascular-active peptides. In fact, in mammals, proteolytic cleavage of proANF gives rise not only to the major form of circulating ANP (ANP1–28), but also to several biologically active peptides from the 98-amino acid N-terminus of the prohormone (proANF 1–30, long-acting sodium stimulator; proANF 31–67, vessel dilator and proANF 79–98, kaliuretic stimulator) (Vesely et al. 1994).

Since 1990, CgA has been identified at heart level in the granules of rat atrial myoendocrine cells (Steiner et al. 1990), and in the cells of the conduction system in colocalization with the α1E subunit of the voltage-gated calcium channel (Weiergraber et al. 2000). More recently, CgA identification was extended to the human ventricular myocardium. Under normal conditions, the peptide is expressed

at low levels which are detectable by PCR and ELISA; but in the presence of dilated and hypertrophic cardiomyopathy, CgA is also immunologically detectable on tissue sections (Pieroni et al. 2007). Interestingly, in the heart, CgA correlates with the cardiac NPs system. In fact, in atrial myocytes and in the conduction cells, the protein is costored with ANP, while in the ventricle it colocalizes with BNP (Pieroni et al. 2007). This evidence is a further indication of the potent secretory ability of the endocrine granules of the heart. They in fact represent a complex secretory machinery in which multiple substances are stored to be released under appropriate stimuli, e.g., myocardial stretch. Intriguingly, in both decompensated and hypertrophic heart, increased plasma CgA levels parallel the increment of circulating BNP (Pieroni et al. 2007). Thus, at cardiac level, the stretch-induced release and/or transcriptional upregulation mechanisms described for NPs could also be operative for CgA (Pieroni et al. 2007).

5.1 The CgA-Derived Vasostatins

Two CgA fragments corresponding to the highly conserved vertebrate domain $CgA_{1–76}$ (VS-1), and the less conserved domain $CgA_{1–113}$ (VS-2) are named vasostatins (VSs) because of their ability to relax vessels which have been precontracted by high endothelin-1 (ET-1) and potassium concentrations (Aardal and Helle 1992). They have been identified in both homeotherm (man, rat, pig, bovine) and poikilotherm (frog) vertebrates. The fragments sequenced so far show a very high percentage of identity, sharing important traits such as the presence, in all species, of a disulfide bridge between C17 and C38 (crucial for their biological activity), and the sequence 50–62 with 100% identity) (Helle et al. 2007).

VS-1 and VS-2, together with the other shorter VS peptides $CgA_{1–40}$, $CgA_{4–57}$, $CgA_{47–66}$ and $CgA_{67–76}$, are naturally generated within the matrix of chromaffin granules and are coreleased with CAs following chromaffin cell stimulation, for example by acetylcholine (Metz-Boutigue et al. 1993). Likely, VSs are generated also at heart level. In fact, several N-terminal fragments containing the VS-1 domain have been found in rat heart extracts ($CgA_{4–113}$, $CgA_{1–124}$, $CgA_{1–135}$ and $CgA_{1–199}$), together with a larger fragment, presumably corresponding to the intact CgA; this is consistent with an intracardiac cleavage of the precursor (Glattard et al. 2006).

VSs are multifunctional peptides, showing, in addition to their vasorelaxant properties, other functions via autocrine, paracrine and/or endocrine mechanisms (see for references Tota et al. 2007b).

As expected on the basis of morphological and biochemical data mentioned above, CgA-derived VSs contribute to the autocrine/paracrine regulation of the heart, as comparatively documented in both homeotherm (rat) and poikilotherm (eel, frog) vertebrates. Their major actions consist in both negative inotropism (eel, frog, rat) and lusitropism (rat), and in the relevant counteraction of β adrenergic-mediated

positive inotropism, which is typically induced by isoprenaline (eel, frog, rat) (Tota et al. 2004; Imbrogno et al. 2004; Cerra et al. 2006; Pieroni et al. 2007).

Negative inotropism and lusitropism are more pronounced in the case of VS-1, which is active at low nanomolar concentration. These effects, as shown in the rat heart, are independent from heart rate and coronary performance and appear comparable in the trabeculate and luminally-supplied eel and frog hearts. Of note, in the three species, in the presence of ISO stimulation, both VS-1 and the longer VS-2 are able to counteract the β-adrenergic positive inotropism and lusitropism (Tota et al. 2004; Imbrogno et al. 2004; Cerra et al. 2006; Pieroni et al. 2007). The inotropic response is obtained with a functional noncompetitive type of antagonism (Cerra et al. 2006). Interestingly, the isolated and perfused frog heart working at physiological loads was used as a bioassay for structure–function analyses aimed to describe the VSs structural requirements involved in both the specific inhibition of cardiac contractility and the counteraction of the β-adrenergic inotropism. Since both effects are most potently elicited by VS-1, CgA7–57 and $CgA1–40_{SS}$, the disulfide bridge appears crucial for the marked negative inotropism whether mechanically activated or stimulated by ISO (Tota et al. 2003). This also indicates that the inotropic activity of the N-terminal domain of CgA is highly conserved and that neither the N-terminal nor the C-terminal group of VS-1 are critical for this activity.

More recently, in the rat heart, VS-1 was found to exert cardioprotection by reducing the effects of ischemia, in a manner which mimics ischemic preconditioning (Cappello et al. 2007). On the whole, the cardiotropic and vasoactive properties of CgA-derived VSs, together with the ischemic preconditioning-like effect suggest that these peptides function as homeostatic stabilizers of the cardiovascular system, particularly under conditions of stress (i.e., sympathetic overstimulation, or cardiac injury).

5.1.1 Action Mechanism/s of VSs

The mechanism of action of VSs is still enigmatic. So far, the identification of classic high affinity receptors remains elusive, while receptor-independent cell penetration (antimicrobial action)- or perturbation (cardiac inotropism)-associated mechanisms were postulated (Tota et al. 2007b; Helle et al. 2007).

Studies on eel, frog and rat hearts indicated aspects of unity and diversity regarding the intracellular cascades underliyng the VS-1-elicited negative inotropism. In eel and rat, but not in frog, these cascades involve β-adrenergic receptors, PTX-sensitive G-proteins, and the NO–cGMP–PKG pathway. Only in the eel they also involve cholinergic M1 receptors. In both eel and frog, the VS-1 negative inotropism is also abolished by the inhibition of potassium and calcium fluxes (and thus of intracellular Ca^{2+} transients), emphasizing the importance of spatially restricted membrane domains in which receptors, modulatory proteins and ion channels may be functionally coupled (Corti et al. 2004; Imbrogno et al. 2004; Cappello et al. 2007). Taken together, all these findings call for caveolae as sites of action for the peptide. In fact, in cardiac cells caveolae represent specialized membrane

microenvironments in which the above signaling molecules are clustered with their substrates (for ref. see Tota et al. 2007b). The involvement of the cytoskeleton in the functional coupling of VS-1 to its intracellular signaling partners was also taken into consideration. In fact, when the cytoskeleton is not correctly functioning, because of the inhibition of either actin polymerization (via cytochalasin D), or vesicles intracellular trafficking (via BDM and wortmannin), or actin association with L-type calcium channels by calmodulin-dependent kinase (via by W7), the VS-1-induced negative inotropism is impaired, being either abolished in the frog, or severely reduced in the eel (Mazza et al. 2007).

5.2 Catestatin

Another CgA fragment which recently came to the attention of cardiac investigators is Cts. In contrast to VSs, its sequence is located close to the C-terminal region of the prohormone, corresponding to human $CgA_{352–372}$, or bovine $CgA_{344–364}$ (Mahata et al. 1997, 2000, 2003, 2004; Mahata et al. 2004).

Cts is a strong inhibitor of CAs release mediated by nicotinic receptor. It non competitively inhibits nicotinic receptors and blocks the calcium-dependent CAs release as well as the acetylcholine-induced desensitization of the nicotinic receptor itself (Mahata et al. 1997). Via histamine receptors, the peptide also induces vasorelaxation, showing antihypertensive characteristics (Kennedy et al. 1998). In fact, circulating levels of human Cts decrease in the plasma of patients with essential hypertension. Genetic ablation of the *Chga* gene in mice increases blood pressure and pretreatment of *Chga*-null mice with Cts prevents blood pressure elevation, indicating a direct role of Cts in preventing hypertension (Krüger et al. 2003). Very recently, the direct cardiovascular effects and mechanisms of action of Cts on myocardial and coronary functions were documented in mammals using the Langendorff-perfused rat heart (Angelone et al. 2008). Cts dose-dependently increased heart rate and coronary pressure and decreased left ventricular pressure (LVP), rate pressure product (RPP) and both positive and negative LV*dP*/*dt*. Cts inhibited phospholamban (PLN) phosphorylation. The inotropic and lusitropic actions being abolished by chemical inhibition of β_2-ARs, Gi/o protein, NO, or cGMP indicate involvement of β_2–ARs–Gi/o protein–NO–cGMP signaling mechanisms. Cts also inhibited ET-1-induced positive inotropism and coronary constriction (Angelone et al. 2008). These studies, which point to Cts as a novel cardiac modulator able to protect the mammalian heart against excessive sympathochromaffin over-activation (e.g., hypertensive cardiomyopathy) were also extended to the amphibian heart. Using the isolated avascular *R. esculenta* heart as a bioassay, in which the interactions between the endocardial endothelium (EE) and the subjacent myocardium can be studied without the confounding effects of the vascular endothelium, Mazza et al. (2008) demonstrated the direct cardiotropic effects of bovine catestatin and its interaction with β-adrenergic (ISO) and ET-1 signaling. In fact, Cts dose-dependently decreased stroke volume and stroke work, with

a threshold concentration of 11 nM, approaching the *in vivo* level of the peptide. Cts reduced contractility by inhibiting phosphorylation of PLN. Furthermore, the Cts effect was abolished by pretreatment with either NOS (L-NMMA) or cGMP (ODQ) inhibitors, or an ET-1 receptor (ET_B) antagonist (BQ788). Cts also noncompetitively inhibited the ISO–dependent positive inotropism. Moreover, Cts inhibited the positive inotropic effect of ET-1, mediated by ET_A receptors without influencing the negative-inotropic ET-1 action mediated by ET_B. Cts action through ET_B was further supported when, in the presence of BQ788, Cts failed to inhibit the positive inotropism of both ISO and ET-1 stimulation and PLN phosphorylation. That is, also the cardiotropic actions of Cts, including the β-adrenergic and ET-1 antagonistic effects documented in frog, support a novel role of this peptide as an autocrine–paracrine modulator of cardiac function, particularly when the stressed heart becomes preferential target of both adrenergic and ET-1 stimuli (Maaza et al. 2008).

Significance of two cardioactive peptides in one precursor- The analogous cardiosuppressive actions of VS-1 and Cts raise the question concerning the biological significance of two peptides processed from one precursor. Further studies will clarify whether, behind such apparently redundant molecular strategy, there is an advantage of having two peptides regulating the same physiological response. Because of coordinate actions, they may act on overlapping or different sites, subserving subtly different functions, e.g., summation and synergism, or distinct spatio-temporal compartmentation (cell- and tissue-specific proteolytic processing and release). With specific reference to cardiovascular prohormones, we have already seen (see Paragraph 5) the example of the two peptide hormones processed from the proANP precursor, i.e., vessel dilator and ANP (Vesely 2006), each of the two proANP gene products have vasodilatory, diuretic and natriuretic properties.

6 The Renin–Angiotensin-System

The Renin Angiotensin System (RAS) is an endocrine, autocrine and paracrine hormonal system which in vertebrates exerts a major control of blood pressure, extracellular fluid osmolarity, and volume homeostasis. The fast action and the short duration of its effects indicate that RAS responds to emergent changes in either the internal or the external environment to initiate a homeostatic reestablishment of the initial conditions. The RAS cascade (Fig. 3) starts with the renin-mediated cleavage of the decapeptide angiotensin I (Ang I) from angiotensinogen. Then, a dipeptide is removed from the C-terminal of Ang I, generating the octapeptide angiotensin II (AngII), which is considered the most physiologically active component of the RAS. Further cleavage processes of the AngII-N-terminus by aminopeptidases generate the vasoactive angiotensin III (AngIII; Ang 2–8), angiotensin IV (Ang IV; Ang 3–8) and Ang 1–7 (see Nishimura 2001 for references). The AngI/AngII conversion is catalysed by a rather nonspecific dipeptidyl-carboxypeptidase, the angiotensin converting enzyme (ACE: Ehlers and Riordan 1990). Alternative to ACE, other

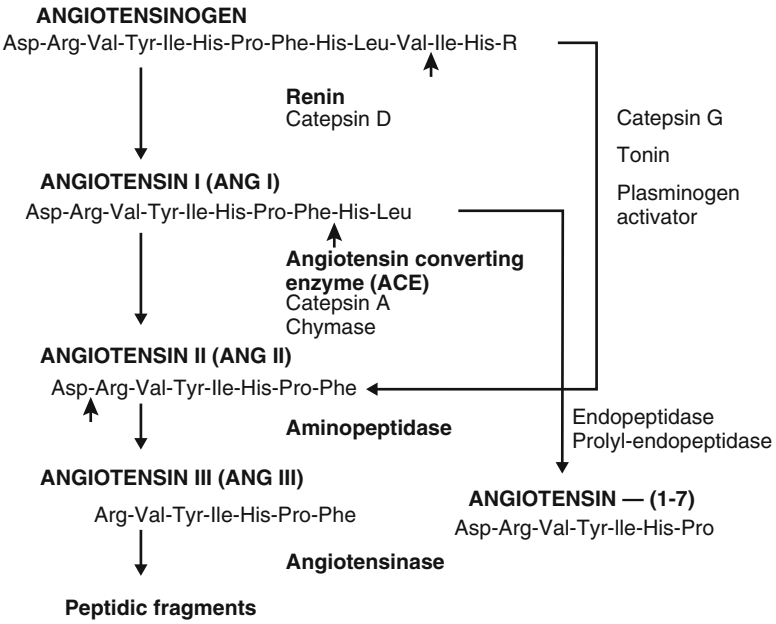

Fig. 3 Enzymatic pathways for AngII production. The scheme is based on information mainly derived from mammalian species. For references see the text

enzymatic pathways are responsible for AngII production. Tonin, cathepsin G, and chymotrypsin generate AngII directly from the substrate, bypassing AngI. Prolyl endopeptidase and other peptidases form Ang 1–7 (Ferrario and Iyer 1998), perhaps both from AngII and directly from Ang I, bypassing again classic pathways. ACE, also called kininase II, links the RAS to its physiological enantiomer, the kallikrein–kinin system (KKS). In fact, ACE cleaves and inactivates bradikinin (BK) (Kobayashi and Takei 1996). Another link between the two systems is represented by kallikrein, which cleaves renin from the precursor prorenin to generate BK from the precursor kininogen (Kobayashi and Takei 1996).

Native AngII is a stable molecule which shows little structural species-specific variations, indicating a very high evolutionary conservation. Changes are encountered at position 1 (Asp/Asx/Asn), 3 (Val/Ile/Pro), and 5 (Ile/Val). AngI shows substitutions also at position 9 (His/Ser/Ala/Asn/Tyr/Gly/Thr) (see Kobayashi and Takei 1996; Nishimura 2001).

Nonmammalian studies have shown that RAS shares with other hormonal systems (i.e., CAs, NPs, CgA) a high phylogenetic continuity. The morphological and biological traits of nonmammalian RASs have been well summarized by a large number of reviews and books which have provided the basis for a critical evaluation of its phylogenetic development (Kobayashi and Takei 1996; Nishimura 2001; Sandberg and Ji 2001). Moreover, recent application of highly sensitive molecular biology techniques has allowed to bridge some important evolutionary gaps,

including the suggested absence of an active RAS in cyclostomes and elasmobranchs. In fact, the failure to detect renin activity and to elicit AngII-elicited vasopressor effects initially suggested that RAS first appeared in teleosts (Nishimura et al. 1973; Takei et al. 1993). However, this hypothesis has been reconsidered after the identification of a unique AngI in cyclostomes (Takei 2000; Rankin et al. 2004) and in *T. scyllia* (Takei et al. 1993).

It both mammals and nonmammalian organisms two RASs are expressed: one is localized in the plasma and regulates immediate cardiovascular requirements; the second is localized in tissues and is mainly involved in long-term modifications. It is commonly assumed that the presence of ACE is a strong, although not conclusive, evidence of tissue AngII production. Based on this, a local RAS has been postulated in the heart of elasmobranchs, sarcopterigians, lungfish, and teleosts (Nishimura et al. 1970, 1973; Takei 2000; Takei et al. 2004a). ACE-like activity is described in the heart of the lamprey *Lampetra fluviatilis* (Cobb et al. 2002), in the branchial heart of the hagfish *Myxine glutinosa* (Cobb et al. 2004), in the heart of the elasmobranchs *Raja erinacea, S. acanthias*, and *Scyliorhinus canicula* (Lipke and Olson 1988; Uva et al. 1992), and of *R. esculenta*, and *X. laevis* (Quassinti et al. 2007). Apart from these findings, major evidence concerning the spatial localization and the enzymatic pattern of the cardiac RAS comes from studies on mammals. Hopefully, this knowledge will be extended to lower vertebrates. In the mammalian heart, the density of ACE in the right atrium is higher than that in the left atrium and in the ventricle. ACE is mainly found in the endocardial layer and the cardiac valves, in the endothelium of large and small coronary arteries and arterioles, while coronary veins almost completely lack it (Yamada et al. 1991; Falkenhahn et al. 1995). This regional expression strongly increases in the presence of elevated load challenges (mechanical stretch), thus indicating an increased local production of AngII (Sadoshima et al. 1993). At least in mammals, in addition to ACE, chymase represents a major AngII converting enzyme activity (Urata et al. 1990). Interestingly, during the last 7 years, the complexity of cardiac RAS has been increased by the identification of an homologue of ACE, called ACE2, which cleaves AngI and AngII into the inactive Ang1–9 and Ang1–7, respectively (for references see Oudit et al. 2003). It also hydrolyzes the kinin metabolites des-Arg10-kallidin (des-Arg10-Lys1-bradykinin) and des-Arg9-bradykinin, thus further linking RAS and KKS (Vickers et al. 2002). In the heart ACE2 is highly expressed in the coronary endothelium (for references see Oudit et al. 2003). The antagonism between ACE and ACE2 modulates the balance between AngII (vasopressor) and Ang 1–7 (vasodilator), with a significant impact on cardiovascular function. Interestingly, ACE2 was also recently found in several poikilotherms such as the zebrafish, the chicken, *Fugu*, *Tetraodon* and *Xenopus tropicalis*. In the heart of transgenic *X. tropicalis* tadpoles ACE2 is expressed on the epicardial surface, and in the truncus arteriosus (Chou et al. 2006), a location which may correspond to the endothelial cell expression found in mammalian arteries, arterioles and venules (Oudit et al. 2003).

6.1 AngII Receptor System

The existence of an active RAS requires the presence of specific binding sites for AngII in target tissues and organs. A growing number of molecular, biochemical and functional studies indicate that AngII receptors (ATs) are present throughout vertebrates. However, the current knowledge on ATs characterization and classification has been provided by the impressive number of studies performed in mammals. Contrarily, information on the pharmacological properties, the signaling pathways and the biological functions of ATs in poikilotherms is fragmentary. Accordingly, before describing ATs in nonmammalian species, it is necessary to summarize basic information obtained on mammals.

Mammalian ATs are classified, based on their affinity for selective nonpeptidic antagonists, as AT1 (losartan, CV11974 and TCV116 sensitive) and AT2 (PD 123177, PD 123319 and CGP 42112 A sensitive). AT1 is composed of at least two subclasses (AT1A and AT1B) which bind the same antagonists (Iwai and Inagami 1992; Chiu et al. 1994). Other ATs subtypes (i.e., AT4) are associated with AngIV binding (see De Gasparo et al. 1995 for extensive review). AT1 and AT2 belong to the superfamily of G-protein coupled receptors, but they utilize different second messengers, thus exerting different final effects. AT1, the best characterized AngII receptors, are responsible for the majority of AngII actions, especially those concerned with osmoregulation, fluid balance, cardiovascular modulation and growth promotion. They are coupled to at least five effector systems (Alexander et al. 1985; Berk and Corson 1997; Griendling et al. 1997). The main postreceptor signal transduction pathways include activation of the slow Ca^{2+} channel (Freer et al. 1976), acceleration of phosphoinositide hydrolysis (Baker and Aceto 1989; Baker et al. 1989) and stimulation of NOS activity (Paton et al. 2001). Contrarily to AT1, AT2 are higly expressed in fetal tissues and rapidly decline after birth, being scarcely expressed in adult mammals (Horiuchi et al. 1999; Inagami et al. 1999; Stroth and Unger 1999). They do not utilize DAG and IP3 signaling and their activation counteracts the AT1-mediated effects, resulting in an antipressor activity and degenerative cellular remodeling (Horiuchi et al. 1999; Inagami et al. 1999). AT2 modulate cGMP levels, serine–threonine phosphatase and arachidonic acid production, (for review, see de Gasparo et al. 1995; Stroth and Unger 1999). Their antigrowth effects are partly mediated by protein tyrosine phosphatase activation, with subsequent inactivation of mitogen-activated protein kinase (Tsuzuki et al. 1996). Both AT1 and AT2 have been identified in the heart. Cardiac AT1 are responsible for most of the AngII-mediated effects on cardiac performance (i.e., chronotropism and inotropism) and rate of protein synthesis in isolated myocyte preparations (Schorb et al. 1993), while AT2 induce opposite effects (Lijnen and Petrov 1999).

Moving from this well established mammalian framework, cloning and ligand affinity studies performed on nonmammalian species indicate that the ATs pool is mostly represented by AT1-like receptors. In some cases (i.e., catfish; Olivares-Reyes et al. 1997), analysis of both signaling cascades and competitive binding abilities has suggested the presence of “atypical” AngII receptors. There is no

available evidence for nonmammalian AT2-type receptors. It is currently believed that a prototypic AT receptor (AT1?), evolved in primitive vertebrates, diverged during phylogeny into more than one type to give mammalian ATs (Nishimura 2001). However, since application of antagonists selective for mammalian receptor subtypes is not appropriate for selective classification of nonmammalian AT receptors, a definitive picture requires the development of new and more selective molecules, and/or the results of further molecular biology studies.

Functional and pharmacological analyses carried out in many poikilotherms indicate the presence of AT with low affinities for the classic ligands, such as losartan and CGP42112A. This is in contrast with the higher affinity for losartan and CGP42112A described for the angiotensin receptor cloned from turkey adrenal (Murphy et al. 1993). ATs were identified in elasmobranchs, teleosts, amphibia and reptiles at the level of tissues and organs involved in cardiovascular and hydromineral homeostasis (see Kobayashi and Takei 1996 and Nishimura 2001 for extensive references).

In elasmobranchs, an AngII-mediated osmoregulatory role is supported by the presence of CGP42112A-sensitive receptors in the rectal gland and the considerable affinities for both losartan and CGP42112A in the interrenal gland (Tierney et al. 1997a; Hazon et al. 1997). Interestingly, in *S. acanthias* ATs might be located at the adrenergic nerve endings and/or in the adrenal medulla (Opdyke and Holcombe 1976; Carroll and Opdyke 1982; Bernier et al. 1999), which suggests a functional link with the adrenergic system. In *S. canicula* the atrium, the ventricular myocardium, and the outer conal layer are able to bind the homologous dogfish Pro3AngII with high-affinity (Cerra et al. 2001). Of note, competition experiments performed with either homologous AngII or CV11974 or CGP42112 (specific ligands for mammalian AT1 and AT2, respectively) indicate a prevalence of CGP42112-selective ANGII binding sites in both the inner and the outer conal layers (Cerra et al. 2001). Although caution is needed when interpreting the binding data obtained with mammalian selective ATs ligands, these results point to an unknown dogfish AT2-like receptor, thus contradicting the view that nonmammalian ATs are mostly AT1-like.

Studies on teleosts have revealed the presence of a single AT isoform in the proximal tubule brush border of *A. anguilla*, and proximal tubular cells from the icefish, *Chiondraco hamatus* (Marsigliante et al. 1994, 1996, 1997), but no data are available on the heart. Cloning of a cDNA encoding for an AT receptor has revealed in *A. anguilla* about 60% homology to the rat AT1A and 35% to rat AT2 subtypes (Tran van Chuoi et al. 1999). However, in spite of these results, molecular and pharmacological characterization of teleost ATs remains unavailable.

In amphibians, an AT1-homologue has also been identified at the level of different tissues and organs, including the heart. In *Xenopus*, the myocardium expresses an AT receptor that shows more than 60% affinity with the mammalian AT1 and is considered its amphibian counterpart. These receptors display pharmacological properties distinct from mammalian AT1 and AT2. They are G protein-coupled and act by increasing cytosolic Ca^{2+} via IP3 (Ji et al. 1993; Bergsma et al. 1993). Their expression is higher than that found in other AngII target tissues (Ji et al.

1993; Nishimatsu et al. 1994) and this contrasts with the more limited presence of AT1 receptors in the mammalian heart (Nishimatsu et al. 1994; Allen et al. 2000). Accordingly, the cardiac responsiveness of the amphibian heart to AngII may remarkably differ from that of mammals.

There is no cloning evidence about a reptile AngII receptor, but ATs with very low affinity for both losartan and PD123319 were identified in cardiac cell membrane of the snake *B. jararaca*. This receptor is not coupled to PLC/phosphatidylinositol biphosphate breakdown adenylyl cyclase inhibition nor activation (Breno et al. 2001).

6.2 AngII Cardiovascular Actions

In all vertebrates, AngII-induced cardiovascular actions result from direct targeting on vascular smooth muscle and cardiac tissues, and from SNS involvement. SNS stimulation is an evolutionary old characteristic of the AngII system since it occurs in agnathans, elasmobranchs, teleosts, amphibians, reptiles, birds and mammals (Carroll and Opdyke 1982).

The identification of both cardiac ACE-like activity (*R. erinacea, S. acanthias*, and *S. canicula*; Lipke and Olson 1988; Uva et al. 1992) and AngII binding (*S. canicula*; Cerra et al. 2001) indicates that a local RAS is operative in the elasmobranch heart. Nevertheless, no corresponding functional information is available, except one study reporting in *S. acanthias* unchanged heart contractility after AngII administration (Opdyke et al. 1982b). However, the receptor data obtained in the atrium, the ventricular myocardium, and the outer conal layer of *S. canicula* strongly suggest that the peptide is able to target the heart, following a chamber-specific pattern (Cerra et al. 2001). The fact that, in another dogfish species, *Scyliorhinus stellaris*, the outer layer of the conus arteriosus is also rich in binding sites for the vasodilator CNP (Cerra et al. 1995), suggests that in elesmobranchs AngII and CNP may behave as functional antagonists in regulating the pulsatile activity of the cardiac outflow region.

Both homologous and heterologous AngII exert notable hypertensive action, as early documented in *S. acanthias* (Opdyke and Holcombe 1976). A number of investigations carried out in different species (i.e., *S. canicula*: Hazon et al. 1989; *T. scyllia*: Hazon et al. 1995; see Anderson et al. 2001 for references) confirmed the initial hypothesis that AngII is a hypertensive agent in elasmobranchs. Moreover, the increase in plasma CAs levels, which parallels AngII administration (Opdyke et al. 1981), and the disappearance of AngII-mediated pressor effects after α-adrenergic inhibition (Opdyke and Holcombe 1976), suggested that SNS activation is required for AngII vascular control. However, this view was reconsidered after the results of more recent studies which revealed that, in *T. scyllia*, α-adrenergic inhibition only delayed slightly the pressor response of homologous AngII, without affecting its amplitude (Tierney et al. 1997b). This suggests that both CAs and ANGII are required for the complete vasopressor effect. In this case, AT receptors located

either at the adrenergic nerve endings or in the adrenal medulla, and at vascular level may contribute to the vasopressor action. The presence of AngII binding sites in branchial blood vessels of *T. scyllia* further confirms a direct vascular targeting of the peptide (Tierney et al. 1997a).

The only available data on Dipnoan cardiovascular function show that in the Australian lungfish, *Neoceratodus forsteri*, application of AngII of either the fish-type [Asn1, Val5] (Sawyer et al. 1976), or the tetrapod-type [Asp1, Val5], or the native type [Asn1, Val5] (Joss et al. 1999), is able to induce a dose-dependent increase in arterial pressure with no effects on heart rate and negligible renal effects.

Evidence for an intracardiac RAS in teleosts comes from the identification of ACE-like activity in the heart of a variety of species, including six air-breathing teleosts (*Heteropneustes fossilis, Clarias batrachus, Channa gachua, Anabas testudineus, Notopterus chitala*, and *Monopterus cuchia*: Olson et al. 1987). This suggested the presence of AngII-dependent intracardiac circuits of regulation. However, whether these circuits are operative in the short-term by modulating the beat-to-beat performance, and/or in the medium/long term by regulating cardiac growth and remodeling, is unknown. In both the American eel *A. rostrata* (Bernier et al. 1999) and the trout *O. mykiss* (Oudit and Butler 1995) the heart is either directly or indirectly stimulated by homologous $[Asn^1, Val^5]$-AngII. In particular, indirect effects are supposed to be mediated either by CAs (Oudit and Butler 1995; Bernier and Perry 1999) or by adrenergic tone modulation (Reid 1992). In fact, the dose-dependent increments in cardiac output and stroke volume, observed in *O. mykiss* after teleost AngII exposure are inverted by α-adrenergic block (Bernier and Perry 1999). The adrenergic involvement is confirmed by both the increased CAs secretion from in situ posterior cardinal vein and the elevated plasma CAs concentrations in chronically cannulated fish after bolus injections of $[Asn^1, Val^5]$-AngII (Bernier et al. 1999). However, *in vitro* perfusion of the isolated working heart of the freshwater European eel *A. anguilla*, indicates that the homologous teleost peptide elicits a direct cardiac action. In fact, teleost AngII induces direct negative chronotropic and inotropic effects but does not affect the Frank-Starling response (Imbrogno et al. 2003). Inotropism is blocked by CV11974, an antagonist with a high specificity for piscine ANGII receptors (see Cerra et al. 2001 and references therein), thus calling for AT1-type of receptors. Negative inotropism and chronotropism contrast with the stimulatory effects reported in trout and in the American eel. Species-specific cardiac responsiveness, different intracardiac AT patterns, and the use of either intact cardiovascular or *in situ* or isolated and denervated working heart preparations may contribute to these discrepancies. For example, in contrast to the *in vitro* experiments, *in vivo* analyses can detect synergism between circulating CAs and RAS. Interestingly, in the European eel, inotropism is unaffected by both adrenergic inhibition (i.e., via propanolol, sotalol, phentolamine) and activation (i.e., via phenylephrine and ISO), arguing against an intracardiac adrenergic involvement, further excluding CAs from the direct cardiac AngII effects (Imbrogno et al. 2003). On the other hand, the study of Imbrogno et al. (2003) revealed that in the eel heart the AngII-induced inotropism occurs via an EE–NO–cGMP–PKG mechanism. In the mammalian vascular endothelium, which

is rich in AT1 receptors, AngII is able to interact with the endothelial specific NOS isoform (for references, see Li et al. 2007). Conceivably, although there is no report concerning the presence of AngII receptors in the EE of teleosts, the obligatory role played by the eel EE in transducing the intracavitary ANG II signal may indicate a prevalent EE location of AT1-like receptors.

Species-specific AngII effects have been documented in teleost vasculature, showing also at this level synergy with the adrenergic system (Oudit and Butler 1995; Bernier and Perry 1999). In trout, AngII regulates minute-to-minute blood pressure (Olson 1992; Kobayashi and Takei 1996). Since venous capacitance curves are not affected by ACE inhibition in vivo (Zhang et al. 1995) and isolated large veins are either refractory or exhibit an endothelium-dependent relaxation to AngII administration (Conklin and Olson 1994a, b), this effect is believed to be almost exclusively related to the vasoconstriction of small resistance vessels (Conklin and Olson 1994b; Olson et al. 1994; Zhang et al. 1995). However, an involvement of the RAS in the control of venous capacitance in trout during exercise has been recently postulated (Sandblom et al. 2006).

Due to their evolutionary position in the transition from aquatic to terrestrial life and to their double life style, amphibians offer a variety of natural models for understanding the multiple functions that AngII can exert at the cardiovascular level. Unfortunately, so far, ACE activity was only found in crude heart homogenates from *R. esculenta* and *X. laevis* (Quassinti et al. 2007) and no further cell-specific characterization has been carried out. In toad isolated atria, AngII administration elicits tachycardia (Slivkoff and Warburton 2003). This cardiac sensitivity is accompanied by a remarkable expression of cardiac AngII receptors whose pharmacological profile and intracellular signaling have been characterized in *Xenopus* (Ji et al. 1993; Nishimatsu et al. 1994). Interestingly, these receptors populate the myocardium more than in mammals, suggesting a potentially higher cardiac sensitivity to the hormone, but it is unknown if they are activated by either circulating or locally released AngII. It is possible that AngII is produced and released by cardiac cells (e.g. endocardium) and via autocrine/paracrin signaling activates this rich population of myocardial receptors with short/long term effects on cardiac performance.

Indeed, despite this very fragmentary information, an important intracardiac role of RAS should be expected. For example, in many species (i.e., frogs, toads) the heart is constantly exposed to the risks linked to dehydration, including abnormal variations of myocardial interstitial fluid composition and osmolarity. In this context, AngII may play a major role because of its influence on the ion–fluid equilibrium through the modulation of thirst-related behavior, cutaneous water gain, and renal handling of ions and water (Hillyard 1999; Johnson and Propper 2000). This is exemplified by the morpho-functional rearrangement observed on the pericardial mesothelium of *R. esculenta* after exposure to homologous AngII (Cerra et al. 2003b). It is likely that, during dehydration, increased AngII production (Hillyard 1999) elevates pericardial permeability, modulating the characteristics of the pericardial fluid and thus of the cardiac interstitium. Moreover, AngII-mediated local mechanisms may contribute to the cardiac vulnerability observed in frog in relation to sexual and seasonal factors (Garofalo et al. 2006).

Like in mammals, in amphibians AngII appears as a component of the vascular compensatory hormonal processes activated in the presence of osmotic changes. For example, AngII-elicited modulation of vascular responsiveness is important in setting back arterial pressure in the presence of hypotesive stresses (i.e., dehydration-induced volume depletion, hypotension caused by sodium nitroprusside administration; *B. marinus*: West et al. 1998). In some cases, the hormone-evoked vasoconstriction resembles the situation found in mammals, being mediated by a direct vascular action (*B. marinus*; Wahlqvist and Campbell 1988). In other cases, a CAs-mediated vascular response is also present (*R. catesbeiana*: Carroll and Opdyke 1982; Harper and Stephens 1985).

Reptiles express all RAS components (Lavras et al. 1978; Zehr et al. 1981; Cipolle and Zehr 1984; Silldorff and Stephens 1992). In the snake *Bothrops jararaca* both RAS-related enzymes and substrates have been characterized and activation of the system was shown to influence cardiovascular and steroidogenic functions (Breno and Picarelli 1992; Lazari et al. 1994). However, except for the identification of AngII receptors in cardiac membranes of *B. jararaca*, there is a lack of information concerning the direct effect of either AngII administration or ATs activation on the heart performance of reptiles. *In vitro* functional studies, mainly carried out at the vascular level, have described an AngII-induced pressor response, but the specific AT receptor type involved has not yet been reported (Breno and Picarelli 1992; Lazari et al. 1994). In both *B. jararaca* (Breno and Picarelli 1992), and *Ptyas korros* (Chiu et al. 1986), AngII effects are blocked when either ACE is inhibited by captopril or the peptide antagonist Sar1,Leu8 AngII is administred (Breno and Picarelli 1992). Moreover, vasoconstriction is reduced by phenoxybenzamine, suggesting that part of the response can be attributed to a direct AngII action and part to an indirect action of the hormone through CAs release (Breno and Picarelli 1992; Chiu et al. 1986).

The reptilian cardiovascular system is characterized by important shunts which control the mixing of oxygen-rich and oxygen-poor blood, providing crucial physiological adjustments. Major cardiac shunts are made by the right-to-left bypass of pulmonary circulation, and by the left-to-right redistribution of the pulmonary oxygenated venous blood into the pulmonary circulation (Burggren et al. 1997). These shunts are under a remarkable neuro-endocrine influence (i.e., CAs), which, through finely regulated adjustments, determine blood direction in response to environmental challenges and different physiological states (see Hicks 2002 for references). In this context, and in absence of available information, studies on how RAS activation is involved in the regulation of reptilian cardiovascular physiology are much required.

7 Other Cardiac Endocrine/Paracrine/Autocrine Substances

From the late 1990s, the discovery of other cardiac products with endocrine/paracrine/autocrine properties has dramatically expanded the concept of the endocrine heart. Table 2 summarizes the major cardiac products so far identified.

Table 2 Major products identified in the heart of poikilotherm and homoitherm vertebrates

Substance	*Site of production*	*Ref.*
Adenylpurines	Endothelial cells and cardiac myocytes	*Shah (1996)*
Adrenomedullin	Cardiac myocytes and fibroblasts (atria and ventricles)	*Kato et al. (2003)*
Aldosterone	Atria and ventricles (rat)	*Delcayre et al. (2000)*
Angiotensin II	Cardiac and vascular RAS	*Dzau (1993)*
Calcitonin gene-related peptide	Atria, sinoatrial and atrioventricular nodes	*Beaulieu e Lambert (1998)*
Catecholamines	ICA (intrinsic cardiac adrenergic cells)	*Huang et al. (1996, 2005)*
CgA and derived peptides	Atrial and ventricular cardiocytes, conduction system	*Steiner et al. (1999)*; *Weiergraber et al. (2000)* *Pieroni et al. (2007)*
CoASSG (Coenzyme A Glutathione disulfide)	Myocardial tissue	*Luo et al. (2006)*
Cytokines	Cardiac myocytes and coronary endothelium	*Kaye et al. (1996)*
Endothelin-1	Coronary endothelium and cardiac tissue	*Shah (1996); Beaulieu e Lambert (1998)*
Ghrelin	Cardiomyocytes	*Iglesias et al. (2004)*
Leptin	Cardiomyocytes	*Purdham et al. (2004)*
Neuropeptide Y	Nodal tissue, atria and coronary vessels	*Beaulieu e Lambert (1998)*
NO	Endothelial cells and cardiac myocytes	*Shah (1996)*
Oxytocin	Cardiac tissue (rat)	*Jankowski et al. (1998)*
PAMP (proadrenomedullin N-terminal 20 peptide)	Cardiac myocytes and fibroblasts (atria and ventricles)	*Kato et al. (2003)*
Prostanoids	Endothelial cells and cardiac myocytes	*Shah (1996)*
Relaxin	Atrial cardiomyocytes (rat)	*Taylor e Clarck (1994)*
Substance P	Sinoatrial and atrioventricular nodes	*Beaulieu e Lambert (1998)*
Urocortin	Cardiomyocytes and non-cardiomyocytes	*Ikeda et al. (2002)*
VIP (Vasoactive Intestinal Peptide)	Sinoatrial node and coronary vessels	*Beaulieu e Lambert (1998)*

However, such a list is far to be complete since novel substances are discovered day by day. Accordingly, beyond its specific hallmark muscle/pump characteristics, the vertebrate heart, continuously reveals itself as a very complex multifunctional and interactive nodal point of information transfer, in which the highly integrated systems (endocrine and nervous) of the body orchestrate diverging and/or converging autocrine/paracrine pathways responsible for the structural and functional integrity of the organ. Because of such complexity, even more than in the past, present day cardiac physiologists may wonder what is the respective importance of “the parts and the whole” in the global behavior of the heart. By reporting here few examples from the more recent literature on poikilotherm vertebrates we will

often refer to mammalian studies since they provide the major data. Among cardiac products of nonmammalian vertebrates, ghrelin, adrenomedullin and neuropeptide Y represent a promising field of research to widen the comparative and evolutionary scenario of the endocrine heart.

Ghrelin is a 28-amino-acid acylated peptide found by reverse pharmacological approach as the natural ligand of the orphan receptor called growth hormone secretagogue receptor (GHS-R) (Kojima et al. 1999). In both mammals and nonmammalian vertebrates, the peptide is involved in the regulation of pituitary hormone secretion, food intake and drinking behavior (Unniappan and Peter 2005). So far, ghrelin has been detected in several elasmobranchs such as the hammerhead shark (*Sphyrna lewini*) and the black-tip reef shark (*Carcharhinus melanopterus*) (Kawakoshi et al. 2007). It is also present in teleosts such as the goldfish (*Carassius auratus*; Unniappan et al. 2002), the eel (*A. anguilla*; Kaiya et al. 2003a), the Mozambique tilapia (*Oreochromis mossambiques*; Kaiya et al. 2003b), the Nile tilapia (*Oreochromis niloticus*; Parhar et al. 2003) and the rainbow trout (*O. mykiss*; Kaiya et al. 2003c). Like in mammals, piscine ghrelin is formed from its precursor, preproghrelin, which consists of three regions: the signal peptide, the mature peptide, and the C-terminal peptide (Unniappan and Peter 2005) which in mammals is known as obestatin (see Garcia and Korbonits 2006). Ghrelin peptides identified in nonmammalian species show different length and different posttranslational processing, including a C-terminal amidation which is present only in the piscine peptide (Unniappan et al. 2002). Although in poikilotherms ghrelin is mainly localized in the brain, the stomach, and the intestine, recent data have also identified ghrelin mRNA in the heart of the Japanese eel (Kaiya et al. 2003a). This first evidence of cardiac ghrelin production in nonmammalian vertebrates was not followed by functional studies aimed to clarify whether the peptide acts as an endocrine mediator, or it influences the heart itself in a paracrine/autocrine fashion. In mammals, cardiac ghrelin, whose release is stimulated by fasting and water intake, modulates cardiac cell energy and protects against hypoxia- and CAs- induced injuries (Li et al. 2006). The peptide also contributes to negatively control lusitropism and inotropism by involving NO, prostaglandin and KCa channels (Soares et al. 2006; see Garcia and Korbonits 2006). Moreover, by acting on peripheral resistances and by inhibiting water intake, it ameliorates cardiac hemodynamics, reducing cardiac load. (see Unniappan and Peter 2005 for references). The identification of GHS-R at the level of either the human myocardium and aorta, or the rat atrium and left ventricle, has suggested that in mammals ghrelin acts as a paracrine/autocrine cardiac hormone (Katugampola et al. 2001).

Adrenomedullin (AM) has been recently recognized as a cardiac hormone, since it has been found in cardiac atria and ventricle of mammals (Kitamura et al. 1993; see Kato et al. 2003). It is synthesized by cardiac myocardiocytes, coronary endothelial cells, and fibroblasts and is released following mechanical stress (i.e., volume and pressure overload) and/or chemical stimulation by humoral agents such as ANGII and ET-1 (Ishiyama et al. 1997; Morimoto et al. 1999; Yamakawa et al. 2000).

So far, AM has been sequenced in nonmammalian vertebrates, including several piscine species (i.e., two pufferfishes *Takifugu rubripes*, and *Tetraodon nigroviridis*, rainbow trout, zebrafish, *Cyprinus*), and birds (*Gallus gallus*) (see Takei et al. 2004b). AM-like material was found in tissues from many species, from fish, to amphibians, reptiles (*Rana perezi*; Collantes et al. 2003; *Pleurodeles waltl*, and *Dermophis mexicanus*: Munoz et al. 2001), and birds (*G. gallus*; Zudaire et al. 2005), However, among nonmammalian vertebrates, only chicken cardiomyocytes were found to express AM in both atria and ventricle (Zudaire et al. 2005). Encouraging functional evidences obtained in the rainbow trout and the Atlantic cod indicate that AM is able to reduce heart rate and dorsal aortic pressure, thus playing a role on the short-term cardiovascular performance (Aota 1995). In mammals, intracardiac AM of both myocyte and fibroblast origin, is a potent inhibitor of cardiomyocytes hypertrophy, fibroblast proliferation and collagen deposition in the extracellular matrix (Kato et al. 2003). It also downregulates ANP gene expression and ANP secretion from cardiomyocytes in vitro (Sato et al. 1997). We can therefore expect that AM-activated autocrine/paracrine processes, together with NPs and RAS, may contribute to the long-term cardiac plasticity of the lower vertebrate heart.

As in the case of other recently discovered cardiac hormones, there is a limited, but promising, literature in nonmammalian vertebrates concerning the cardiac production and release of Neuropeptide Y (NPY). It belongs to a peptidic family which also includes Peptide YY (PYY) and Pancreatic Polypeptide. NPY and PYY were isolated and sequenced in many vertebrate species, including poikilotherm vertebrates (see Holmgren and Jensen 2001). NPY, the major member of the family and the most conserved during its 450 million years of evolution, is present in both central and peripheral nervous system of a large number of homeotherm and poikilotherm vertebrates. It is involved in appetite and blood pressure modulation and exerts stimulatory effects on heart performance (see Holmgren and Jensen 2001). The cardiac production of NPY in nonmammalian vertebrates was postulated after the discovery of the peptide in the eel heart (Uesaka 1996). More recently, NPY mRNA was found in the heart of the orange spotted grouper (*Epinephelus coioides*) (Chen et al. 2005). In the eel, a homologous eel NPY extracted by heart homogenates is able to increase the contractile force of the isolated atrium in a dose-dependent manner, without altering the beating rate. These effects, obtained by increasing intracellular free Ca^{2+}, are synergistic with those obtained by CAs (Uesaka 1996). This is not surprising since, as shown in mammals, NPY-induced cardiac stimulation occurs either directly by inducing coronary constriction and facilitating CAs-induced vasoconstriction, or indirectly by both enhancing afterload and potentiating the effects of other cardiostimulators, including CAs (Uesaka 1996). The functional association between NPY and CAs, which concurs to NPY-mediated cardiac control, is also present in elasmobranchs. In the coronary vessels of the skate *R. rhina*, NPY potentiates the effect of noradrenaline (Holmgren 1995). In the dogfish, contrarily to noradrenaline which enhances cardiac output by increasing stroke volume, NPY increases cardiac output by increasing heart rate. Combined administration of NPY and norepinephrine increases pressure development via

elevation of inositol 1,4–5-triphosphate level, although the two substances did not change pressure if independently administered (Xiang 1994).

8 A Major Autocrine/Paracrine Sensor-Integrator: The NOS–NO System

Although it cannot be properly considered as a cardiac hormone, the highly diffusible gas Nitric Oxide (NO) finds its place in this chapter because its pleiotropic actions (for recent reviews in a comparative and evolutionary perspective, see Tota et al. 2007a, Tota and Trimmer, 2007) include also its role as a potent autocrine and paracrine modulator of cardiovascular physiology. Its production by the family of spatially compartmentalized Nitric Oxide Synthase (NOS) isoenzymes (namely endothelial, inducible and neuronal NOS: eNOS, iNOS, nNOS, respectively), provides the heart with a finely tuned tonic modulation of contractility, energetics and substrate metabolism, influencing cell growth and survival. At the same time, it works as a very sensitive spatio-temporal autocrine/paracrine organizer through connection–integration processes, thereby playing a role in networks configuration. In fact, as largely demonstrated in mammals, the intracardiac NOS–NO system is positioned at the cross-road of many extrinsic and intrinsic neuro-endocrine pathways, including those activated by a variety of autacoids. Recent investigations, here reported, started to extend these concepts to the heart of several poikilotherm vertebrates, in particular teleost fish and amphibians.

In the hearts of these animals at least two differently located NOS isoforms, eNOS and iNOS, both constitutively expressed, were detected (see for ref. Tota et al. 2005). In several ecophysiologically and phylogenetically distant species of fish, such as the European eel (*A. anguilla*), two Antarctic teleosts, the hemoglobinless *Chionodraco hamatus* (icefish) and its red-blooded ecotype counterpart *Trematomus bernacchii*, as well as the evolutionary distant lungfish *P. dolloi* (Pellegrino et al. 2004; Amelio et al. 2006, 2008), eNOS is highly expressed at the level of the ventricular EE. A comparable pattern of expression is also detectable in the amphibian heart, as shown in *R. esculenta*, in which the enzyme is localized at the EE cells (Sys et al. 1997). Contrarily to eNOS, in fish iNOS is mainly, if not exclusively, expressed at the level of the ventricular myocytes (Amelio et al. 2006). Unlike the compact myocardium of the mammalian heart, in which the EE has comparatively limited extension, the avascular or poorly vascularized fish and amphibian hearts exhibit an impressively large EE surface which covers the intricate network of myocardial trabeculae of the cardiac wall (Tota 1983; Tota and Gattuso 1996). This endocardial tissue represents a major source for endocardial eNOS–NO release (Tota et al. 2005). Moreover, like in the mammalian heart, in the coronary-supplied fish hearts, eNOS is expressed not only in the EE, but also at the level of the vascular endothelium, as exemplified by the subepicardial vessels of *T. bernacchii* (Amelio et al. 2006) and the coronaries of the tuna ventricle (*Thunnus thynnus thynnus*: Tota et al. 2005). According to the mammalian literature (for references and

comments see Seddon et al. 2007), both the EE- and the vascular endothelium-generated NO are considered a paracrine factor, since, once released, it may rapidly diffuse to influence the performance of the subjacent myocardium. In teleosts (i.e., *A. anguilla*, and *C. hamatus*), activation of cardiac constitutive NOSs (mainly eNOS) is responsible for a basal production of nanomolar concentrations of NO (Pellegrino et al. 2004). In the unstimulated (basal conditions) hearts of *A. anguilla* (Imbrogno et al. 2001) and *Salmo salar* (Gattuso et al. 2002), NO, endogenously produced by the NOS authentic substrate (L-arginine), induces negative inotropic effects. Similar cardiodepression is also caused by exogenous NO donors (*A. anguilla*: Imbrogno et al. 2001; Atlantic salmon embryos: Eddy et al. 1999; *R. esculenta*: Sys et al. 1997). In addition to the basal cardiac nitrergic control, NO is involved in the modulation of signal-tranduction pathways triggered by physical and/or chemical stimuli (e.g., cholinergic, see below). For example, in *A. anguilla* (Imbrogno et al. 2001) and *S. salar* (Gattuso et al. 2002), NO modulates the cardiac heterometric response (i.e., Frank-Starling mechanism), allowing the heart to better sustain filling pressure changes. In all poikilotherms studied so far, both NO-dependent basal inotropic modulation and the heterometric response require the activation of soluble GC, the consequent release of cGMP, and the activation of PKG (for references see Tota et al. 2005). This is in agreement with the mammalian knowledge which indicates that the cGMP pathway is utilized by paracrine NO, largely produced by eNOS located in the coronary endothelium, to hasten myocardial relaxation, reduce myocardial oxygen consumption, inhibit β-adrenergic inotropic responses and affect the Frank-Starling mechanism. Moreover, autocrine NO may also directly act on ion channels and pumps, thanks to the subcellular location of eNOS and nNOS (Seddon et al. 2007, and references therein). The inotropic and lusitropic effects exerted by autocrine NO, released either by the caveolar eNOS and the nNOS of the sarcoplasmic reticulum, require cGMP signaling (Seddon et al. 2007). In teleosts and amphibians, the cardiac NO–cGMP signaling is also involved in the cross-talk with other major regulatory signalings. For example, the biphasic inotropic responses to cholinergic stimulation observed in *A. anguilla* and *R. esculenta* are mediated by NO–cGMP mechanism (Gattuso et al. 1999; Imbrogno et al. 2001). In addition, in *A. anguilla* this signaling system is required to transduce the negative inotropic effects elicited by β3-AR stimulation (Imbrogno et al. 2006) and exposure to endoluminal AngII (Imbrogno et al. 2003). In the eel, but not in the frog heart, the negative inotropism induced by VSs peptides (as reported above) is mediated by an active NO–cGMP signal transduction mechanism (Imbrogno et al. 2004; Corti et al. 2004). This information is summarized in Table 2. According to these findings, in teleost and frog hearts, used as paradigms of cold-blooded vertebrate hearts, several cardioregulatory hormones examined in this chapter converge on NOS/NO and recruit cGMP signaling to exert their effects (Imbrogno et al. 2003, 2004, 2006).

The interactions between the NOS–NO system and other cardiomodulators follow defined spatio-temporal criteria and involve specialized regions both at tissue (i.e., EE *vs* myocardium) and cellular level (i.e., membrane caveolar microdomains). As epitomized by the eel and frog hearts (see Table 3), the EE integrity is a

Table 3 Involvement of EE–NO in the modulation of cardiac performance of eel and frog. For references see (Tota et al. (2005 2007a, b); (Imbrogno et al. 2001, 2003, 2006)

Cardiac performance modulator	Eel	Frog
Acetylcholine (positive inotropism)	+	+
Acetylcholine (negative inotropism)	−	+
Angiotensin II	+	?
Endothelin-1 (positive inotropism)	?	+
Endothelin-1 (negative inotropism)	?	+
Vasostatin I	+	−

+ involvement;
− no involvement;
? no detected

prerequisite for cholinergic, ANGII and VSs modulation of inotropism (Imbrogno et al. 2001, 2003, 2006). It is conceivable that the EE-located eNOS contributes to the cross-talk between intracavitary physical and chemical stimuli and the EE – NO – cGMP system. Indeed, such enzyme location at the interface between the blood and the subjacent myocardium facilitates NO to function as a fine-tuned autocrine–paracrine endoluminal sensor-transducer. At the cellular level, the selective compartmentation of eNOS within the caveolar membrane microdomains may allow the enzyme to interact with other caveolar proteins (i.e., AT1, muscarinic and β3-AR receptors) providing an integrated regulation of cardiac performance.

9 Concluding Remarks

Knowledge of the structural and functional basis of the heart as an endocrine organ has made possible major advances in the understanding of vertebrate cardiovascular homeostasis and brain–heart connections, providing a new dimension of physiology.

Pioneering work on heart regulation by intracardiac CAs, costored with proteinaceous material in the electron-dense specific secretory granules, paved the long way to the discovery of the cardiac NPs, followed by the more recent identification and characterization of a surprising number of new endocrine and paracrine principles. Interestingly, some of them (the Chromogranin A-derived cardiosuppressive and "antiadrenergic" Vasostatin 1 and Catestatin) revealed themselves as major components of the electron-dense material of the specific cardiac secretory granules, hence highlighting how a morphological feature may hide the secret beauty of intricate molecular loops only later uncovered (namely, the form is the plastic image of the function). Similar to this, a large number of lessons are offered by the comparative and evolutionary knowledge related to the endocrine heart of cold-blooded vertebrates. An obvious one is that only a historical approach (development, phylogenetic conservation and diversification, as well as adaptive plasticity) can suggest ways that incipient endocrine agents, with their molecular loops, have evolved as important cardiac modulators and how they have become critical intermediates during

evolutionary transitions or in a distinct phylogenetic lineage. Another lesson is more pragmatic and of a particular guide for those researchers in the specific field. Indeed, the dynamic balance between cardiac hormones, receptors, autocrine/paracrine pathways (including the NOS/NO system) and the short-, medium- and long-term physiological implications of their relationships appears now more and more intricate. A better understanding of the old evolutionary roots of these networks, and how they have evolved from relatively less complicated designs, can help to disentangle the experimental complexity unraveling intricate cardiac pathways. Even if there are many serious gaps due to discontinuous information available, we have provided together with the literature, also some paradigmatic examples which hopefully will help the reader to adopt the Claude Bernard (1865) principle ("There are also experiments in which it is proper to chose certain animals which offer favorable anatomical arrangements or special susceptibility to certain influences. This is so important that the solution of a physiological or pathological problem often depends solely on the appropriate choice of the animal for the experiment so as to make the result clear and searching") so that he will find some ideas presented in this chapter useful.

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Stoking the Brightest Fires of Life Among Vertebrates

Raul K. Suarez and Kenneth C. Welch

Abstract Hummingbirds and nectarivorous bats in flight display some of the highest rates of aerobic metabolism among vertebrates. Analysis of the pathway of oxygen, i.e., the "oxygen transport cascade", reveals the concerted upregulation of capacities for O_2 flux from the external environment, through the respiratory and cardiovascular systems, into muscle mitochondria. Pathways for aerobic energy metabolism are highly conserved, but enzymatic capacities for carbohydrate and fatty acid oxidation, as well as for aerobic ATP synthesis, are also upregulated in concert. Despite evidence indicating sufficient capacities for fatty acid oxidation to support hovering, repeated bouts of hover-feeding in hummingbirds and nectar bats involve the oxidation of carbohydrate. Recent studies reveal that recently ingested sugar directly fuels flight, giving rise to the concept of the "sucrose oxidation cascade". The ecological and bioenergetic advantages conferred by sugar oxidation during foraging are discussed.

1 Introduction

Kleiber's "fires of life", referring to the processes involved in aerobic energy metabolism (Kleiber 1961), must rely on a supply of carbon substrates while consuming O_2 and producing CO_2. It is therefore appropriate that a volume concerning respiratory physiology should include a chapter dedicated to the subject of fuel use. Here, we shall examine aerobic pathways of muscle energy metabolism, how they are fueled, and how they operate during exercise in species that achieve some of the highest known mass-specific metabolic rates among vertebrates.

Small hummingbirds in steady, hovering flight display mass-specific rates of oxygen consumption $(\dot{V}O_2)$ (Bartholomew and Lighton 1986; Lasiewski 1963; Suarez

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Table 1 Metabolic rates of some animals during exercise

	Mass (g)	VO_2 (ml O_2/g × h)	References
Honeybee	0.078	101.0	Suarez et al. (1996)
Rufous hummingbird	3.4	38.3	Suarez et al. (1990)
Nectar bat	11.7	21.6	Winter et al. (1998)
Etruscan shrew	2.4	24.1	Fons and Sicart (1976)
Human athlete	70,000	4.3	Blomstrand et al. (1986)

Not intended as a comprehensive list, the purpose of the table is to facilitate comparison of rates sustained during routine hovering in rufous hummingbirds (*Selasphorus rufus*) and nectar bats (*Glossophaga soricina*) with summit metabolic rates of Etruscan shrews (*Suncus etruscus*) at low temperature, human athletes during exercise at VO_2max, and honeybees (*Apis mellifera*) in hovering flight. In the case of exercising animals, because >90% of VO_2 is accounted for by locomotory muscles, data concerning muscle mass allow the estimation of VO_2 per unit muscle mass. When RQ values are known, flux rates through pathways of carbohydrate or fatty acid oxidation and ATP turnover rates can be calculated

et al. 1990) about tenfold higher than those observed in humans engaged in maximal, aerobic exercise (i.e., VO_2 max) (Blomstrand et al. 1986) (Table 1). During molting, or in response to mass-gain (Epting 1980), or when exposed to low-density, normoxic air (Chai and Dudley 1995), hummingbirds are able to increase their hovering metabolic rates further by about 50%, thereby achieving the highest known mass-specific, aerobic metabolic rates among vertebrates. Nectarivorous bats during hovering flight display mass-specific VO_2 values (Voigt and Winter 1999; Welch et al. 2008; Winter et al. 1998) about 40–50% lower than hummingbirds but are, nevertheless, impressive when compared with even smaller mammals, e.g., similar to summit metabolic rates measured in shrews exposed to low ambient temperature (Fons and Sicart 1976). To burn most brightly in these animals, the fires of life require the highest flux rates of O_2 and CO_2, and rates of fuel catabolism known among vertebrates. During high-intensity, aerobic exercise, >90% of whole-body metabolic rate is accounted for by locomotory muscles (Suarez 1992; Taylor 1987). This greatly facilitates mechanistic studies concerning the relationships between biochemical flux capacities and actual (physiological) flux rates. This is especially true of hummingbirds, in which two sets of flight muscles, the pectoralis and supracoracoideus, consist exclusively of fast-twitch, oxidative fibers (Grinyer and George 1969; Lasiewski et al. 1965; Mathieu-Costello et al. 1992; Rosser and George 1986). These features allow the use of whole-body VO_2 and VCO_2 values for the estimation of rates of oxidation of carbon substrates in a single cell-type (Suarez et al. 1990).

2 Pathways of Fuel Oxidation

Among the major achievements of twentieth century biology was the elucidation of the major pathways involved in the oxidative metabolism of carbohydrates, fats and amino acids. Comparative biochemists and physiologists then built upon this

foundation, exploring natural variation on the themes established by Meyerhof, Warburg, Krebs, and their successors. This body of work has revealed that pathways for the catabolism of various substrates occur as modular units. These modules are found in various combinations in homologous cell types across species. In locomotory muscles, broad patterns of quantitative variation in enzymatic flux capacities across species are now well-documented. The stage is now set for studies of functional significance in the context of both ecology and evolution, the primary focus of this chapter.

In evolving to become small and to hover while feeding on floral nectar, hummingbirds and nectarivorous bats have both invaded a niche previously occupied exclusively by insects (Dudley 2000). The flight muscles of hummingbirds (Suarez et al. 1986, 1990) and nectarivorous bats (Suarez et al., unpublished) possess high enzymatic flux capacities for the oxidation of carbohydrates and long chain fatty acids. They use the same, highly conserved metabolic modules found in all vertebrate endotherm red muscles and hearts (Fig. 1). These include the

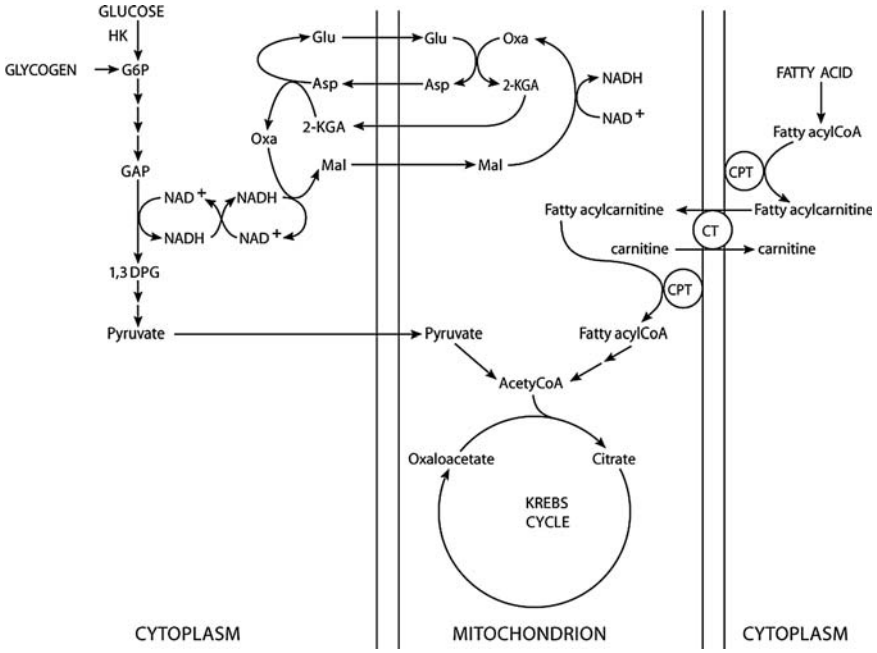

Fig. 1 Pathways of carbohydrate and fatty acid oxidation in hummingbird flight muscles. A number of the modules referred to in the text are apparent: glycolysis, the malate–aspartate shuttle (*both on the left*), fatty acid oxidation (*on the right*), the Krebs cycle (*bottom*). Metabolic pathways are highly simplified and are redrawn from Suarez et al. (1990). Abbreviations: *HK*; hexokinase, *G6P*; glucose 6-phosphate, *GAP*; glyceraldehyde 3-phosphate, *1,3 DPG*; 1,3-diphosphoglycerate, *NAD*$^+$; nicotinamide adenine dinucleotide (oxidized), *NADH*; nicotinamide adenine dinucleotide (reduced), *Oxa*; oxaloacetate, *Mal*; malate, *2-KGA*; 2-ketoglutarate, *Glu*; glutamate, *Asp*; aspartate, *CPT*; carnitine palmitoyltransferase, *CT*; carnitine acyltranslocase. CPT II is shown facing the matrix-side of the inner mitochondrial membrane

glycolytic pathway, the malate-aspartate shuttle for maintenance of high cytoplasmic $[NAD^+]/[NADH^+]$ ratios during high rates of glycolytic flux, a carnitine-dependent pathway for long-chain fatty acid oxidation, and high mitochondrial capacities for flux through the Krebs cycle, electron transport, proton pumping and oxidative phosphorylation (Suarez et al. 1986, 1991). It is both relevant and interesting to consider how a journal editor once commented that it was unfortunate that a new enzyme or pathway had not been found that explains why hummingbirds display such high metabolic rates. That evolution often works by making use of what is available through inheritance, rather than by reinventing biochemical machinery de novo whenever needed, should not be the cause of disappointment. In the case of the flight muscles of hummingbirds and bats, evolutionary processes have enhanced enzymatic flux capacities through known pathways of carbohydrate and fatty acid catabolism. Of particular interest is the upregulation of hexokinase (HK) activities; the resulting high capacities for glucose phosphorylation show the contrast between the flight muscles of hummingbirds and bat species with high sugar diets and those of birds of other species (Crabtree and Newsholme 1972b) and non-nectarivorous bats (Yacoe et al. 1982). However, hexokinase is not even considered to be a glycolytic enzyme by some biochemists (Fell 2000). It will be seen further on that the results of comparative physiology probably warrant a revision of this view.

Evolution has similarly enhanced pathways for long-chain fatty acid oxidation through increased enzyme levels. These are the same enzymes catalyzing reactions in the same pathway found in the red and cardiac muscles of other species of birds and mammals (Crabtree and Newsholme 1972a). Thus, carnitine palmitoyl transferase (CPT) activities (Fig. 1) in hummingbird flight muscles (Suarez et al. 1990) are extraordinarily high in comparison with other vertebrate skeletal muscles (Crabtree and Newsholme 1972a), and mitochondria isolated from this tissue display high rates of carnitine-dependent oxidation of palmitoylcoenzyme A (Suarez et al. 1986).

The biochemical evidence indicating high capacities for carbohydrate and fatty acid catabolism, mitochondrial respiration, and oxidative phosphorylation (Suarez et al. 1986, 1991) provide independent support of morphometric data (see below) that reveal extraordinarily high oxidative capacities in hummingbird flight muscles. But at what rates do these pathways actually operate in vivo?

3 Metabolic Rates During Flight

Hummingbirds and nectarivorous bats are capable of energetically expensive hovering, a mode of flight typically used to feed on floral nectar. Bartholomew and Lighton (1986) developed an elegant method for the measurement of O_2 consumption and CO_2 production rates during hover-feeding that has since been used in many studies of hummingbird flight energetics and adapted for use with nectarivorous bats. These respirometric data allow identification of the fuel(s) oxidized, as well as the estimation of flux rates through the relevant pathways of fuel oxidation (Suarez et al. 1990) and rates of ATP turnover (Welch et al. 2007). These

estimates also apply to forward flight because hummingbirds display relatively flat power curves (i.e., the energetic cost of flight is relatively constant) over a wide range of flight speeds (Berger 1985). Similarly, in small, nectarivorous bats, the power requirements for stationary hovering are close to those required for forward flight at intermediate speed (Voigt and Winter 1999).

Carbohydrate oxidation occurs with a respiratory quotient ($RQ = VCO_2/VO_2$) of 1.0, while fatty acid oxidation yields an RQ value of 0.71. In rufous hummingbirds, *Selasphorus rufus*, the species most extensively studied, hovering at $RQ = 1.0$ requires a rate of carbohydrate oxidation of 13.7 µmole glucosyl units per g muscle per minute (Suarez et al. 1990). On the other hand, the rate of fatty acid oxidation required to support hovering when $RQ = 0.71$ is 3.8 µmole palmitate per g muscle per minute.

These estimated flux rates can be compared with enzymatic flux capacities or V_{max} values to yield further insights. However, further discussion should be preceded by a brief outline of some basic principles. V_{max} values, measured in vitro under optimal conditions, equal $[E] \times k_{cat}$, where $[E]$ is enzyme concentration and k_{cat} is the catalytic efficiency or turnover number of the enzyme molecules. The theory and rationale underlying comparisons between V_{max} values and metabolic flux rates is well-developed and rigorous (Newsholme and Crabtree 1986) (but, unfortunately, commonly misunderstood). The measurement of V_{max} values is not (and should not be) based on the naive and mistaken notion that metabolic enzymes necessarily operate at V_{max} in vivo. Rather, V_{max} values at non-equilibrium steps in pathways establish upper limits to flux, while the fractional velocities (i.e., fraction of V_{max}) at which the enzymes catalyzing these reactions operate are values that can be determined empirically. For example, the V_{max} for glycogen phosphorylase in rufous hummingbird flight muscles is 31.2 µmole glucosyl units per g muscle per minute (Suarez et al. 1986). Although unremarkable in comparison with glycogen phosphorylase activities found in the muscles of other vertebrates (Crabtree and Newsholme 1972b), this is more than twofold higher than the required rate of carbohydrate oxidation (Suarez et al. 1990). However, HK V_{max} values are extraordinarily high in rufous hummingbird flight muscles; if the oxidation of glucose (rather than glycogen) supports hovering flight, HK would operate at about 75% of its maximal capacity (Suarez et al. 1990). On the other hand, the V_{max} for carnitine palmitoyl transferase is 7.2 µmole per g per minute (Suarez et al. 1990). It should be noted that this V_{max} value was obtained using Triton X-100 during homogenization. Because there are two forms of CPT involved in fatty acid oxidation and this detergent inactivates one form (CPT I), but not the other (CPT II) (Woeltje et al. 1987), the V_{max} value of 7.2 µmole per g per minute represents CPT II activity. Therefore, it is this form of the enzyme that operates at 47% of V_{max}. This exercise yields two important insights: The first is that high flux rates are made possible by high levels of enzyme expression at key steps in metabolism. The second is that, in addition to high flux capacities at these steps, some enzymes must operate at high fractional velocities (Suarez et al. 1997).

Although biochemical data specific to nectarivorous bats are not yet available, Yacoe et al. 1982 conducted a comparative biochemical study of enzymatic flux

capacities in the flight muscles of a number of bat species of varying diets and life histories. In addition to enzymatic data indicating generally high aerobic capacities, it was found that HK activities in fruit-eating (frugivorous) bats are in the neighborhood of V_{max} values in rufous hummingbirds. In light of these data, it is reasonable to expect that HK activities in nectarivorous bats would be similarly high, and that mechanistic insights derived from hummingbirds might apply to these animals as well.

4 The Supply of Oxygen

During high-intensity, aerobic exercise, the steady-state rate of O_2 consumption of mitochondria in the locomotory musculature is equal to the rate of O_2 flux from the external environment through the respiratory and cardiovascular systems (Weibel 1984). Control of flux through the "oxygen transport cascade" is shared by various steps in the pathway of O_2 (di Prampero 2003; Jones 1998). Therefore, high rates of aerobic metabolism would not be expected to be based solely on the upregulation of biochemical capacities (Fig. 2). In hummingbirds, high rates of flux through the respiratory system are made possible, in part, by lung O_2-diffusing capacities higher than those of mammals of similar mass (Dubach 1981). Hummingbird hearts are much larger than what is predicted on the basis of the scaling of avian hearts (Bishop 1997). Their blood has high hematocrit, O_2-carrying capacity and -unloading efficiency (Johansen et al. 1987). Heart rates have been clocked at about 1,300 per minute (Lasiewski 1964), leading to estimates of cardiac outputs of five

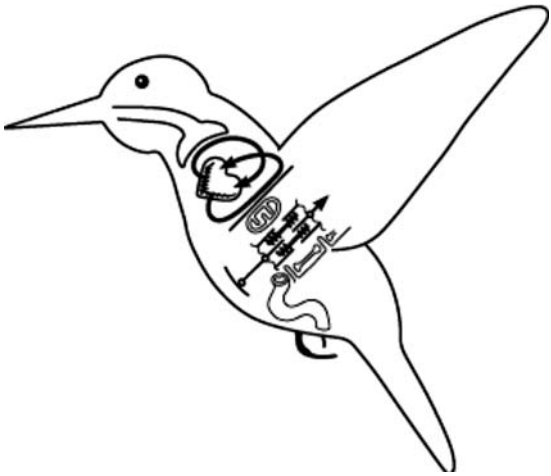

Fig. 2 Diagram showing up-regulated parts of the hummingbird oxygen transport and sucrose oxidation cascades responsible for high rates of dietary energy intake and expenditure. It is redrawn from Suarez (1998) and was originally modified from a cartoon of a frog (Weibel 1985)

times body mass per minute and whole-body red blood cell circulation times of about 1 s (Johansen 1987). The flight muscles possess high capillary content that, in combination with small diameter muscle fibers, leads to high capacities for O_2 flux through the carrier-free zone between red blood cells and the muscle cell membranes (Mathieu-Costello et al. 1992; Suarez et al. 1991). Within the flight muscle fibers are found high myoglobin content (Johansen et al. 1987) and mitochondria occupying about 35% of cell volume (Suarez et al. 1991). These are among the highest observed among vertebrate locomotory muscles (Suarez 1996), while cristae surface areas of $58\,m^2$ per cm^3 of mitochondrial volume approach theoretical upper limits (Srere 1985). A significant fraction of the mitochondria are localized near the sarcolemma; the clustering of mitochondria close to capillaries may further increase capacities for O_2 flux (Mainwood and Rakusan 1982).

Bats are subject to the constraints imposed by mammalian evolutionary design. Nevertheless, studies concerning their cardiorespiratory systems reveal remarkable parallels with hummingbirds. These include larger hearts and cardiac outputs, high hematocrits and O_2-carrying capacities, pulmonary structures enhancing O_2 uptake, and high capillary densities in the flight muscles (reviewed in Maina 2000). Thus, hummingbirds and small bats are the natural analogues of human-engineered, high-performance automobiles possessing high-capacities for the delivery of both fuel and O_2.

5 Fuel Use During Flight: The Sucrose Oxidation Cascade

The high-energy density of triacylglycerol and its storage in unhydrated form (unlike glycogen) in muscles, liver and adipose tissue once led to the generalization that the energy for avian flight comes mainly from fatty acid oxidation (Blem 1976). In hummingbirds, long-term, migratory flight is preceded by premigratory fattening (Odum et al. 1961) and interrupted by refueling stops involving high rates of intake of dietary sucrose as well as high rates of fat synthesis (Carpenter and Hixon 1988; Carpenter et al. 1983). Migratory flight is known to deplete fat stores, so there is no doubt that most of the energy for hummingbird migration comes from fatty acid oxidation. Given high capacities for the oxidation of both carbohydrate and fat in hummingbird flight muscles (and, given the relatively minor contribution made by protein oxidation to vertebrate exercise metabolism), under what circumstances is each fuel used?

When they wake up after their overnight fast and hover to take their first sucrose meal in the morning, hummingbirds display RQ values close to 0.7, indicating that the flight muscles derive most of their energy from fatty acid oxidation (Suarez et al. 1990). Nectarivorous bats, on the other hand, fast in the daytime and feed at night. Recent results obtained with the nectar bat (*Glossophaga soricina*) also show RQ values close to 0.7 during the first feeding bouts after the daytime fast (Welch et al. 2008). In both hummingbirds and nectar bats, repeated hover-feeding visits to a sucrose dispenser (modified to function as a flow-through respirometer) result in

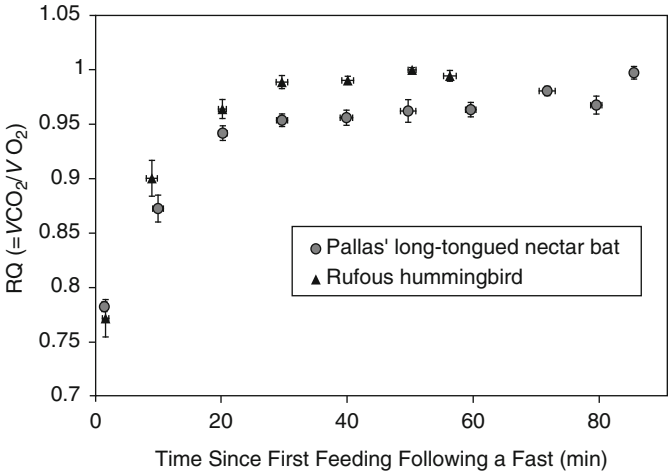

Fig. 3 Respiratory quotient ($RQ = VCO_2/VO_2$) of rufous hummingbirds (*Selasphorus rufous*) and nectar bats (*Glossophaga soricina*) as a function of time after the first feeding bout following nighttime and daytime fasts respectively. Fasting results in RQ values close to 0.7, indicating fatty acid oxidation. Repeated hover-feeding results in rapid increases to values close to 1.0, indicating switching from fatty acid oxidation to carbohydrate oxidation. Points represent means and standard errors. Data are from Welch et al. (2008)

rapid, progressive increases in RQ values until these stabilize to about 1.0, indicating that carbohydrate serves as the main oxidative fuel for flight when dietary sucrose is available (Suarez et al. 1990; Welch et al. 2008, b) (Fig. 3). But what is the nature of this carbohydrate?

To address this question, experiments were conducted that took advantage of the difference in $^{13}C/^{12}C$ ratios of sucrose molecules produced by plants through C3 (sugar beet) or C4 (sugar cane) photosynthesis. Beet sugar contains a lower $^{13}C/^{12}C$ ratio (expressed relative to a standard as $\delta^{13}C$) than cane sugar. Thus, hummingbirds and nectar bats reared on a diet containing beet sugar incorporate beet sugar carbon into their stores of carbohydrate and fat, and soon expire CO_2 with a low $\delta^{13}C$, reflecting that of beets. When these animals are fasted and then allowed to hover-feed, their RQ values are initially close to 0.7, as expected, while the $\delta^{13}C$ value of their expired CO_2 remains low and "beet-like". In these experiments, the fasted animals are then provided cane sugar, which has a higher $\delta^{13}C$ resulting from C4 photosynthesis. As the RQ rises during repeated feeding from about 0.7 to 1.0, the $\delta^{13}C$ also increases from values reflecting the oxidation of fat made from beet sugar to higher $\delta^{13}C$ values indicating the direct use of recently ingested cane sugar (Welch et al. 2006, 2008) (Fig. 4). Calculations based on these results reveal that close to 100% of the fuel oxidized during repeated foraging in hummingbirds comes directly from recently ingested sucrose, while recently ingested sucrose fuels close to 80% of hovering metabolism in the case of nectar bats.

The high flux rates from dietary sucrose (a disaccharide consisting of glucose and fructose) to expired CO_2 inspire comparison with the oxygen transport cascade,

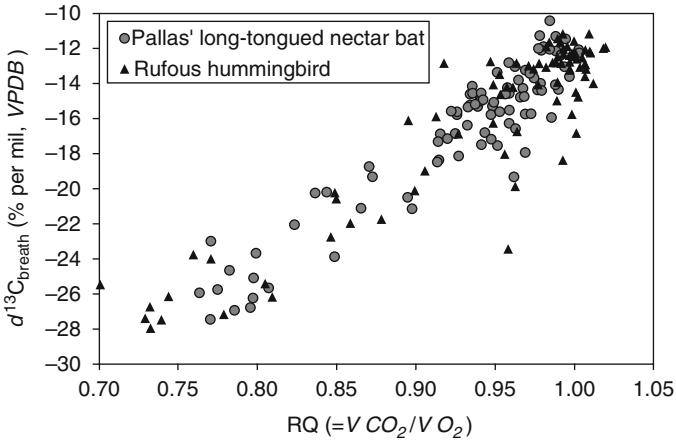

Fig. 4 $\delta^{13}C$ values of expired CO_2 in hovering breath samples collected from rufous hummingbirds (*Selasphorus rufous*) and nectar bats (*Glossophaga soricina*), plotted as a function of RQ. Low $\delta^{13}C$ values when RQ $\approx$ 0.7 result from the lower $^{13}C/^{12}C$ ratio of beet sugar provided in the maintenance diet, while high $\delta^{13}C$ values when RQ $\approx$ 1.0 result from the $^{13}C/^{12}C$ ratio of cane sugar provided in experiments. The increase in $\delta^{13}C$ values as RQ goes from 0.7 to 1.0 indicates that recently ingested dietary sugar accounts for most of the carbohydrate oxidized during hover-feeding. Points represent individual measurements. Data are from Welch et al. (2008)

i.e., the pathway of O_2 from the external environment, through the respiratory and cardiovascular systems, to the muscle mitochondria where reduction of the oxygen atoms to H_2O takes place. In the oxygen transport cascade, the driving force for O_2 flux is the large pO_2 gradient between the external environment and the mitochondria, which serve as O_2 sinks. In the case of dietary sugar, the "sucrose oxidation cascade" involves high rates of ingestion, hydrolysis by intestinal sucrase (McWhorter and Martinez del Rio 2000), transport across the intestinal epithelium via both carrier-mediated and paracellular pathways (McWhorter et al. 2006), entry into the blood and, ultimately, oxidation in the mitochondria which serve as carbon sinks that convert organic carbon molecules derived from dietary sucrose into CO_2. The pathway is not as simple as in the case of O_2. In humans, about half of ingested fructose is converted to glucose by the liver which then appears in the blood (Delarue et al. 1993). Skeletal muscles possess lower capacities for fructose transport (Kristiansen et al. 1997) and oxidation (Zierath et al. 1995) relative to glucose. Both glucose and fructose can serve as substrates for HK-catalyzed phosphorylation, yielding glucose 6-phosphate and fructose 6-phosphate, respectively. Thus, irrespective of whether hummingbird and nectar bat flight muscles directly oxidize dietary fructose at significant rates or not, the stable carbon isotope experiments provide further evidence that most carbon must go through the HK step in hummingbirds and nectar bats as they fuel foraging flights with carbohydrate. An unresolved issue concerns the carbon fluxes to and from glycogen. Given the high metabolic rates fueled by carbohydrate, it is likely that liver and muscle glycogen pools would not last long (about 5 min, at most) if these animals were to rely exclusively

on glycogen to fuel flight (Suarez et al. 1990). In addition, the carbon stable isotope results reveal that the exogenous sugar turns over rapidly within the pool of metabolized substrates (Welch and Suarez 2007). Thus, a viable working hypothesis is that liver glycogen serves to buffer blood glucose concentrations while flight muscle glycogen buffers the pool of hexose-phosphates in glycolysis during foraging flights. In this scenario, muscle glycogen phosphorylase and glycogen synthase fluxes would change dynamically and in reciprocal fashion, but all sucrose-derived carbon appearing in the blood would still have to go through the HK reaction in exercising muscles. These animals, while ingesting sucrose and expiring sucrose-derived CO_2 at high rates, now begin to appear more as the natural analogues of high-performance aircraft engaged in aerial refueling. It is interesting to consider how, in nature, this aerial refueling contributes to plant pollination and plant biodiversity.

6 In Vitro, In Vivo and Beyond

According to Chantler (1982), "the most noble aim of the biochemist, often discussed when inebriate, seldom when sober, is to relate the in vitro to the in vivo". Studies concerning hummingbirds and, more recently, nectar bats have revealed that the highest rates of aerobic metabolism observed among vertebrates result from the concerted upregulation of capacities for fuel and O_2 fluxes in multiple organ systems and at multiple levels of biological organization. At the biochemical level, key enzymes are expressed at higher concentrations and operate at higher fractional velocities than in other species. But Chantler's dictum can be taken even further, and into the realm of ecology and evolution. Premigratory rufous hummingbirds are known for their ability to ingest sucrose at rates high enough to enable fat deposition at rates of up to 10% of body mass per day (Carpenter and Hixon 1988; Carpenter et al. 1983). Their livers possess high acetylcoenzyme A carboxylase activities (Suarez et al. 1988), allowing dietary sucrose in excess of daily energy needs to be rapidly converted to fatty acids and esterified to fat. However, fat synthesis costs ATP, so it appears that one way hummingbirds behave "efficiently" to maximize net energy gain is to engage in short foraging bouts and to oxidize dietary sugar. This avoids the use of fat, but also avoids the energetic inefficiency of synthesizing fat from dietary sucrose, and then using fat to fuel foraging flight. This implies that the greater energetic efficiency resulting from the direct use of dietary sugar, and the consequent enhancement of net daily energy gain, might be one of the benefits of territorial behavior. If this is the case, an intriguing suggestion is that metabolic biochemistry may have contributed to the evolution of optimal foraging behavior or even the evolution of territoriality in hummingbirds (Suarez and Gass 2002).

The latest estimates of the number of ATP molecules synthesized per oxygen atom consumed (i.e., the *P/O* ratio) indicate that about 15% less oxygen is required per mole of ATP synthesized when glucose is oxidized as compared with fatty acid oxidation (Brand 2005). Part of the mechanistic basis for this difference can be seen

in the difference in *P/O* ratios displayed by isolated, coupled mitochondria isolated from hummingbird flight muscles oxidizing pyruvate, as compared with palmitoyl-CoA plus carnitine (Suarez et al. 1986). An interesting question is whether this substrate-dependent difference in *P/O* ratio makes any difference to whole animals in the context of what they do in nature. A given mass of hummingbird requires the same amount of energy to hover, irrespective of whether its flight muscles oxidize carbohydrate or fat. This leads to the prediction that hovering VO_2 should decline by 15% as hummingbirds transition from the fasted $(RQ = 0.71)$ to the fed $(RQ = 1.0)$ state. Recent data obtained using rufous and Annas (*Calypte anna*) hummingbirds support this hypothesis (Welch et al. 2007); this is the first report of the direct influence of substrate-dependent *P/O* ratios on whole animal performance. Migratory rufous hummingbirds refuel at high-altitude, subalpine meadows where they experience the combined effects of low air density and hypoxia (Gass et al. 1999; Welch and Suarez 2008). Thus, territorial hummingbirds maximizing net energy intake by oxidizing dietary sugar may derive additional benefits at high altitude from the 15% lower requirement for O_2.

7 Concluding Remarks

It seems unlikely that there would be reductionist, single gene, or single enzyme-based explanations for how nature's fires of life burn so brightly in hummingbirds and nectar bats. Rather, these amazing animals are the result of the concerted upregulation of flux capacities in multiple organ systems and multiple levels of organization. This suggests that a quantitative, integrative "systems approach" would be more fruitful in advancing understanding than simple reductionism. The well-known, highly conserved and modular nature of metabolic pathways among vertebrates may suggest that the study of vertebrate metabolic biochemistry is no longer interesting or worthwhile, except when applied to human disease states. On the contrary, the great reliance on dietary sucrose as a direct fuel for exercise metabolism during hummingbird and nectar bat foraging is an excellent illustration of the significance of quantitative variation in biochemical flux capacities across species. Studies of intermediary metabolism and its regulation in these animals may yield useful insights into diseases that tend to afflict humans who eat too much carbohydrate, do not exercise and deposit too much fat.

Comparative physiology involves the exploration of functional biodiversity in the natural world. At the core of this research program are studies of functional integration in ecological and evolutionary contexts. The work on hummingbirds has led to insights concerning the ultimate (evolutionary) upper limits to aerobic capacities (Suarez 1998) as well as information concerning the environmental factors and responses to them that allow (or prevent) energy balance or net energy gain (Gass et al. 1999; Suarez and Gass 2002). It is not difficult to imagine how the outcome of studies at the interfaces between physiology, biochemistry and ecology

might prove useful, especially in a period characterized by declining biodiversity and global climate change.

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Part III
Respiratory Physiology of Birds: Metabolic Control

Prenatal Development of Cardiovascular Regulation in Avian Species

J. Altimiras, D.A. Crossley II, and E. Villamor

Abstract The pulsatile rhythm of the avian embryonic heart is not under autonomic control until late in development, nor are the blood vessels that nourish the different vascular beds of the growing embryo and fetus. Thus, during early development cardiovascular control is mostly dependent on the release of local or systemic vasoactive and cardioactive molecules. It is only in late development that the rapid reflex regulatory mechanisms that characterize adult cardiovascular control start functioning. The current review focuses on how the transition from an aneural cardiovascular system to a neural adult-like system occurs in the chicken fetus, which is the best (and at times the only) known avian species. First, we review the appearance of the different molecular components of a regulatory loop, i.e., nerve fibers, neurotransmitters or receptors. Second, we take a look at the functional integration and maturation of the different afferent and efferent pathways. Third and last, we offer a general overview of humoral and local effectors of cardiovascular control.

Abbreviations

αAR	α adrenoceptor
ACE	Angiotensin converting enzyme
ANP	Atrial natriuretic peptide
AT	Angiotensin II
AT1R	Angiotensin type 1 receptor
AT2R	Angiotensin type 2 receptor
βAR	β adrenoceptor

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BDNF	Brain-derived neurotrophic factor
BNP	B-type natriuretic peptide
BRS	Baroreflex sensitivity
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CAM	Chorioallantoic membrane
CNP	C-type natriuretic peptide
CNS	Central nervous system
CO	Carbon monoxide
CPI-17	Protein kinase c potentiated inhibitor protein-17 kDa
DA	Ductus arteriosus
EC_{50}	Half maximal effective concentration
EHDF	Endothelium-derived hyperpolarizing factor
ET-1	Endothelin-1
ETC	Electron transport chain
HH	Hamburger–Hamilton stage
HPV	Hypoxic pulmonary vasoconstriction
MLC_{20}	Myosin light chain
MLCK	Ca^{2+}-calmodulin-dependent myosin light chain kinase
MLCP	Myosin light chain phosphatase
NO	Nitric oxide
NP	Natriuretic peptide
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2
PKC	Protein kinase C
pO_2	Partial pressure of oxygen
ROS	Reactive oxygen species
sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside

1 Introduction

We began conceptualizing this chapter with the ambition of providing a comprehensive overview of the current understanding of cardiovascular regulation during ontogeny and maturation in birds. However, with the exception of the domestic fowl, the information on other species is very incomplete. Therefore, in order to provide the greatest detail available, this chapter will be based exclusively on chicken embryos/fetuses as a model of avian development. The main focus will be on cardiovascular regulatory mechanisms. For a review on the ontogeny of different cardiovascular variables such as heart rate, blood pressure or cardiac output we refer to Tazawa and Hou (1997).

Before we go further it is appropriate to settle a terminological debate, perhaps of little importance, that has long existed in embryological and developmental

Table 1 Relative ages of chicken embryos/fetuses in relation to incubation time and the standard Hamburger–Hamilton stages (Hamburger and Hamilton 1951)

Relative age	Incubation time (d)	HHstages
0	0	1–11
0.1	2	12–22
0.2	4	23–28
0.3	6	29–34
0.4	8	35–36
0.5	10	37–38
0.6	13	39–40
0.7	15	41–42
0.8	17	43–44
0.9	19	45
1.0	21	46

Incubation time (d) has been rounded to the nearest integer value

studies of the chicken as experimental model. That is, should we refer to the prenatal stages of the chicken as embryo or as fetus? Here we adopt the medical terminology that refers to an "embryo" as the organism in the first third of gestation/incubation, while a "fetus" is the organism from the end of the first third until the time of birth/hatching (Larsen 2001). In the chicken the fetal phase starts after the completion of organogenesis by day 8 (HH stage 34, Sissman 1970).

Further, to facilitate fruitful comparisons with other species, we have normalized all incubation stages of the chicken to relative ages (0–1, rounded to 0.05 steps for convenience). Therefore, we refrain from using the Hamburger–Hamilton embryological staging nomenclature (Hamburger and Hamilton 1951), days of incubation or embryonic days. Table 1 presents a simple conversion table that might be of assistance to those more familiar with days of incubation or HH stages. For simplicity, relative age will be often expressed as a simple number without qualifiers such as "relative age" or "incubation time" or "development".

To understand the regulatory mechanisms of the heart and the vasculature and the involvement of the autonomic nervous system, a clear distinction must be made between the actual presence of the different elements of the regulatory pathway (i.e., a nerve fiber or a given receptor) and the functionality of the entire pathway. We start with a review of the time period in which elements of the control mechanisms appear, and continue with a review of the functional onset of tonic control and how this is related to cardiovascular homeostasis. The last part of the chapter is an introduction to the role of humoral and local effectors on the developing chicken, an area in which more research is needed before a thorough revision can be made.

2 Ontogeny of the Control of the Heart Via the Autonomic Nervous System

2.1 Autonomic Innervation of the Heart

The superior cardiac branch and the sinal branch of the vagus nerve (X) reach the truncus and the atria around 0.2 (Kuratani and Tanaka 1990), and contact with all cardiac chambers is reached by 0.35. Sympathetic cardiac nerves projecting from the sympathetic ganglia reach the heart region around 0.5 relative age (Higgins and Pappano 1979; Kirby et al. 1980) and penetrate the myocardium of the fetal heart at 0.75 of development (Verberne et al. 1999). The exact origin of these sympathetic fibers is either from the first pair of the thoracic ganglia (Kirby et al. 1980) or from cervical ganglia (Verberne et al. 1999). Thus, there is a difference in the ontogeny of parasympathetic and sympathetic fibers. The former have a very early onset in comparison with the latter.

Functional studies using field stimulation methods later qualified the conclusions obtained from anatomical studies. Using atrial field stimulation in combination with autonomic drugs, a cholinergic-dependent negative chronotropic response was shown at 0.6. The response appeared earlier (0.5 fetuses) with physostigmine pretreatment (Pappano and Löffelholz 1974). Conversely, an adrenergic-dependent positive chronotropic response to field stimulation was first evident at the time of hatching (Pappano and Löffelholz 1974), but the response could be elicited as early as 0.5–0.6 with tyramine (Crossley 1999; Pappano 1975), a drug that potentiates the release of catecholamines from postganglionic neurons. The effects of tyramine increased with age until 0.9–1 (Crossley et al. 2003b).

These results exemplify the typical sequence of maturation of an autonomic efferent regulatory pathway:

1. Placement of the required regulatory elements: nerve fibers reaching the target tissues and appearance of receptors on the target tissues
2. Maturation of the synaptic coupling between postganglionic neurons and target tissues. In the parasympathetic pathway described above, the blockade of cholinesterases with physostigmine facilitated an earlier onset of chronotropic activity with field stimulation by allowing the accumulation of acetylcholine in the synaptic space. A similar observation can be made for the sympathetic pathway and the increased response to tyramine between 0.5 and 0.9
3. Physiological release of neurotransmitters in response to suitable stimuli

In conclusion, there is a latent period from the time when nerve fibers reach the target organ to the time when nerves are capable of releasing their respective neurotransmitter.

2.2 Cholinergic and Adrenergic Receptors on the Heart

The presence of cholinergic and adrenergic receptors in the pacemaker region of the heart allows the modulation of heart rate (chronotropic effects), while its presence in the ventricle is responsible for changes in the force of contraction (inotropic effects).

Classical studies have demonstrated the existence of muscarinic cholinergic receptors at 0.1–0.15 in the pacemaker areas of the fetal chicken heart (Coraboeuf et al. 1970; Cullis and Lucas 1936; Dufour and Posternak 1960; Hsu 1933; Pappano et al. 1973; Pappano and Löffelholz 1974). When stimulated, these receptors trigger a negative chronotropic effect that is eliminated by pretreatment with atropine, and this occurs early on in the absence of autonomic innervation (reviewed by Pappano 1977).

The timing and localization of postsynaptic β-adrenergic receptors (βAR) in pacemaker regions mirrors that of cholinergic receptors. A βAR-mediated chronotropic response is evident at 0.1 (Berry 1950; Fingl et al. 1952; Hsu 1933; McCarty et al. 1960). The sensitivity of pacemaker tissue to epinephrine is unchanged from 0.6 to 0.85 and decreases from 0.85 to 0.95 (Löffelholz and Pappano 1974). The decrease may be related to the receptor desensitization caused by high circulating catecholamines, which in turn are produced in response to tissue hypoxia and oxygen diffusion limitations in the egg (Crossley et al. 2003b; Wittman and Prechtl 1991). While the exact subtype has yet to be systematically determined, teratological studies suggest that both β_1 and β_2 receptors are present (Lenselink et al. 1994).

In addition to the early presence in atrial and pacemaker tissue, the inotropic response obtained upon pharmacological stimulation suggests that βAR are present

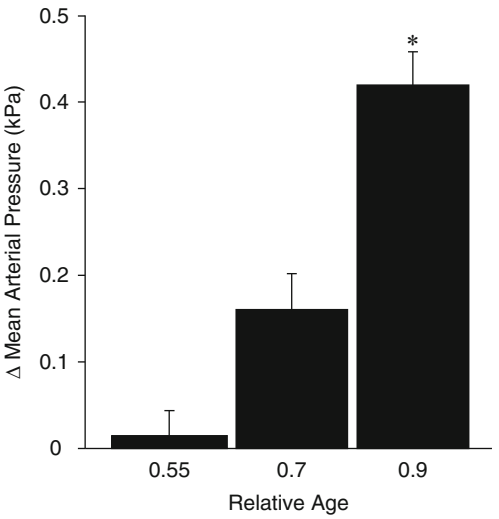

Fig. 1 Change in mean arterial pressure in response to tyramine administration ($10\,\mathrm{mg\,kg^{-1}}$) in fetal chickens at 0.55, 0.7 and 0.9 ($N = 5$). Significant differences indicated by an *asterisk*

by 0.2 of incubation in the fetal ventricle (Frieswick et al. 1979; Higgins and Pappano 1981; McCarty et al. 1960; Shigenobu and Sperelakis 1972). Receptor-binding studies at 0.7 and 0.9 indicate a decrease in the number of βAR from 12 fmol μg protein^{-1} to 8 fmol μg protein^{-1} (Altimiras and Lindgren 2007). Despite the drop in βAR density, the EC_{50} of fetal ventricular tissue to isoproterenol and adrenaline increases from 0.75 to 0.9 before falling prior to hatching (Altimiras and Lindgren 2007; Higgins and Pappano 1981). This is also shown by the increased changes in mean arterial pressure after the administration of tyramine at 0.9, in comparison to earlier stages as shown in Fig. 1 (Crossley 2003b).

3 Ontogeny of the Control of Vascular Contractility

3.1 Developmental Changes in the Mechanisms Controlling Vascular Reactivity

The fetal circulation is designed to meet the requirements of a rapidly growing organism existing at a low pO_2 relative to postnatal life. Therefore, the assembly of a blood vessel into a well-organized and functional structure is essential for organ growth and development. In mature animals, vascular smooth muscle and endothelial cells, both highly specialized cells, have the principal function of regulating blood vessel tone, blood pressure, and blood flow distribution (Owens et al. 2004; Rzucidlo et al. 2007). During development, however, endothelial and vascular smooth muscle cells play a key role in blood vessel morphogenesis. These cells exhibit high rates of proliferation, migration, and production of extracellular matrix, components that make up a major portion of the vessel wall. These processes occur while the newly forming vessels are simultaneously acquiring the capacity to regulate vascular tone (Owens et al. 2004; Rzucidlo et al. 2007). Smooth muscle contraction and relaxation are determined by phosphorylation/dephosphorylation at Ser^{19} of the 20 kDa myosin light chain (MLC_{20}). MLC_{20} phosphorylation is mediated by the Ca^{2+}-calmodulin-dependent MLC kinase (MLCK), which, in turn, is activated by the increase in cytosolic Ca^{2+} (Cogolludo et al. 2007b; Ganitkevich et al. 2002; Somlyo and Somlyo 2003; Webb 2003). Cytosolic Ca^{2+} is increased through Ca^{2+} release from intracellular stores (sarcoplasmic reticulum) as well as entry from the extracellular space through Ca^{2+} channels. In addition to the Ca^{2+}-dependent activation of MLCK, the state of MLC_{20} phosphorylation is further regulated by MLC phosphatase (MLCP), which removes the high-energy phosphate from MLC_{20} to promote smooth muscle relaxation. MLC_{20}, MLCK and MLCP are expressed in the chicken vascular smooth muscle at least as early as 0.5 of incubation (Ogut and Brozovich 2000). At 0.4 incubation, the chicken aorta shows tonic contractile properties in response to an increase in cytosolic Ca^{2+} (Ogut and Brozovich 2000), and the developmental increase in the level of MLC_{20} phosphorylation reaches a plateau from 0.75 onwards (Ogut and Brozovich 2000).

Several G-protein receptor-coupled agonists (including adrenergic agonists) inhibit MLCP, leading to an increase in MLC phosphorylation and contraction without changes in the cytoplasmic Ca^{2+} concentration. This mode of regulation is termed Ca^{2+} sensitization and is an essential process for agonist-induced contraction of smooth muscle (Cogolludo et al. 2007b; Ganitkevich et al. 2002; Somlyo and Somlyo 2003; Webb 2003). At least two signaling pathways are involved in the inhibition of MLCP. First, inhibition via phosphorylation of the MLCP regulatory subunit, MYPT1, which is thought to involve RhoA/Rho-kinase-dependent pathways. The second mechanism of MLCP inhibition is through phosphorylation of the smooth muscle-specific MLCP inhibitor protein, CPI-17 (Protein Kinase C potentiated inhibitor protein-17 kDa, Cogolludo et al. 2007b; Ganitkevich et al. 2002; Kitazawa et al. 2004; Somlyo and Somlyo 2003; Webb 2003). Under physiological conditions, the three mechanisms — Ca^{2+} release, influx, and sensitization — often act in concert. Interestingly, CPI-17 is undetectable in chicken smooth muscles (aorta, mesenteric artery, gizzard and small intestine; Kitazawa et al. 2004), and protein kinase C (PKC) activation does not evoke significant contraction in adult chicken arteries (Kitazawa et al. 2004). In addition, the Rho kinase inhibitors Y-27632 and hydroxyfasudil produce a marked impairment of receptor-dependent and receptor-independent contractions in vessels of chicken fetuses (femoral artery and ductus arteriosus from 0.7 and 0.9 Blanco et al. 2007; Villamor et al. 2008a). The effect of Rho Kinase inhibitors increases with incubation age, suggesting a developmental augmentation in the RhoA/Rho kinase-mediated increase in Ca^{+2} sensitivity of the contractile apparatus. Therefore, the deficiency of CPI-17 in chicken smooth muscle make it a useful model for studying not only the role of CPI-17 but also other potential mechanism(s) regulating Ca^{2+}-sensitivity in smooth muscle contractility.

3.2 Adrenergic Receptors on the Chicken Vasculature

Epinephrine administration increase arterial pressure in the fetus after 0.15 (Girard 1973; Hoffman and Van Mierop 1971) and αAR antagonists trigger hypotension in intact 0.4 fetuses (Crossley 1999), which demonstrates the early presence of adrenoceptors in the fetal cardiovascular system. Additional studies with specific AR agonists and antagonists show that both αAR and βAR are present in the vascular tree as early as 0.3 of incubation (Koide and Tuan 1989; Saint-Petery and Van Mierop 1974). At 0.6 αAR are present in the mesenteric circulation, and they may be present earlier (Rouwet et al. 2000). Between 0.7 and 0.9 incubation, the contractile reactivity to α_1AR and receptor-independent stimulation increases in the femoral and carotid arteries of chicken embryos (Le Noble et al. 2000). In contrast, preductal or postductal pulmonary arteries do not show α-adrenergic-induced contraction at any age (Villamor et al. 2002; Ågren et al. 2007).

Contractile responses to perivascular nerve stimulation have been demonstrated in late-gestation chicken femoral arteries but not in carotid or pulmonary arteries. Because constrictor responses to exogenous norepinephrine are typically obtained

before neurogenic responses (Le Noble et al. 2000), the sympathetic control of arterial vascular resistance is limited to the late phases of fetal life in chickens. βAR relaxation has also been demonstrated in different vessels such as the femoral artery, and the sensitivity and responsiveness increased with incubation age (Blanco et al. 2007).

In summary, the data available to date support the idea that there is a progressive increase in the adrenergic influence on the vasculature that plays a critical role during *in ovo* life, as discussed in Sect. 4.3.

3.3 Cholinergic Receptors and the Endothelial Control of Vascular Reactivity

Possibly the most momentous change in the field of vascular biology in the past 50 years has been the discovery and elucidation of the endocrine/paracrine roles of the endothelium (Alexander and Dzau 2000). In simple but elegant experiments, Furchgott and Zawadzki found that relaxation with muscarinic agonists in precontracted vessels was only possible if endothelial cells were present (Furchgott and Zawadzki 1980). Several endothelium-derived relaxing and contracting factors have been found, including nitric oxide (NO), prostaglandins, thromboxane A_2, endothelin-1 (ET-1), carbon monoxide (CO), and a yet-unidentified factor called endothelium-derived hyperpolarizing factor (EHDF) (Baragatti et al. 2007; Busse et al. 2002). Therefore, it is now widely recognized that the endothelium is not merely a passive, blood-compatible surface but also plays a primary role in the local modulation of vascular function and structure.

Stimulation of muscarinic receptors by acetylcholine evokes an endothelium-dependent relaxation in systemic (aorta, femoral, carotid, mesenteric) and pulmonary arteries of the chicken fetus (Le Noble et al. 2000; Martinez-Lemus et al. 2003; Nishimura et al. 2003; Rouwet et al. 2000; Villamor et al. 2002). The timing of the responses varies between organs, as early as 0.6 in mesenteric arteries but with few changes between 0.7 and 0.9 in femoral or carotid arteries. Endothelium-derived NO appears as the main mediator of this relaxation but EDHF might be also involved (Le Noble et al. 2000; Villamor et al. 2002). Inhibition of the production of prostaglandins (i.e., cyclooxygenase blockade) does not affect acetylcholine-evoked relaxation of systemic or pulmonary arteries (Le Noble et al. 2000; Villamor et al. 2002). Although technical limitations have restricted the studies of endothelium-dependent relaxation to vessels from more mature fetal chickens (starting at 0.6), NO appears as a critical regulator of fetal circulation during earlier stages of development as well. At 0.15 of incubation NO synthase mRNA is expressed in the sinus venosus, ventricle, outflow tract, pharyngeal arch arteries, and aorta of the chicken (Groenendijk et al. 2005). Isolated cardiomyocytes taken from animals at 0.5 relative age respond to both sodium nitroprusside (SNP), an NO donor, and L-arginine, the NO precursor, indicating that the NO/cGMP pathway is functional in the heart at this stage of development (Takahashi et al. 2001; Ungureanu-longrois et al. 1997).

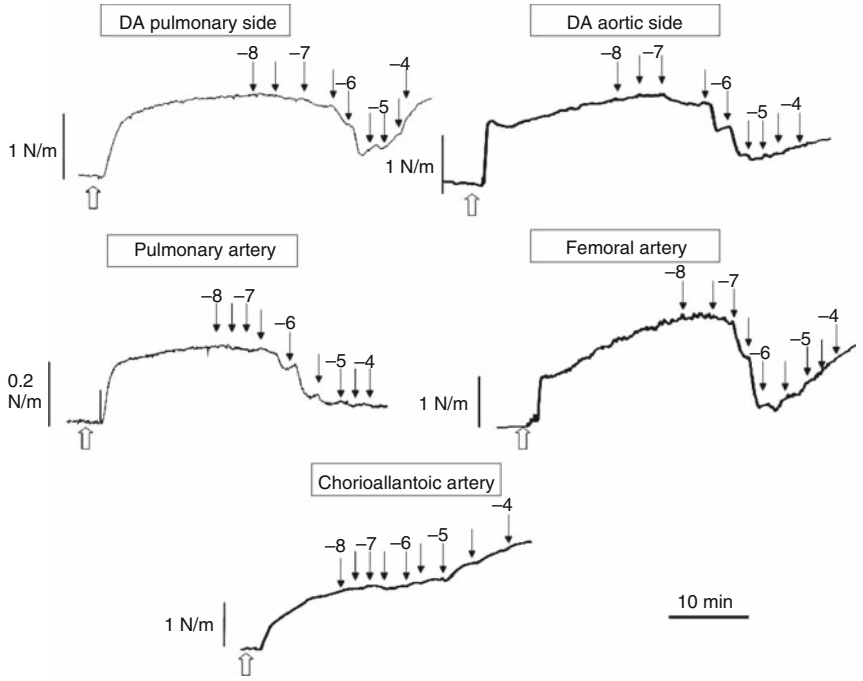

Fig. 2 Typical tracings of isometric tension vs time illustrating the effect of acetylcholine in arteries with intact endothelium from 0.6 fetuses. Vessels were contracted with 62.5 mM KCl (*white arrow*). Concentrations of acetylcholine are shown as log *M*. Note that acetylcholine induced relaxation at low concentrations and (in some vessels) contraction at higher concentrations. In the chorioallantoic artery, no relaxant effects of acetylcholine were observed

NO also causes a clear hyperemia of the CAM vasculature as early as 0.5 (Dunn et al. 2005) and *in vivo* hypotension at 0.45 (Altimiras and Crossley 2000). However, isolated chorioallantoic arteries do not respond to acetylcholine with relaxation but with contraction (Fig. 2). *In vivo* studies have also demonstrated that NO donors elicit a marked decrease in ventricular preload, possibly due to venodilation, without affecting arterial resistance as early as 0.15 (Bowers et al. 1996).

In several mammalian species endothelium-dependent relaxation, particularly in the pulmonary circulation, is reduced during fetal life and transiently compromised after birth (Abman et al. 1991; Boels et al. 1999; Villamor et al. 2003) even if the release of endogenous NO seems necessary for a smooth transition of the pulmonary circulation at birth (Abman 1999; Abman et al. 1990). In the chicken, endothelium-dependent relaxation of pulmonary and systemic arteries remain unchanged during the last phase of incubation, which includes the gradual transition to postnatal life (i.e. during the processes of internal and external pipping) (Le Noble et al. 2000; Villamor et al. 2002). Therefore, the transient impairment of pulmonary endothelial function described in mammalian neonates is absent in the chicken.

Other putative mediators of endothelium-dependent relaxation that regulate vascular tone in fetal chickens have been studied in addition to acetylcholine. Adenosine plays a role in the angiogenic response of the chorioallantoic membrane to hypoxia at 0.5 and 0.65 and decreases whole-body structural vascular resistance in a dose-related manner at 0.5–0.7 (Adair et al. 1989). Thus, the presence of purinergic receptors in the fetal vasculature has at least two roles: to regulate CAM vascularization as well as to regulate vascular tone. The role of other vasoactive compounds acting through the release of endothelial mediators (such as angiotensin II) or other endothelium-derived vasoactive mediators (such as ET-1) is discussed in Sect. 5 of this chapter.

3.4 Vascular Reactivity of the Ductus Arteriosus

All air-breathing vertebrates possess a ductus arteriosus (DA) that connects pulmonary and systemic arterial blood flow. This connection closes permanently at a certain stage in development, or develops the capacity to close and reopen depending on the physiological needs (Bergwerff et al. 1999). Fetal mammals have a single DA while fetal birds have two DA, each acting to shunt a major portion of the cardiac output from the right heart away from the non-ventilated lung into the descending aorta (Bergwerff et al. 1999; Clyman 2006; Smith 1998). Therefore, the *in ovo* or *in utero* patency of DA is essential for prenatal life. Once hatching or birth takes place, the lungs are ventilated, and require an increase in pulmonary blood flow that is achieved through a dramatic decrease in pulmonary vascular resistance and by closing the DA (Bergwerff et al. 1999; Clyman 2006; Smith 1998).

Although the isolated DA is sensitive to a wide range of contractile agonists, the main factors maintaining *in utero* patency of the mammalian DA are low O_2 tension, high levels of circulating prostaglandin (PG)E_2, and locally produced PGE_2 and PGI_2 (Clyman 2006; Clyman et al. 1978; Smith 1998). In addition, the major factor actively stimulating DA contraction at birth is an increase in O_2 tension. This stimulus has a profound effect on the DA, both directly and by modulating its response to vasodilators and vasoconstrictors (Smith 1998; Smith and McGrath 1988, 1993, 1995). The DA acquires vasoactive competence early in development (Bergwerff et al. 1999; Clyman 2006; Smith 1998) and changes in responsiveness with advancing gestational age. These changes have been extensively characterized in numerous mammalian species including man, lamb, mouse, rat, guinea pig, dog, and rabbit (Sutendra and Michelakis 2007). Very recently, the changes in DA reactivity during *in ovo* development and transition to *ex ovo* life have been analyzed in two avian species: the chicken (Ågren et al. 2005, 2007, 2008; Villamor et al. 2008a, b) and the emu (Dzialowski and Greyner 2008). The chicken DA responds to a wide range of vasoactive agonists including O_2, prostanoids, potassium channel blockers, NO, catecholamines, ET-1, adenylate cyclase activators, guanylate cyclase activators, phosphodiesterase inhibitors, and Rho kinase inhibitors (Ågren et al. 2005,

2007, 2008; Villamor et al. 2008a, b). As in the mammalian DA, the multiplicity of vasoactive factors is at odds with the relatively simple physiological role of the DA (Smith 1998). The main vasoconstrictor of the mammalian DA, the postnatal increase in O_2 tension, also plays a relevant role in the closure of the DAs of chicken and emu (Dzialowski and Greyner 2008; Ågren et al. 2007). However, the main vasodilator of the mammalian DA, PGE_2, only triggers weak vasodilation of the chicken and emu DA, and it even stimulates vasoconstriction in the chicken DA at high concentrations (Ågren et al. 2005; Dzialowski and Greyner 2008). In common with mammalian DA, the chicken DA undergoes a process of maturation to prepare the task of postnatal closure. This process is characterized by an increase in the contractile and a decrease in the relaxing capacity of the vessel. Thus, the contractions induced by O_2, membrane depolarization, thromboxane A_2, ET-1 and αAR agonists increased between 0.7 and the end of incubation, whereas the relaxations evoked by acetylcholine, the NO donor sodium nitroprusside, PGE_2, βAR agonists, and adenylate cyclase stimulators decreased (Ågren et al. 2005, 2007, 2008; Villamor et al. 2008a, b).

The endothelium is an important modulator of the vascular tone of the chicken DA during *in ovo* life and during its closure at hatching. Acetylcholine induces a concentration-dependent response in DA in fetal chickens. Low concentrations induce endothelium-dependent relaxation of the chicken DA mediated via NO and EDHF. High concentrations induce an endothelium-dependent contraction (Ågren et al. 2008). Oxygen-induced contraction of the DA is also modulated by the endothelium, a response that increases with inhibition of NO synthase or soluble guanylate cyclase, and decreases in the presence of ET-1 receptor blockers (Ågren et al. 2007). Endothelial damage is common to numerous vascular diseases but, interestingly, occurs as a normal developmental process in the DA. When examined by scanning electron microscopy, the endothelium of the DA from the fetus prior to internal pipping (0.9) shows a smooth and continuous surface. In contrast, the intimal surface of DAs harvested from externally pipped embryos (0.95) has an irregular endothelial lining with protrusion and detachment of endothelial cells, leaving large areas of exposed subendothelial tissue (Ågren et al. 2008). This process of endothelial detachment is accompanied by a marked impairment in NO production and endothelium-mediated relaxation (Ågren et al. 2008).

One of the most relevant features of the chicken DA is the presence of a marked morphological and functional heterogeneity along its path between the pulmonary artery and the aorta (Ågren et al. 2007, 2008; Bergwerff et al. 1996, 1999) (Fig. 3). Specifically, the pulmonary side has the structure of a muscular artery and responds to O_2 with contraction, whereas the aortic segment has the morphology of an elastic artery and relaxes in response to O_2 (Ågren et al. 2007, 2008; Bergwerff et al. 1996, 1999) (Fig. 3). In addition, αAR agonists induce larger contractions when administered to the pulmonary side, while acetylcholine, SNP, and the NO-independent stimulator of soluble gualylate cyclase (sGC) BAY 41-2272 evoke significant larger relaxations in the pulmonary than in the aortic side (Ågren et al. 2008; Ågren et al. 2007). In contrast, the βAR agonist isoproterenol, the adenylate cyclase activator

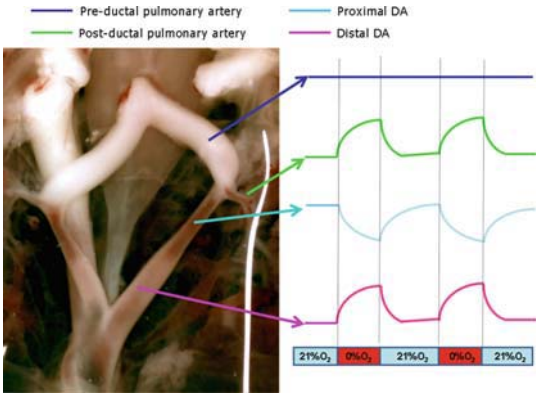

Fig. 3 At 0.9 the chicken fetus presents a series of neighbor vessels with a marked difference in the response to O_2. The pre-ductal extrapulmonary artery does not respond to changes in oxygenation, whereas the post-ductal intrapulmonary arteries contract in response to hypoxia and relax in response to normoxia. The pulmonary side of the ductus arteriosus (DA) contracts in response to normoxia and relaxes in response to hypoxia, whereas the aortic side of the DA shows a similar pattern than the post-ductal pulmonary artery, i.e. hypoxic vasoconstriction and normoxic relaxation

forskolin, and the phosphodiesterase 3 inhibitor milrinone induce larger relaxations in the pulmonary side of the vessel (Ågren et al. 2005). This may indicate that the pulmonary side of the chicken DA is more sensitive to the vasodilators acting through cAMP, whereas the aortic side is more sensitive to cGMP-mediated relaxation.

3.5 Oxygen Sensing in Chicken Fetal Vessels

The DA belongs to a specialized system of O_2-sensitive organs and tissues in the body that includes the pulmonary arteries, the carotid body, and the neuroepithelial body among others. These tissues share striking similarities in their response to changes in O_2 tension (Aaronson et al. 2006; Sutendra and Michelakis 2007; Weir et al. 2002, 2005). The proposed mechanism for DA closure includes an acute phase in which minutes of exposure to postnatal normal O_2 levels result in DA constriction. This mechanism is thought to be intrinsic to the DA smooth muscle cells (Michelakis et al. 2000, 2002; Sutendra and Michelakis 2007; Thebaud et al. 2004; Tristani-Firouzi et al. 1996; Weir et al. 2002, 2005) and, at least in the human or the rabbit DA, it includes a sensor, the electron transport chain of the mitochondria (ETC). The ETC increases production of reactive oxygen species (ROS), particularly H_2O_2, in response to changes in O_2 levels. This mediator (i.e., the freely diffusible H_2O_2) can reach the cell membrane and decrease the opening

of O_2- and redox-sensitive K^+ channels (such as Kv1.5 and Kv2.1). This causes depolarization of smooth muscle, opening of the voltage-gated Ca^{2+} channels, increase in $[Ca^{2+}]_i$ and vasoconstriction (Michelakis et al. 2000, 2002; Thebaud et al. 2004; Tristani-Firouzi et al. 1996; Weir et al. 2002, 2005). The mitochondria-ROS-K^+ channels axis is the basis of O_2 sensing in many other O_2-sensitive tissues (Sutendra and Michelakis 2007; Weir et al. 2005), suggesting the evolutionary preservation of the O_2-sensing mechanism (Cobeño et al. 2008; Cogolludo et al. 2007a; Sutendra and Michelakis 2007). The contraction of the chicken DA to O_2 is markedly blocked by the ETC inhibitors rotenone, myxothiazol and antimycin A, by the H_2O_2 scavenger polyethylenglycol-catalase, and by the Kv channels inhibitors 4-aminopyridine (non-selective) and DPO-1 (Kv1 selective) (Cobeño et al. 2008; Cogolludo et al. 2007a). Furthermore, exogenous H_2O_2 mimicked the responses induced by O_2 (no effect at 0.7, and contraction and relaxation in pulmonary and aortic sides of the DA by 0.9 and 0.95 respectively: Cobeño et al. 2008; Cogolludo et al. 2007a). Altogether, these results indicate that the mitochondria-ROS-K^+ channels are responsible for O_2-induced contraction in the chicken DA. However, and similarly to the situation of the mammalian DA (Hong et al. 2006; Kajimoto et al. 2007), Rho-kinase inhibitors blunt the normoxic contraction of the chicken DA (Villamor et al. 2008a), which means that other pathways such as the calcium sensitization mechanism may be also important in DA closure.

As another O_2-sensitive vessel, the pulmonary arteries, typically contract when exposed to hypoxia (Russell et al. 2008). Hypoxic pulmonary vasoconstriction (HPV) is a highly conserved adaptive physiological mechanism that optimizes oxygen saturation of pulmonary venous blood by increasing pulmonary vascular resistance in poorly aerated lung regions (Aaronson et al. 2006; Michelakis et al. 2004; Moudgil et al. 2005; Russell et al. 2008; Weir et al. 2005; Villamor et al. 1997). In contrast, the systemic vasculature frequently responds to hypoxia with vasodilation in an effort to maintain adequate tissue oxygenation (Russell et al. 2008). For example, femoral arteries of 0.9 fetuses respond to hypoxia with relaxation (Ruijtenbeek et al. 2002). HPV has been demonstrated in adult chicken extrapulmonary arteries pre-constricted with KCl (Russell et al. 2008), although other authors have reported a lack of response without pre-constriction. It is well known that mammalian pulmonary arteries respond very little to hypoxia while at passive resting tension, and that HPV is strongly enhanced by some level of pre-constriction (Aaronson et al. 2006). Interestingly, we have observed a consistent and reproducible response to hypoxia in intrapulmonary arteries of fetal and juvenile chickens at passive resting tension (Villamor, unpublished observations). In contrast, extrapulmonary arteries do not respond to hypoxia under those conditions. Therefore, as illustrated in Fig. 3, the chicken fetus presents a series of neighbor vessels (i.e., the pre- and post-ductal pulmonary arteries, the aortic and the pulmonary sides of the DA) with a marked difference in the response to O_2. The mechanisms that initiate, differentiate and regulate this variety of vascular responses to O_2 warrant further investigation.

4 Functional Integration of Autonomic Cardiovascular Regulation

4.1 Ontogeny of Afferent Pathways

The main sensory areas that trigger cardiovascular reflexes in chickens are:

(1) The carotid bodies involved in chemoreception, and
(2) Specialized mechanosensory nerve endings in the adventitial layer of the aortic arch involved in baroreception

There is no anatomical evidence of carotid sinuses in birds. The homologous region would be the bifurcation of the common carotid with the subclavian artery, but at this location the vessel wall is not thinner and vessel diameter is not expanded as it is in mammals (Ábrahám 1969).

The embryology and maturation of the reflexogenic areas are reasonably well-known from early studies and their innervation patterns have been described. The carotid bodies constitute the primary loci for peripheral chemoreceptors sensing arterial oxygen and carbon dioxide tensions and pH. They are located in the inferior part of the neck that is contiguous with the thoracic cavity, in contrast to mammals, where the carotid bodies are located in a cervical position. This may be due to the elongation of the chicken neck, since other structures located cervically in mammals, such as the bifurcation of the common carotid artery and the nodose ganglion (also called distal vagal ganglion), are also found in the thoracic inlet in the chicken (Wakley and Bower 1981).

The carotid bodies appear around 0.25 and migrate to an adult-like location by 0.4 (Murillo-Ferrol 1967), at which time they consist of mesenchyme-like cells. At 0.6 a large number of granule-containing cells are dispersed in the parenchyma (Kameda 1994), coinciding with a peak for serotonin immunoreactivity (Kameda 1990). These granule-containing cells are denominated glomus cells or Type I cells and they are responsive to chemical stimuli such as the partial pressure of oxygen and carbon dioxide. The first detection of synaptic junctions between long axons and glomus cells is also observed at 0.6 (Kameda 1994). At 0.7 the glomus cells express most of the features found in mature glomus cells (Kameda 1994) but further maturation that extends to the post-hatching period cannot be discarded. In rats, the carotid body increases in size postnatally and glomus cells continue to proliferate after birth (Wang and Bisgard 2005). The responsiveness of glomus cells to hypoxia, as indicated by an increased catecholamine secretion, also increases postnatally and is coupled to a decrease in the constitutive (hypoxia-independent) release (Donnelly 2005). Glomus cells are also found in the wall of the common carotid artery and other vessels of the outflow tract, but their role in chemosensitivity has not been studied (Kameda 2002).

As structures derived from the second–third aortic arch, one would expect the carotid bodies to be innervated from the glossopharyngeal (IX) cranial nerve, as happens in mammals. This is not the case, and the sensory innervation of the chicken

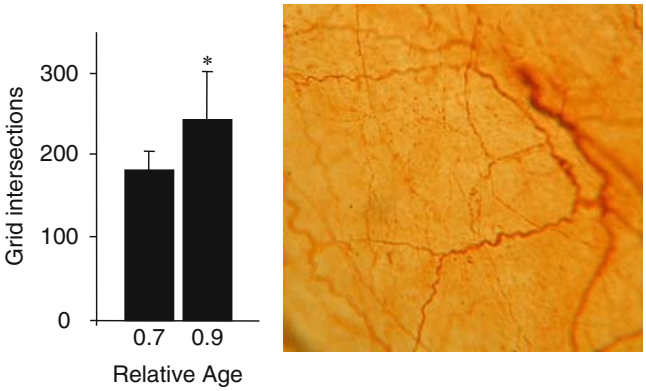

Fig. 4 Innervation density for neurofilament immunostained whole mount sections of the aortic arch of chickens at two stages of development. Innervation density is measured as the number of intersections between nerves and a square grid. A *star* indicates a significant difference between ages

carotid bodies travels along the vagus nerve (X) and one of its branches, the recurrent laryngeal nerves (Jones and Johansen 1972; Murillo-Ferrol 1967). It is possible, however, that some glossopharyngeal nerve fibers move into the vagus via the anastomosis of Staderini that connects the vagal trunk to the petrosal ganglion before it continues caudally, although there is no experimental evidence for it (Whittow and Sturkie 2000).

An additional vagal branch, the aortic nerve (also called depressor nerve, Nonidez 1935), carries axons of sensory neurons to the adventitial layer of the aorta, where the nerve fibers branch to fine free nerve endings patterned as "flower-spray" or "end-net" structures (Ábrahám 1969; Cheng et al. 1997). In rats, these structures are unequivocally identified as baroreceptive nerve endings based on the expression of mechanosensitive channels (γ-subunit of the epithelial sodium channel) in axons from nodose ganglion cells (Drummond et al. 1998, 2001). In chickens, an increase in the innervation density of the aorta from 0.7 to 0.9 has been shown (Altimiras and Crossley 2007), see Fig. 4.

Nonidez's early anatomical study also described a depressor nerve of the carotid in an area homologous to the mammalian carotid sinus (Nonidez 1935). Even if birds have no carotid sinuses, the observation could indicate the existence of other baroreceptive areas independent of the baroreceptors in the aortic arch. However, it is generally accepted that the nerve endings in that location are not mechanosensitive, because the nerve fibers terminate in a plexus that penetrates down to the media of the vessel instead of the adventitial "flower-spray" or "end-net" patterns of typical mechanosensitive nerve endings (Ábrahám 1969).

In contrast to mammals, therefore, the axons of the main cardiovascular afferent neurons are bundled in the vagus nerve and have the cell bodies in a common location, the nodose ganglion. Similar to all peripheral sensory neurons in vertebrates, nodose ganglion neurons are pseudo-unipolar. Their axons bifurcate shortly

after emerging from the cell body. While one branch grows peripherally towards the heart and other viscera, the other branch grows centrally and establishes synaptic connections within the central nervous system (CNS), most importantly with the nucleus of the solitary tract.

Nodose ganglion neurons derive from cells of the nodose placode while Schwann cells and supporting cells of the ganglion are provided by neural crest cells (Harrison et al. 1994). The primordium of the ganglion is visible at 0.15. Following a proliferation period the ganglion reaches the largest number of cells at 0.3, after which time cell numbers will drop to a half by the time of hatching due to programmed cell death (Harrison et al. 1994). The development of the ganglion occurs simultaneously with the projection of neuronal axons to the target tissues and the CNS. When the axons are growing early in development the survival of these neurons is independent of the presence of neurotrophins, but as they reach the target tissues they become dependent on brain-derived neurotrophic factor (BDNF) for survival. Such dependence is reflected in the mRNA expression of the catalytic domain of TrkB, a receptor tyrosine kinase which acts as a BDNF receptor. trkB mRNA increases progressively from 0.15 to 0.2, coinciding with the maximum proliferation of nodose ganglion neurons (Robinson et al. 1996). The neurons are also susceptible to other trophic factors such as nerve growth factor at later stages (0.4, Hedlund and Ebendal 1980).

Altogether it seems that the afferent pathways connecting the reflexogenic areas of the cardiovascular system with the central nervous system are established within the first half of incubation. A maturation of the sensitivity to the time of hatching is probably the case for the carotid bodies, but little information is available for the baroreceptive areas. In the fetal lamb, afferent baroreceptor sensitivity measured from the carotid sinus nerve decreased with age from 0.7 to 0.9, simultaneously with the developmental increase in blood pressure (Blanco et al. 1988). A potential explanation of the results would be that the enhanced sensitivity in earlier fetuses is aimed to stimulate the maturation of the central pathways involved in the reflex, but this hypothesis needs to be tested experimentally.

4.2 Onset of Tonic Control of the Heart

The importance of cholinergic and adrenergic tonic activity to maintain baseline cardiovascular function in fetal chickens has been studied *in vivo* by administering receptor antagonists at different stages of development. Atropine, an antagonist of cholinergic muscarinic receptors, induces no chronotropic effects at any developmental age in White Leghorn chickens (Crossley and Altimiras 2000; Haque et al. 1995; Pickering 1895; Saint-Petery and Van Mierop 1974; Tazawa et al. 1992). Thus, although cholinergic receptors are present in early embryos and the parasympathetic efferent arm is functional by 0.6 (Pappano et al. 1973, see Sect. 2 of this chapter), the White Leghorn fetus develops in the absence of a cholinergic or parasympathetic tone.

It is important to emphasize that the absence of a cholinergic tone does not rule out the possibility that the parasympathetic nervous system can be recruited if baseline cardiovascular function is disturbed. In fact, continuous recordings of instantaneous heart rate in fetal chickens have documented decelerations in fetal heart rate which are probably due to an increase in parasympathetic activity (Akiyama et al. 1999; Höchel 1998; Kato et al. 2002; Tazawa et al. 2002).

A later study in broiler chickens (a chicken strain primarily used for meat production) demonstrated a cholinergic tone on heart rate that started at 0.6 (Chiba et al. 2004), almost at the same time that field stimulation studies can induce changes in spontaneous cardiac contraction frequencies (Pappano et al. 1973). The basis for such strain-specific differences defies explanation but it is worthy of further studies, because it indicates a large degree of plasticity of cardiovascular regulatory mechanisms between strains.

In contrast, a tonic adrenergic stimulation is present throughout fetal development. An adrenergic tone on both heart rate and systemic arterial pressure appears relatively early (Crossley and Altimiras 2000; Koide and Tuan 1989; Saint-Petery and Van Mierop 1974; Tazawa et al. 1992) and is dependent on αAR and βAR with differential effects on cardiac and vascular tissue (Crossley 1999). A βAR-positive chronotropic tone appears at 0.3 incubation (Girard 1973; Saint-Petery and Van Mierop 1974) and is critical to the maintenance of basal baseline function (Crossley 1999; Crossley and Altimiras 2000; Tazawa et al. 1992). βAR chronotropic tone increases in magnitude with fetal development, elevating baseline heart rate 10% at 0.4 to 20% at 0.95 (Crossley 1999). The tone originates entirely from circulating catecholamines, as the elimination of the sympathetic nervous terminals with 6-hydroxydopamine or ganglionic blockade with hexamethonium has no impact on control fetal heart rate (Crossley 1999; Tazawa et al. 1992).

At the same time, αAR antagonists depress heart rate at 0.4 (Crossley 1999) and continue to do so at later developmental stages (Crossley and Altimiras 2000; Koide and Tuan 1989; Tazawa et al. 1992). The magnitude of the αAR tone is maximal from 0.6 to 0.95 but is absent at hatching (Crossley 1999). The bradycardic effects must be due to indirect effects related to the strong αAR-vasodilation that follows phentolamine administration (an αAR antagonist), because αARs are absent from the chicken heart (Chess-Williams et al. 1991). Vasodilation leads to blood pooling in the CAM, reduction in venous return, and decrease in cardiac output and heart rate.

4.3 Onset of Tonic Control of the Vasculature

The total and regional peripheral resistance of the vasculature is primarily regulated by the sympathetic nervous system, which releases catecholamines from both the sympathetic nerve terminals and the adrenal medulla. Because vascular smooth muscle is endowed with both αAR and βAR (Saint-Petery and Van Mierop 1974), the net response to catecholaminergic stimulation will depend on the balance

between the number and sensitivity of vasoconstrictor αAR and vasodilator βAR (Altimiras and Crossley 2001; Guimaraes and Moura 2001).

A powerful αAR vascular tone is present in fetal chickens (0.3) and persists to 0.95 (Crossley 1999; Crossley and Altimiras 2000; Girard 1973; Koide and Tuan 1989; Saint-Petery and Van Mierop 1974; Tazawa et al. 1992). The receptor subtype responsible for maintaining vascular tone seems to be α_1AR because of the similar responses obtained using an non-specific αAR antagonist (phentolamine) and an α_1AR specific antagonist (prazosin, Crossley and Altimiras 2000). As the fetus grows and matures its dependence on αAR-mediated vasoconstriction increases from a meager 10% change in resting arterial pressure to over 55% in the last days of development (Crossley 1999).

αAR vasoconstriction prevails in the skeletal muscles and has limited effects on the heart, intestines, and yolk sac, as shown by the distribution of microspheres away from the carcass with the infusion of αAR agonists (Mulder et al. 2001). Thus, αAR mechanisms contribute to the maintenance of basal vascular tone and to the redistribution of the cardiac output, and these mechanisms are important for defending blood flow to the brain and heart during hypoxemic conditions (Mulder et al. 2001).

The αAR vasoconstrictor tone is opposed by a βAR vasodilator tone that also has an early appearance in embryonic chickens at 0.3 (Saint-Petery and Van Mierop 1974). The magnitude of the βAR vascular tone increases from 0.35 to 0.6 (Crossley 1999; Girard 1973), mostly due to the proliferation and expansion of the extraembryonic vasculature of the CAM, which reaches its maximum by 0.7 (Romanoff 1967). βAR vascular tone remains stable up to 0.75 (Haque et al. 1995; Koide and Tuan 1989; Tazawa et al. 1992) and increases late in development with a maximal expression by 0.9–0.95 (Crossley and Altimiras 2000). A maximal response to βAR antagonists at the same stage has been shown in other chicken strains (Crossley, personal unpublished results) and emus (Crossley et al. 2003a). βAR vascular tone is absent during external pipping (Crossley 1999).

The maximal expression of αAR tone coupled to the absence βAR tone vascular tone in late development is critical to ensure proper delivery of oxygen to embryonic tissues at the time when CAM gas exchange is switched to lung gas exchange, a process that occurs in a rather short period of time (Menna and Mortola 2002).

The αAR and βAR vascular tones are entirely attributed to circulating catecholamines given that neither sympathectomy with 6-hydroxydopamine nor ganglionic blockade with hexamethonium alters resting arterial pressure in fetal chickens (Crossley 1999; Crossley and Altimiras 2000; Crossley et al. 2003b; Tazawa et al. 1992).

4.4 Maturation of Baroreflex Regulation

The baroreflex is the most important short-term regulator of blood pressure in vertebrates. Birds are no exception (Bagshaw and Cox 1986). Nerve activity from barosensitive areas in the common outflow tract relays information of the phasic

(within a cardiac cycle) and tonic (between cycles) changes in blood pressure via nodose ganglion neurons to the CNS, primarily the nucleus of the solitary tract. Baroreceptive and other sensory afferents, e.g., chemoreceptive, are processed and an integrated response is ultimately directed to the heart and the vasculature via the autonomic nervous system.

The cardiac baroreflex response involves parasympathetic vagal efferents and, to a lesser extent, sympathetic efferents that change heart rate and cardiac output in a reciprocal manner to the blood pressure changes. The peripheral response involves sympathetic efferents to the vascular smooth muscle that modify peripheral resistance (see Sects. 3.2 and 4.3 of this chapter). For example, if pressure increases sympathetic activity decreases and vasodilation ensues. The two complementary mechanisms have been denominated the cardiac limb and the peripheral limb of the baroreflex respectively.

Little is known about how the peripheral limb of the baroreflex develops. In adult birds, the peripheral limb contributes to baroreflex regulation by acting in synergy with the cardiac limb, and remains active even when the heart rate response subsides (Jones 1973; Smith and Jones 1992). So, even if changes in peripheral resistance have a longer latency than cardiac responses, the longer activation makes the peripheral limb more effective in blood pressure regulation (Jones 1973). In the fetus, neurogenic constrictor responses in femoral arteries are only observed around the time of internal pipping and the onset of lung ventilation (Le Noble et al. 2000), implying that changes in peripheral resistance mediated by the sympathetic nervous system are limited to the late fetal stages (>0.9). A distinct change in renal sympathetic nerve activity related to blood pressure alterations is also observed in fetal lambs at 0.9 gestation. In fact, peripheral gain in fetuses is several-fold larger than in newborns or 6-week old lambs (Segar et al. 1992).

The ontogeny of the cardiac limb of the baroreflex has been studied in more detail. The standard experimental approach to estimate baroreflex sensitivity (BRS), the so-called Oxford method, is based on the vascular administration of vasoactive substances to elicit blood pressure changes, typically a nitric oxide donor to vasodilate and an αAR agonist to vasoconstrict, so that reflex responses in heart rate can be measured (Smyth et al. 1969). Using this method, the earliest baroreflex responses in White Leghorns are seen at 0.85 (Altimiras and Crossley 2000) but only 17% of the fetuses show a change in heart rate after experimental manipulation of blood pressure. A day later (0.9), the proportion of animals showing a baroreflex response climbs to 33%, and the gain of the baroreflex increases progressively fivefold between 0.9 and hatching (Altimiras and Crossley 2000). In broiler chickens gain at 0.9 is similar, $23\,\mathrm{kPa}^{-1}\ \mathrm{min}^{-1}$ vs $21\,\mathrm{kPa}^{-1}\ \mathrm{min}^{-1}$ in White Leghorns (Altimiras and Crossley 2007).

The Oxford method interferes with the peripheral limb of the reflex (Maloney et al. 1977) and has been criticized for delivering a stimulus to the baroreceptors poorly comparable to physiological blood pressure variations (Parati 2005). This methodological limitation may be of importance during the early onset of the baroreflex when BRS is expected to be lowest. Thus, in a recent study, we followed BRS in fetuses from 0.8 to 0.95 using a sequence procedure of the spontaneous BRS

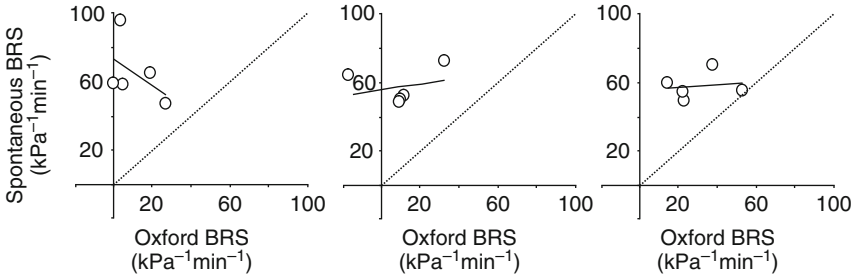

Fig. 5 Correlation between Oxford BRS and spontaneous BRS at different stages of development (0.85, 0.9 and 0.95 from left to right respectively). The *dotted line* corresponds to the line of equality if both methods yielded the same BRS estimate

method (Bertinieri et al. 1985). The procedure is based on the analysis of the correlation between heart rate and blood pressure during episodes (called sequences) when blood pressure changes spontaneously (see Laude et al. 2004 for a comparison of the performance of different procedures). The method also allows a sequential assessment of BRS in the same animal over time, which is not feasible with the Oxford method due to potential cumulative effects of the drugs.

Interestingly, spontaneous BRS is relatively constant from 0.8 to 0.95 and averages 59.8 kPa^{-1} min^{-1}. This value is several-fold larger than the Oxford BRS measured in the same fetuses, which increased progressively from 10.9 kPa kPa^{-1} min^{-1} at 0.8 to 30 kPa^{-1} min^{-1} at 0.95 (Elfwing 2007). The lack of correlation in the alternative BRS estimates at different fetal ages is shown in Fig. 5.

BRS differences with different methods are probably related to the small parasympathetic tone in fetuses (Chiba et al. 2004). While the spontaneous method focuses on heart rate changes that compensate for minute blood pressure changes ($>$0.36 kPa) within a few heart beats ($>$3 beats), the Oxford method elicits larger pressure changes ($>$0.75 kPa) on a longer time scale (60–90 s). Thus, small increases or decreases in blood pressure lead to an enhanced or diminished parasympathetic tone that causes, respectively, a compensatory bradycardia or tachycardia. Because parasympathetic tone in fetuses is small, larger pressure changes such as those induced pharmacologically cannot be defended to the same extent, resulting in a lower BRS.

Thus, the emerging but still speculative picture indicates that baroreflex regulation in chickens is absent during most of fetal life and appears at 0.8 at the latest (Elfwing 2007), a little earlier than previously reported (Altimiras and Crossley 2000). Before the onset of a functional baroreflex, blood pressure homeostasis can be alternatively adjusted by slower mechanisms regulating blood volume such as the renal fluid blood pressure control mechanism (Guyton 1991; Guyton et al. 1972) which in the chicken fetus would imply the transfer of fluid to the allantois, the reservoir that collects waste in the embryo (Hoyt 1979).

The slow time response of the renal mechanism may suffice for the early embryo, which is shielded from gravitational stress by the amniotic cavity. However, the

onset of pseudo-respiratory movements first (around 0.8, Kuo 1937) and proper lung respiration at 0.9 would increase the magnitude of blood-pressure variations, and the parasympathetic nervous system would start contributing more to the buffering of blood pressure changes. Finally, during the proper lung ventilation phase the sympathetic nervous system could set in operation and complete the baroreflex response to the adult-like scenario.

5 Effects of Humoral and Local Effectors: Angiotensin, Endothelin-1 and Natriuretic Peptides

In addition to cholinergic and adrenergic mechanisms, other humoral and local mediators affect cardiovascular function in the fetus. The most studied in fetal chickens are angiotensin II (AT) and local effectors such as ET-1 and natriuretic peptides (NP).

AT is a critical regulator of cardiovascular function in adult chickens (Hasegawa et al. 1993; Nakamura et al. 1982; Nishimura et al. 1982; Stallone et al. 1990). AT injection triggers a biphasic response in which there is an initial vasodilation mediated by NO and a secondary αAR-mediated vasocontriction (Nishimura et al. 1982). However, the function of AT during fetal life is not well understood.

The components of the renin–angiotensin system are present relatively early in fetal chicken development. Angiotensin-converting enzyme (ACE) is measurable in freshly laid eggs and ACE mRNA increases dramatically over the first 54 h of development (Savary et al. 2005). At the completion of the first day of fetal development the yolk sac contains mRNA encoding for ACE, angiotensinogen, renin, and AT receptors (Savary et al. 2005). Thus, the mechanisms for both angiotensin synthesis and signal transduction are present early in fetal chicken development.

AT receptor mRNA is found in cardiac tissue, branchial arch tissue, and mesonephric tissue between 0.15 and 0.2 (Kempf and Corvol 2001). AT triggers an angiogenic response of the CAM vasculature at 0.35 (Le Noble et al. 1991, 1993), and receptor-binding assays have quantified the number of AT receptors in the CAM at 0.5 (Moellera et al. 1996). The enzymatic activity of ACE is present in the chicken aorta as early as 0.5 incubation, and it increases with development (Topouzis et al. 1992). Although AT induces relaxation in isolated aortic rings from 0.9 fetuses (Nishimura et al. 2003), the effects are due to the lack of intact sympathetic nerves, so the vasoconstrictive response is absent. Taken together, these results imply that angiotensin has a role in chicken vascular regulation that needs to be clarified with *in vivo* studies. Preliminary work demonstrates that AT injection induces a clear hypertensive response (Fig. 6) that notably lacks the adult initial vasodilation (Crossley, personal observations).

AT also induces a positive inotropic effect on the heart at 0.85 (Freer et al. 1976) and cardiac hypertrophy from 0.35 to 0.9 (Aceto and Baker 1990; Baker and Aceto 1990; Mathew et al. 2004). The hypertrophic response is due to the activation of the AT type 1 receptor (AT1R) and upregulation of MLC (Mathew et al. 2004).

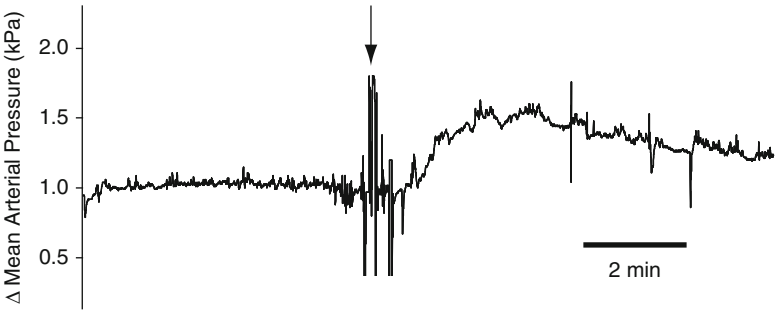

Fig. 6 Effects of an arterial injection of angiotensin II$(1{,}000\,\mu g\,kg^{-1})$ in a chicken fetus at 0.7. The *arrow* indicates the point of injection

Although AT1R is the main receptor type, AT2R is also present in the heart at 0.35 (Rabkin 1996). Cardiac hypertrophy could be coupled to both direct actions of AT on the heart as well as its actions on the fetal vasculature.

ET-1 is also ubiquitously found in the cardiovascular system of chicken fetuses (Kempf et al. 1998). The mRNA for ET-1 receptor subtypes is detected in the wall of the vitelline vessels, the myocardium and the outflow tract as early as 0.15 (Groenendijk et al. 2007), while the converting enzymes for ET-1 can first be detected at 0.2 (Ballard 2002; Hall et al. 2004). The role of ET-1 as a cardiovascular regulator is further confirmed by the hemodynamic alterations that occur after *in vivo* administration of ET-1 receptor antagonists at 0.2 (Groenendijk et al. 2007) and by the positive inotropic effect of ET-1 on cultured cardiomyocytes from 0.5 fetuses (Bézie et al. 1996).

Isolated aortic rings (from 0.7 fetuses) and pulmonary arteries (from 0.9 fetuses) contract in response to ET-1 in a concentration-dependent manner (Martinez-Lemus et al. 2003; Villamor et al. 2002, 2004; Wingard and Godt 2002). Active wall tension in response to ET-1 increases before hatching, and this can be of critical importance for the transition to *ex ovo* life (Martinez-Lemus et al. 2003; Villamor et al. 2004). As mentioned above, ET-1 also plays a determinant role in the reactivity of the DA. Further investigations are necessary to characterize the nature of the receptors and the transduction pathways involved in the vascular responses to ET-1.

The NP family consists of a group of structurally related peptides that are involved in the regulation of sodium and water balance, and cardiovascular homeostasis (Takei 2000; Toop and Donald 2004; Trajanovska et al. 2007) In mammals, three NP subtypes have been isolated, the first two of which are atrial NP (ANP) and B-type NP (BNP), which are produced primarily within cardiac myocytes and released into the circulation in response to a volume overload. The third type is the C-type NP (CNP) that is a paracrine or autocrine factor in the brain and periphery (Takei 2000; Toop and Donald 2004; Trajanovska et al. 2007). Four NP genes have been identified in the chicken genome (Akizuki et al. 1991; Houweling et al.

2005; Trajanovska et al. 2007). These genes encode one BNP, two CNPs and a recently identified NP with an unusual sequence (termed chicken RNP due to its predominant expression levels in the chicken kidney) (Trajanovska et al. 2007). In chicken embryos, NP decreases vitelline arterial pressure and increases vitelline venous diameter as early as 0.2, so NP receptors must be present in the vasculature at that stage (Nakazawa et al. 1990). Further studies of the relaxant effects of chicken NP have been performed using only adult vessels, so our understanding of the fetal response is limited (Trajanovska et al. 2007). Isolated cardiomyocytes also respond to NP at 0.5 (Bézie et al. 1996) and 0.7 (Koide et al. 1996), suggesting that the receptor is present in the ventricle. Further, isolated ventricular cardiomyocytes respond to ET-1 with an increase in the expression of NP mRNA (Bézie et al. 1996). Thus, ET-1 and ANP interact to regulate cardiomyocyte contractility in a paracrine/autocrine fashion, and maintain basal cardiovascular function in fetal chickens.

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Control of Breathing in Birds: Implications for High-Altitude Flight

G.R. Scott and W.K. Milsom

Abstract For birds that fly at high altitude, breathing must increase substantially to accommodate the dual oxygen transport requirements of exercise and hypoxia. Here we review the literature on control of breathing in birds, with particular emphasis on the adaptive trends seen in high-altitude flying species. Increases in breathing during high-altitude flight result from neurally mediated reflexes arising from multiple sites. The locomotor system stimulates breathing directly during exercise via both feedforward stimulation from brainstem locomotor centers and feedback stimulation from exercising muscles. O_2-sensitive chemoreceptors in the carotid body also stimulate breathing during hypoxia, whereas CO_2/pH-sensitive chemoreceptors can restrain breathing if the hypoxic ventilatory response produces a secondary hypocapnia. Theoretical modeling suggests that an enhanced capacity to increase breathing should be adaptive for high altitude flight. Empirical research suggests that the high-altitude flying bar-headed goose can indeed increase breathing significantly more than low-altitude birds during hypoxia at rest, loading more oxygen into the blood. This is probably caused by a reduction in the sensitivity of CO_2/pH-sensitive chemoreceptors to hypocapnia, and/or a reduction in hypoxic metabolic suppression and its depressive effects on breathing. Although this suggests that alterations in respiration control are an important component of the suite of adaptations to high altitude in birds, future studies are needed on control of breathing during flight, especially at altitude. A greater appreciation of the genetic basis for differences in the oxygen transport pathway that occur in high-altitude species will lead to a greater understanding of the evolution of physiological performance.

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1 Introduction

High-altitude environments pose many challenges to life. As elevation increases air gets colder and dryer, and the weather can be otherwise unpredictable. Perhaps the most stressful characteristic of high altitudes is the scarcity of oxygen upon which most living organisms depend. For every kilometre one ascends, the barometric pressure falls over 10%, such that at the peak of the world's highest mountains the oxygen pressure is only one quarter of that at sea level. For humans and most other terrestrial animals, these levels of oxygen are too low to support much activity. However, for the most elite bird species, severe hypoxia is not only tolerated, but experienced while performing the most energetically costly form of vertebrate locomotion: flight.

Birds are in general more tolerant of hypoxia than mammals, which has been well-reviewed elsewhere (Sutton et al. 1990). The avian parabronchial lung has a unique cross-current structure (Maina 2002), and, because of its theoretically higher gas exchange efficiency, researchers once thought it contributed to the exceptional hypoxia tolerance of birds. More recent theoretical studies (Scheid 1990) argue that avian lung anatomy is not as important to the hypoxia tolerance of birds as other physiological characteristics, such as control of blood circulation (Faraci 1986) and tissue capillarity (Mathieu-Costello 1990). Nevertheless, the general tolerance of birds to hypoxia does not entirely explain the amazing high-altitude feats of some birds in nature, such as the trans-Himalayan migrations of bar-headed geese (*Anser indicus*) and demoiselle cranes (*Anthropoides virgo*), or the incredible elevations attained by some soaring vultures (e.g., Rüppell's griffon, *Gyps rueppellii*) (Faraci 1986). For these intriguing species, additional physiological explanations are needed.

The challenge that high-altitude flying birds must overcome is the need to supply enough oxygen to support the high aerobic requirements of flight exercise in an oxygen-poor environment. For these species, O_2 must flow at high rates along the pathway from air to mitochondria despite minimal oxygen gradients. This oxygen transport pathway has several steps, beginning with ventilation, providing air to the gas exchange surface, followed by diffusion of O_2 into the blood, circulation of this blood to tissues, diffusion of O_2 into tissues, and finally O_2 utilization and ATP production by mitochondria (Weibel 1984).

As the first step in the O_2 pathway, breathing plays an essential role in oxygen transport. This review will examine control of breathing in birds, with particular emphasis on the adaptive trends seen in high-altitude flying species. We therefore focus our discussion on how breathing is controlled by exercise and blood gases (O_2 and CO_2/pH), without discussing some other mechanisms of control that have been reviewed in detail elsewhere (Bouverot 1978; Fedde 1990). We then follow with a review of control of breathing in high-altitude flying birds, particularly our own work on bar-headed geese. Our interest is to better understand the mechanisms of physiological evolution, and as such we pay special attention to neural control mechanisms that could form the basis of interspecific differences.

2 Ventilatory Responses to Exercise

Exercise has a strong influence on breathing in all vertebrates; breathing generally increases to accommodate the augmented O_2 demands of exercise. Flight exercise in birds requires that metabolism increases substantially, although the metabolic costs of steady flight change remarkably little across a wide range of speeds (Ward et al. 2002; Engel et al. 2006a; Bundle et al. 2007). To assess how ventilation changes to meet the increase in metabolism during steady flight in birds, we performed a similar synthesis of the available literature to that of Bernstein (1987). Information from his data set as well as data from more recent literature (Table 1 in Appendix) were used to determine the relationship between both oxygen consumption rate $(\dot{V}_{O_2})$, or total ventilation $(\dot{V}_{Tot})$, and body mass (M_b), both in resting birds and birds flying steadily in a wind tunnel (Fig. 1).

Both at rest (1) and during steady flight (2) $\dot{V}_{O_2}$ scales allometrically with body mass in birds. The quotient of these relationships represents the metabolic scope for steady flight: metabolic rate increases approximately 11-fold from rest to steady flight, and this change is nearly independent of body mass (Fig. 1a).

$$\text{Resting birds}: \quad \dot{V}_{O_2} = 0.76\, M_b{}^{0.728}, \tag{1}$$

$$\text{Flying birds}: \quad \dot{V}_{O_2} = 8.53\, M_b{}^{0.757}. \tag{2}$$

Total ventilation also scales allometrically with body mass in birds, but in contrast to oxygen consumption rate it has a slightly smaller scaling exponent both at rest (3) and during steady flight (4). Flight increases $\dot{V}_{Tot}$ approximately eightfold above resting levels, and this change is also nearly independent of body mass (Fig. 1b).

$$\text{Resting birds}: \quad \dot{V}_{Tot} = 0.49\, M_b{}^{0.653}, \tag{3}$$

$$\text{Flying birds}: \quad \dot{V}_{Tot} = 3.94\, M_b{}^{0.667}. \tag{4}$$

From the above regressions it is apparent that while total ventilation increases substantially during flight, it does so in slight disproportion to metabolism [see also (5)]. The smaller change in $\dot{V}_{Tot}$ (eightfold) compared to $\dot{V}_{O_2}$ (11-fold) suggests that the air convection requirement $(\text{ACR} = \dot{V}_{Tot}/\dot{V}_{O_2})$ decreases during flight. A reduced ACR is often accompanied by an increase in oxygen extraction by the lungs, which could be caused by changes in effective ventilation or pulmonary perfusion that support an increase in gas exchange efficiency during exercise.

$$\frac{\dot{V}_{Tot,Flight}/\dot{V}_{Tot,Rest}}{\dot{V}_{O_2,Flight}/\dot{V}_{O_2,Rest}} = 0.72\, M_b{}^{-0.015}. \tag{5}$$

The increase in breathing during flight is probably initiated by both central locomotor regions (i.e., feed-forward control) and afferent neural feedback resulting from muscle exercise. Activation of brainstem sites that control locomotion (pontomedullary reticular formation; Steeves et al. 1987) causes ventilation to increase, primarily by increasing breathing frequency (Funk et al. 1989). Breathing generally

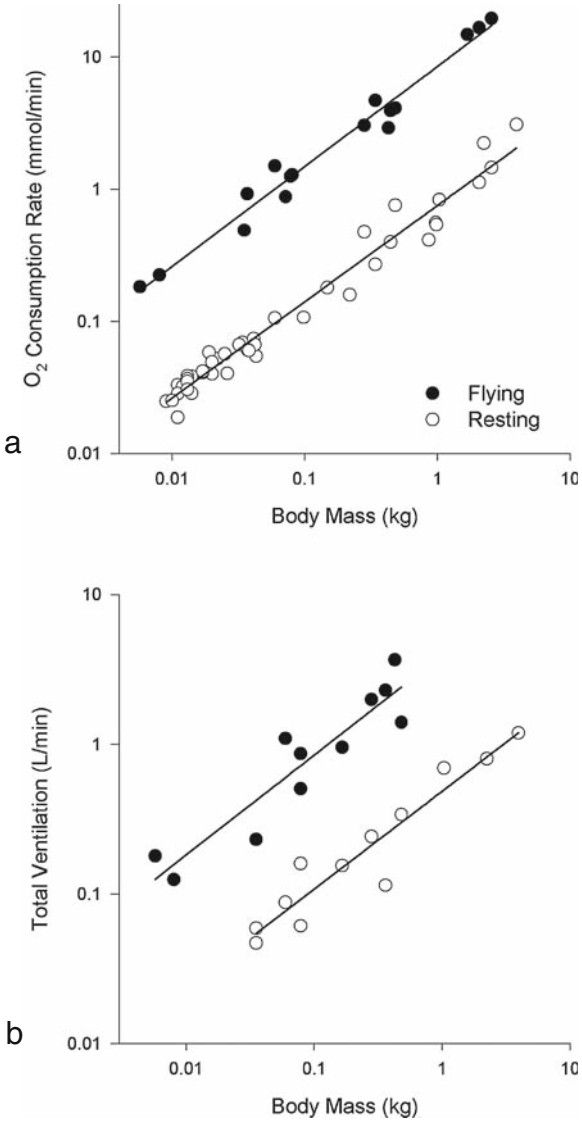

Fig. 1 Oxygen consumption rate (**a**) and total ventilation (**b**) at rest and during steady flight in wind-tunnels for various bird species of different body masses. See text for allometric regression equations ($R^2 \geq 0.89$). Data were collected from the published sources detailed in Table 1 of the Appendix

becomes coordinated with wingbeat during flight (Boggs 1997; Funk et al. 1997), which is also promoted by activating brainstem locomotor regions (Funk et al. 1989). Both the increase in ventilation and the coordination of breathing with wingbeat can occur in absence of afferent neural feedback, indicating that there is some

feed-forward control of ventilation (Funk et al. 1992b). The specific neural pathways involved are unknown in birds, but in mammals they are thought to involve parallel descending control to both spinal locomotor pathways and brainstem respiration networks (Feldman and McCrimmon 2003). Passive wing movements will also influence the coordination of wingbeat and breathing, indicating that there is neural feedback control of ventilation from metaboreceptors or mechanoreceptors in the muscles, chest wall, or wings (Funk et al. 1992a). In contrast, neural feedback from chemoreceptors (due to altered blood gases during exercise, such as elevated arterial CO_2 tensions) probably plays only a small role in the exercise ventilatory response (Kiley and Fedde 1983).

3 Ventilatory Responses to Decreasing O_2

Breathing is sensitive to hypoxia in all vertebrate classes (Milsom and Burleson 2007). Ventilation increases substantially in birds during hypoxia (Figs. 2 and 3), due to increases in both breathing frequency and (to a lesser extent) tidal volume. As in other vertebrate classes, oxygen chemoreceptors in the carotid body of birds are thought to initiate the ventilatory response to hypoxia (Milsom and Burleson 2007). This organ is innervated by the vagus in birds, and is located near the vagal nodose ganglion at the cervicothoracic border (Kameda 2002). The ventilatory responses to both hypoxia and hyperoxia are eliminated or drastically reduced in ducks whose carotid bodies have been denervated (Jones and Purves 1970; Bouverot et al. 1979).

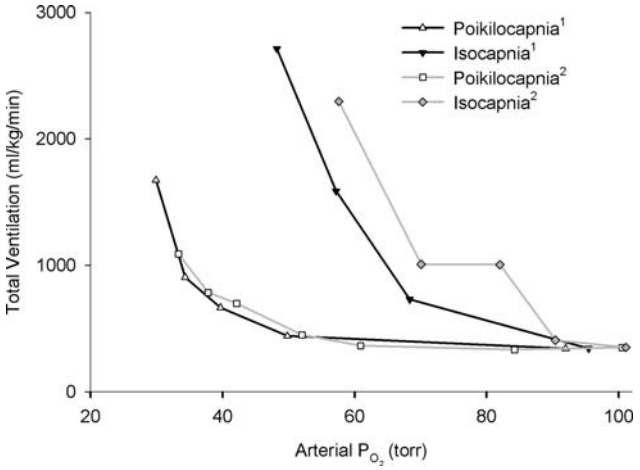

Fig. 2 Isocapnic (constant CO_2; *filled symbols*) and poikilocapnic (uncontrolled CO_2; *open symbols*) hypoxic ventilatory responses of pekin ducks. Data from Scott and Milsom (2007) are represented by triangles [1] and data from Powell et al. (2000a) are represented by squares and diamonds [2]. With poikilocapnic hypoxia the increase in breathing causes a secondary respiratory hypocapnia that attenuates the ventilatory response. When blood CO_2 levels are experimentally maintained during isocapnic hypoxia the ventilatory response to hypoxaemia alone is unveiled

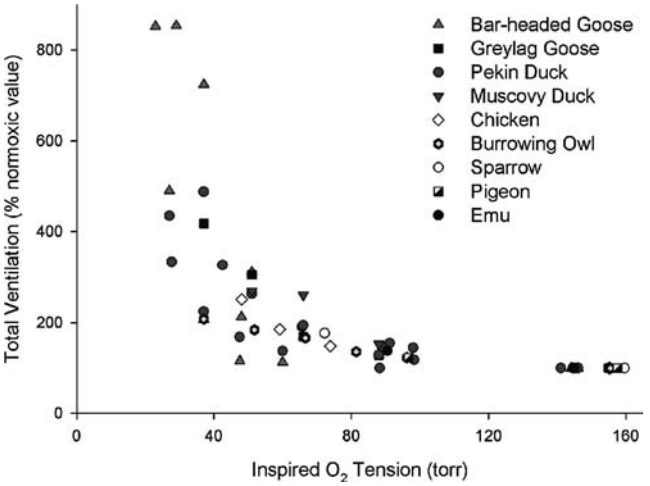

Fig. 3 The ventilatory response to environmental (poikilocapnic) hypoxia (expressed relative to resting ventilation in normoxia) in several species of birds. The proportional increase in total ventilation is substantially greater in the high-altitude-flying bar-headed geese at inspired O_2 tensions below 40 Torr. Data were collected from many published sources (Tucker 1968; Bouverot et al. 1976, 1979; Colacino et al. 1977; Bouverot and Sebert 1979; Black and Tenney 1980; Brackenbury et al. 1982; Kiley et al. 1985; Fedde et al. 1989; Shams and Scheid 1993; Schmitt et al. 2002; Powell et al. 2004; Kilgore et al. 2008; Scott and Milsom 2007). Data for Muscovy ducks are from our own unpublished observations

Furthermore, single afferent fibres from the vagus nerve increase their discharge frequency in response to hypoxia in both ducks and chickens (Bouverot and Leitner 1972; Hempleman et al. 1992). The majority of O_2-sensitive fibres in the vagus originate from the carotid body, although some, thought to be involved in cardiovascular reflexes, originate from the aortic bodies as well (Nye and Powell 1984).

The mechanism of O_2 sensing in avian carotid bodies is uncertain. However, there appear to be multiple O_2-sensing proteins in mammalian glomus cells, the putative chemoreceptive cells in the carotid bodies. Haem-containing enzymes that use O_2 as a substrate could act as sensors (metabolic hypothesis of O_2 sensing), such as the mitochondrial cytochromes, haem oxygenase, nitric oxide synthase, or NADPH oxidase (Prabhakar 2006). Membrane K^+ channels that are inhibited by hypoxia, and thus cause cell depolarization, could also sense O_2 (membrane hypothesis of O_2 sensing). Furthermore, these metabolic and membrane hypotheses are not mutually exclusive, and could interact (Prabhakar 2006). Following the initial detection of hypoxic stimuli, neurotransmitter release from glomus cells probably causes the increase in afferent nerve activity; as in mammals, the glomus cells of birds contain multiple neurochemicals, such as serotonin and catecholamines (Kameda 2002), that could take part in O_2 signalling.

Initial detection and transduction of hypoxic stimuli by the carotid bodies is just one component of the hypoxic ventilatory chemoreflex. Several additional physiological components of the chemoreflex can modulate breathing during hypoxia,

whose effects are often time-dependent. The hypoxic ventilatory response therefore has multiple time domains that can be caused by events occurring at multiple potential sites in the chemoreflex (i.e., carotid body, respiratory interneurons, respiratory motor nuclei, etc.) (Powell et al. 1998). For example, after the immediate rise in breathing at the onset of hypoxia, ducks experience short-term potentiation (STP) of breathing frequency that slowly increases total ventilation over several minutes (Mitchell et al. 2001), which may serve to fine-tune the initial acute response. Birds are also known to experience ventilatory acclimatization to hypoxia (VAH), which occurs several hours to days after the onset of hypoxia. VAH is thought to occur by both carotid body and central nervous system mechanisms (Powell et al. 2000b), and can either increase or decrease ventilation over time depending on the severity or duration of hypoxia exposure (Bouverot et al. 1979; Black and Tenney 1980; Powell et al. 2000a, 2004).

The hypoxic ventilatory response is also influenced by secondary changes in other blood gases. As discussed below, CO_2/pH is a strong respiratory stimulus in birds. The initial increase in breathing during hypoxia increases CO_2 loss, which causes a secondary hypocapnia. This occurs during environmental hypoxia exposure, when CO_2 levels in the blood are not experimentally controlled (i.e., poikilocapnic hypoxia), and inhibits the ventilatory response to decreased O_2 (Fig. 2). A better representation of the ventilatory response to hypoxia per se is therefore obtained during isocapnic hypoxia exposure, which is an experimental treatment that involves adding CO_2 to the inspired gas to keep CO_2 levels in the blood constant. Because blood CO_2 levels are not allowed to fall, the isocapnic hypoxic ventilatory response is typically much greater than the response to poikilocapnic hypoxia (Fig. 2).

4 Ventilatory Responses to Changing CO_2/pH

Breathing is in general very sensitive to changes in CO_2/pH in terrestrial vertebrates (Milsom 2002). In birds, hypercapnia and/or acidosis cause dramatic increases in ventilation; this is primarily due to increases in tidal volume, but breathing frequency often increases as well. Homologous CO_2/pH-sensitive respiratory chemoreceptors influence breathing across vertebrates, and are located both peripherally (sensing CO_2/pH in arterial blood or airway gas) and in the central nervous system (Milsom 2002). In birds, central chemoreceptors have the largest influence on total ventilation during changes in CO_2/pH, followed by the carotid bodies (Sébert 1979; Milsom et al. 1981). Intrapulmonary chemoreceptors have only slight effects on total ventilation but have a large influence on breathing pattern, by enhancing tidal volume and reducing breathing frequency in response to increasing CO_2 (Fedde et al. 1982; Hempleman and Posner 2004; Milsom et al. 2004; Dodd et al. 2007).

Despite their strong influence on ventilatory responses to CO_2/pH (Sébert 1979; Milsom et al. 1981), the location and mechanisms of function of central CO_2 chemoreceptors are poorly understood in birds. Central CO_2-sensitive neurons are abundant throughout the brainstem in mammals (Nattie 1999; Putnam et al. 2004), although the primary mammalian respiratory CO_2 chemoreceptors are most likely located in the retrotrapezoid nucleus (Mulkey et al. 2004) and/or the medullary raphe (Taylor et al. 2005). Central CO_2 chemoreceptors appear sensitive to CO_2, extracellular pH, and/or intracellular pH per se, and these stimuli can cause neural cell depolarization or hyperpolarization (Okada et al. 1993; Wang et al. 1998; Putnam et al. 2004). These chemoreceptors make extensive connections with neurons throughout the central respiration network, and are believed to interact with other chemosensory inputs (e.g., from carotid body chemoreceptors; Day and Wilson 2007).

Just as is the case during hypoxia, the firing rate of single afferent fibers from the carotid body increases during hypercapnia (Bouverot and Leitner 1972; Nye and Powell 1984; Hempleman et al. 1992). Carotid body denervation does not reduce steady state ventilation during hypercapnia, due to the continued stimulation of central chemoreceptors, but it drastically decreases the rate of response (Jones and Purves 1970; Dodd et al. 2007). This suggests that an important role of the carotid bodies in birds is in the immediate response to hypercapnia/acidosis, possibly before changes occur in cerebrospinal fluid CO_2/pH. The mechanisms of CO_2/pH sensing in the carotid bodies of birds are unknown. In mammals, depolarization of receptor cells is believed to occur by the inactivation of acid-sensitive potassium channels, by intracellular acidification, or by the activation of calcium channels by intracellular CO_2 (Putnam et al. 2004; Tan et al. 2007).

Intrapulmonary chemoreceptors (IPCs) are distributed throughout the lungs of birds and diapsid reptiles (Milsom et al. 2004). In birds, they are distributed along the parabronchi, but are more common in caudal regions of the lung, and are vagally innervated (Scheid et al. 1974; Nye and Burger 1978). IPC discharge is highest at low CO_2 levels, discharge declines as CO_2 levels increase, and, due to their location, IPCs can respond to both venous (pulmonary arterial) and airway CO_2 (Fedde and Peterson 1970; Powell et al. 1978, 1980; Scheid et al. 1978; Tallman and Grodins 1982; Hempleman and Bebout 1994). IPC activity inhibits inspiration such that tonic hypercapnia reduces the inhibitory effects of IPCs on breathing. However, CO_2 fluctuates during the breathing cycle, increasing during expiration, which leads to a decrease in IPC discharge at end-expiration that should promote initiation of the next breath (Fedde and Scheid 1976). Due to these functional properties IPCs appear to play an analogous role to mammalian pulmonary stretch receptors in the non-compliant avian lung.

The mechanism of sensing and signal transduction by avian intrapulmonary chemoreceptors has been well-studied (Hempleman and Posner 2004) (Fig. 4). CO_2 diffusing into the receptive ending of the IPC from either the airway or the pulmonary blood promotes intracellular acidification by the carbonic anhydrase catalyzed formation of H^+ and HCO_3^- (Hempleman et al. 2000). The magnitude of the decrease in intracellular pH resulting from this reaction is a function of the

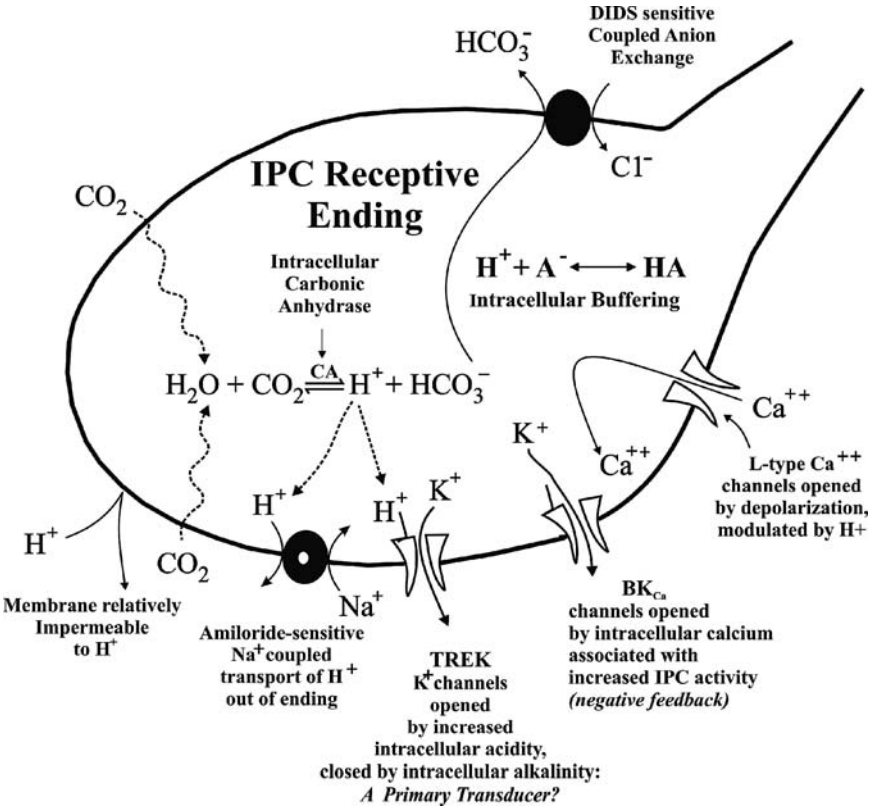

Fig. 4 Model of CO_2 sensing by avian intrapulmonary chemoreceptors (IPCs). CO_2 diffusing into the receptive ending of an IPC promotes intracellular acidification, which hyperpolarizes the receptor by opening TWIK-related K^+-channels (TREK) and closing L-type Ca^{2+} channels, and thus decreases IPC discharge. Discharge can be modified by membrane H^+ or HCO_3^- transport, and also depends on intracellular buffering (see text for other details). The model is based on Hempleman and Posner (2004), Hempleman et al. (2006), and Bina and Hempleman (2007)

balance between membrane H^+ and HCO_3^- transport (Shoemaker and Hempleman 2001; Hempleman et al. 2003). The intracellular acidification is then thought to hyperpolarize the receptor by opening TWIK-related K^+-channels (TREK), and this hyperpolarization also promotes the closing of L-type Ca^{2+} channels (Hempleman et al. 2006; Bina and Hempleman 2007). Increasing CO_2 levels in an IPC-receptive ending therefore results in a decrease in IPC discharge. Negative feedback modulation of IPC discharge may also occur at low CO_2 levels, because the rise in intracellular Ca^{2+} (due to influx through L-type Ca^{2+} channels) associated with IPC excitation also causes Ca^{2+}-dependent K^+ channels (BK_{Ca} channels) to open (Hempleman et al. 2006).

Similar to the hypoxic ventilatory response, time-dependent physiological mechanisms can modulate the ventilatory response to changes in CO_2/pH. After the acute

increase in breathing during hypercapnia in pekin ducks, ventilation declines progressively over time due to parallel reductions in breathing frequency (Dodd and Milsom 1987; Dodd et al. 2007). This ventilatory roll-off is caused by metabolic pH compensation, which alleviates the initial respiration acidosis caused by elevated blood CO_2 tensions. Ventilatory roll-off of the hypercapnic ventilatory response is probably mediated by central CO_2 chemoreceptors, because denervating the carotid bodies or the intrapulmonary chemoreceptors has no effect on the decline in breathing (Dodd et al. 2007).

5 Control of Breathing and Adaptation to High Altitude

For birds flying at high altitude, breathing more could enhance oxygen loading, and thus attenuate the effects of hypoxia. However, this will only be adaptive (in a Darwinian sense) if it influences flight performance (i.e., metabolism and oxygen transport). One means of determining whether this is so is to determine what limits performance in low-altitude birds, which should identify the physiological traits where changes have the greatest potential for enhancing oxygen transport and metabolic rate. We have taken this approach using theoretical modelling of the oxygen transport pathway in low-altitude ducks (Scott and Milsom 2006). The benefit of this modelling technique is that physiological traits can be varied individually, so their influence on the whole pathway can be assessed without compensatory changes in other traits. We found that while changes in ventilation have modest effects on oxygen consumption rate in normoxia and moderate hypoxia (12% inspired O_2, equivalent to roughly 3,000 m elevation), the influence of ventilation is much greater in severe hypoxia (5% inspired O_2, roughly 9,000 m elevation) (Scott and Milsom 2006). This is well-illustrated by calculating the physiological control coefficient of each step in the oxygen transport pathway, defined here as the fractional change in oxygen consumption rate (i.e., fractional change in overall pathway flux) divided by the fractional change of any given step in the pathway (Hochachka and Burelle 2004). The control coefficients for all steps in the oxygen transport pathway will sum to 1. The proportion of control that ventilation has over the oxygen transport pathway increases substantially as the severity of hypoxia increases, such that it becomes the most influential step in the pathway (Fig. 5). The influence of pulmonary diffusion capacity also increases in severe hypoxia, and while flight muscle diffusion capacity (which includes such variables as capillary density) maintains a high proportion of control, the influence of circulatory variables (cardiac output and haemoglobin concentration) declines substantially in severe hypoxia (Fig. 5). Overall, these findings suggest that a heightened capacity to increase ventilation could benefit performance substantially during severe hypoxia, and help sustain the metabolic rates needed to support flight at high altitude (Scott and Milsom 2006).

The potential adaptive benefit of increased ventilatory capacity predicts that high altitude-adapted species should have the capacity to breathe more than low altitude species during hypoxia. In support of this prediction, we have found that

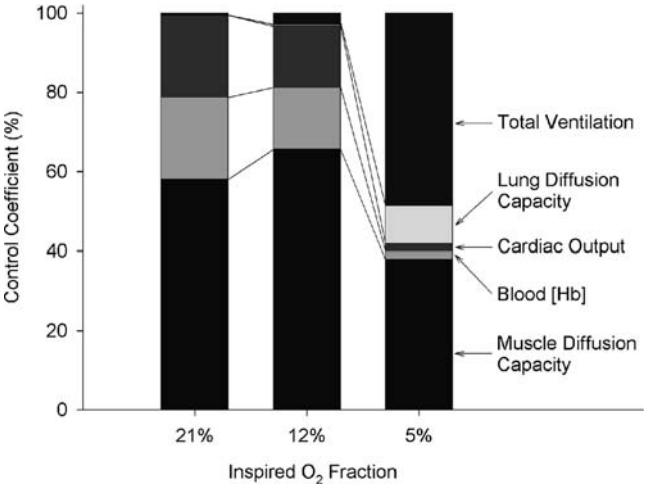

Fig. 5 Physiological control coefficients for metabolic rate in low-altitude waterfowl, in normoxia, moderate hypoxia (12% inspired O_2, simulating 3,000 m elevation), and severe hypoxia (5% inspired O_2, simulating 9,000 m elevation). Control coefficients are defined here as the fractional change in oxygen consumption rate divided by the fractional change of any given step in the oxygen transport pathway, and were calculated using the data and theoretical model from Scott and Milsom (2006) using a haemoglobin P_{50} of 25 Torr. The influence of the respiratory system on metabolic rate (i.e., the proportion of physiological control), particularly of ventilation, increases dramatically during hypoxia, while the influence of the circulatory system decreases

the high-altitude flying bar-headed goose breathes substantially more than low-altitude waterfowl during severe poikilocapnic hypoxia, and consequently loads more O_2 into the blood (Scott and Milsom 2007). To determine whether this characteristic was a result of differences in neutral phylogenetic variation or body size, we collected data on the ventilatory responses of as many species as possible from the literature. Bar-headed geese from our studies and those of previous researchers (Black and Tenney 1980) increase breathing substantially more during severe hypoxia than all other birds (Fig. 3). The difference in bar-headed geese is apparent at inspired O_2 tensions below approximately 40 Torr. This corresponds to an elevation 8–9 km above sea level, which is the elevation of many Himalayan peaks over which bar-headed geese fly on their biannual migration between south and central Asia (Swan 1970; Javed et al. 2000).

There appear to be multiple mechanistic differences accounting for the enhanced poikilocapnic hypoxic ventilatory response (HVR) in bar-headed geese. The isocapnic HVR is not enhanced in this species compared to low-altitude waterfowl, which suggests that bar-headed geese may be less sensitive to hypocapnia, rather than more sensitive to hypoxaemia (Scott and Milsom 2007). The apparent reduced sensitivity to hypocapnia seems to have the greatest influence on early time domains of the poikilocapnic HVR (e.g., acute response, short-term potentiation, etc.), because the difference in breathing between bar-headed geese and greylag geese (*Anser anser*) diminishes over time during prolonged hypoxia exposure (Scott et al. 2008).

The hypercapnic ventilatory responses of bar-headed geese and low-altitude waterfowl are similar, however, so differences in CO_2 sensitivity are either restricted to hypocapnic conditions, or are dependent on the presence of hypoxia. The relationship between metabolism and breathing may also account for part of the enhanced poikilocapnic HVR in bar-headed geese: metabolism is higher in this species during hypoxia, partly because body temperature depression is reduced, while air convection requirements ($\dot{V}_{Tot}/\dot{V}_{O_2}$) are similar to those in low-altitude waterfowl (Scott and Milsom 2007; Scott et al. 2008). Bar-headed geese may therefore experience less hypoxic metabolic suppression and the resulting depression of breathing. The overall effect of these mechanisms on breathing is to augment tidal volume in bar-headed geese, rather than breathing frequency, which should result in more effective ventilation of pulmonary surfaces.

While these data suggest that the control of breathing during poikilocapnic hypoxia is different in bar-headed geese at rest, the question remains as to what happens when oxygen consumption increases during steady flight (Ward et al. 2002): do bar-headed geese sustain an enhanced ventilatory response to hypoxia during flight, despite the additional increase in breathing due to exercise? Air convection requirements increase going from rest-to-running exercise in both bar-headed geese and pekin ducks, and this increase is similar in normoxia and hypoxia (Kiley et al. 1985; Fedde et al. 1989), suggesting that hypoxia and exercise normally have additive effects on ventilation. If so, this would necessitate exceptionally high rates of ventilation during high-altitude flight in bar-headed geese.

6 Genetic Basis for Physiological Evolution

The expression of complex physiological phenotypes is dependent on the coordinated action of numerous gene products, potentially acting on multiple intracellular pathways that interact within individual cells as well as between different cell types. Thus, not surprisingly, evolution of complex physiological systems generally requires that many genes evolve in concert (Montooth et al. 2003; Nikinmaa and Waser 2007). For this reason, understanding the genetic basis of how physiological systems evolve can be simplified by incorporating approaches to understand firstly the physiological and cellular mechanisms of adaptation. Furthermore, this strategy appreciates the "black box" of cellular and physiological systems (so-called subordinate traits) between organismal performance and genotype.

Our evidence suggests that an enhanced capacity to breathe can be adaptive for flight at high altitude, and that control of breathing has evolved in high-altitude-adapted birds (specifically bar-headed geese). However, the genes underlying adaptation of the respiration system to high altitude are presently unknown. The possibility that breathing in bar-headed geese is less sensitive to hypocapnia, which contributes to the enhanced poikilocapnic ventilatory response of this species, suggests that mechanisms of CO_2 sensing or signal integration may have evolved. As described above, CO_2 chemoreception can occur at carotid body, pulmonary,

and possibly multiple central chemoreceptors. The apparent sensitivity to hypocapnia of each of these chemoreceptors could be reduced by altering the molecular mechanisms of CO_2 sensing, the mechanisms transducing initial CO_2 signals into neural output, or multiple subsequent mechanisms in the respiratory chemoreflex pathways that have the capacity to change respiratory motor output. As a hypothetical example, if the intrapulmonary chemoreceptors of bar-headed geese were less active during low CO_2, possibly through genetic changes to TREK K^+ channels or Na^+, H^+ exchangers (Fig. 4), tidal volumes would be enhanced, and IPC discharge would increase less during poikilocapnic hypoxia, reducing the inhibitory effects of hypocapnia on ventilation. This and other possible explanations for the ventilatory response of bar-headed geese can now be assessed with physiological experiments, and could provide strong insight into candidate genes that might be the basis for physiological evolution in high-altitude birds.

7 Conclusions

Breathing must increase substantially during flight at high altitude to accommodate the dual oxygen transport demands of exercise and hypoxia. This is orchestrated by neural control mechanisms arising from the locomotory control system, as well as both O_2- and CO_2/pH-sensitive chemoreceptors. An enhanced capacity to increase ventilation appears to be important in birds adapted for high-altitude flight. Certainly, bar-headed geese at rest can increase breathing more than low-altitude birds during hypoxia and therefore load more oxygen into the blood. This appears to be due to a reduced sensitivity of breathing to hypocapnia, as well as reduced hypoxic metabolic suppression and its associated depressive effects on breathing. Future work should determine whether breathing is further enhanced in bar-headed geese during the elevated metabolic requirements of flight and, if so, the physiological differences that support it. A greater understanding of the differences in the oxygen transport pathway in bar-headed geese will lead to a greater appreciation of the genetic bases of differences in physiological function and the evolutionary changes underlying the incredible migration of this species.

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Appendix

See Table 1

Table 1 Oxygen consumption rates and total ventilation at rest and during steady flight in wind-tunnels for various bird species of different body masses

Common name	Latin name	M_b (kg)	V_{Tot} (l min^{-1})	V_{O_2} (mmol min^{-1})	References
Resting birds					
American tree sparrow	*Spizella arborea*	0.019		0.059	Rezende et al. (2002)
Asian blue quail	*Coturnix chinensis*	0.043		0.055	Rezende et al. (2002)
Austral pygmy-owl	*Glaucidium nanum*	0.098		0.107	Rezende et al. (2002)
Baltimore oriole	*Icterus galbula*	0.032		0.067	Rezende et al. (2002)
Bar-headed goose	*Anser indicus*	2.238	0.802	2.235	Scott and Milsom (2007)
Bar-headed goose	*Anser indicus*	2.560		1.455	Ward et al. (2002)
Barnacle goose	*Branta leucopsis*	2.070		1.129	Ward et al. (2002)
Black-billed magpie	*Pica pica*	0.165	0.155		Boggs et al. (1997)
Black-capped chickadee	*Poecile atricapillus*	0.013		0.037	Rezende et al. (2002)
Bobwhite quail	*Colinus virginianus*	0.218		0.159	Rezende et al. (2002)
Budgerigar	*Melopsittacus undulatus*	0.035	0.047		Bernstein (1987)
Budgerigar	*Melopsittacus undulatus*	0.035	0.059		Bernstein (1987)
Budgerigar	*Melopsittacus undulatus*	0.038		0.060	Rezende et al. (2002)
Chestnut teal	*Anas castanea*	0.969		0.562	Rezende et al. (2002)
Chipping sparrow	*Spizella passerina*	0.011		0.028	Rezende et al. (2002)
Common redpoll	*Carduelis flammea*	0.014		0.039	Rezende et al. (2002)
Dark-eyed junco	*Junco hyemalis*	0.017		0.042	Rezende et al. (2002)
Domestic pigeon	*Columbia livia*	0.360	0.114		Bernstein (1987)
Domestic pigeon	*Columbia livia*	0.442		0.400	Bernstein (1987)
Domestic pigeon	*Columbia livia*	0.340		0.270	Peters et al. (2005)
Downy woodpecker	*Picoides pubescens*	0.025		0.057	Rezende et al. (2002)
Eastern goldfinch	*Carduelis tristis*	0.013		0.039	Rezende et al. (2002)
Eastern kingbird	*Tyrannus tyrannus*	0.037		0.061	Rezende et al. (2002)

Eastern wood-pewee	*Contopus virens*	0.014		0.029	Rezende et al. (2002)
Evening grosbeak	*Coccothraustes vespertina*	0.059	0.088	0.106	Bernstein (1987)
Field sparrow	*Spizella pusilla*	0.013		0.035	Rezende et al. (2002)
Fish crow	*Corvus ossifragus*	0.280	0.242	0.477	Bernstein (1987)
Giant hummingbird	*Patagona gigas*	0.020		0.040	Rezende et al. (2002)
Grey catbird	*Dumetella carolinensis*	0.034		0.069	Rezende et al. (2002)
Greylag goose	*Anser anser*	3.945	1.189	3.094	Scott and Milsom (2007)
House finch	*Carpodacus mexicanus*	0.022		0.052	Rezende et al. (2002)
House sparrow	*Passer domesticus*	0.026		0.040	Rezende et al. (2002)
House wren	*Troglodytes aedon*	0.010		0.025	Rezende et al. (2002)
Japanese quail	*Coturnix japonica*	0.148		0.181	Rezende et al. (2002)
Little penguin	*Eudyptula minor*	0.983		0.541	Rezende et al. (2002)
Mountain chickadee	*Poecile gambeli*	0.011		0.033	Rezende et al. (2002)
Rose-breasted grosbeak	*Pheucticus ludovicanus*	0.041		0.074	Rezende et al. (2002)
Rufous-collared sparrow	*Zonotrichia capensis*	0.020		0.049	Rezende et al. (2002)
Rufous-tailed plantcutter	*Phytotoma rara*	0.042		0.067	Rezende et al. (2002)
Silvereye	*Zosterops lateralis*	0.011		0.019	Rezende et al. (2002)
Common starling	*Sturnus vulgaris*	0.078	0.061		Bernstein (1987)
Common starling	*Sturnus vulgaris*	0.079	0.160		Engel et al. (2006b)
Swamphen	*Gallinula porphyrio*	0.857		0.414	Rezende et al. (2002)
Titmouse	*Baeolophus griseus*	0.017		0.041	Rezende et al. (2002)
Warbling vireo	*Vireo gilvus*	0.013		0.031	Rezende et al. (2002)
White-breasted nuthatch	*Sitta carolinensis*	0.020		0.049	Rezende et al. (2002)

(continued)

Table 1 (Continued)

Common name	Latin name	M_b (kg)	V_{Tot} (l min^{-1})	V_{O_2} (mmol min^{-1})	References
White-necked raven	*Corvus cryptoleucus*	0.480	0.340	0.758	Bernstein (1987)
Yellow warbler	*Dendroica petechia*	0.009		0.025	Rezende et al. (2002)
Yellow-rumped warbler	*Dendroica coronata*	0.012		0.033	Rezende et al. (2002)
Zebra finch	*Poephila guttata*	0.012		0.032	Rezende et al. (2002)
Flying birds					
Glittering-throated emerald	*Amazilia fimbriata*	0.006	0.180	0.183	Bernstein (1987)
Bar-headed goose	*Anser indicus*	2.560		19.686	Ward et al. (2002)
Barnacle goose	*Branta leucopsis*	2.070		16.695	Ward et al. (2002)
Barnacle goose	*Branta leucopsis*	1.680		14.820	Butler et al. (2000)
Evening grosbeak	*Coccothraustes vespertina*	0.059	1.093	1.504	Bernstein (1987)
Sparkling violetear	*Colibri coruscans*	0.008	0.125	0.225	Bernstein (1987)
Domestic pigeon	*Columbia livia*	0.360	2.298		Bernstein (1987)
Domestic pigeon	*Columbia livia*	0.442		3.946	Bernstein (1987)
Domestic pigeon	*Columbia livia*	0.340		4.699	Peters et al. (2005)
White-necked raven	*Corvus cryptoleucus*	0.480	1.400	4.128	Bernstein (1987)
Fish crow	*Corvus ossifragus*	0.280	1.991	3.043	Bernstein (1987)
Ring-billed gull	*Larus delawarensis*	0.427	3.659	2.924	Bernstein (1987)
Budgerigar	*Melopsittacus undulatus*	0.035	0.232	0.489	Bernstein (1987)
Budgerigar	*Melopsittacus undulatus*	0.037		0.924	Bundle et al. (2007)
Cockatiel	*Nymphicus hollandicus*	0.081		1.294	Bundle et al. (2007)
Black-billed magpie	*Pica pica*	0.165	0.953		Boggs et al. (1997)
Rose-coloured starlings	*Sturnus roseus*	0.072		0.878	Engel et al. (2006a)
Common starling	*Sturnus vulgaris*	0.078	0.504	1.256	Bernstein (1987)
Common starling	*Sturnus vulgaris*	0.079	0.864		Engel et al. (2006b)

M_b, body mass; V_{Tot}, total ventilation; V_{O_2}, oxygen consumption rate

Part IV
Mammalian and Human Physiology

Peripheral Chemoreceptors in Mammals: Structure, Function and Transduction

P. Kumar

Abstract Peripheral chemoreceptors are localised in cervical, thoracic and abdominal regions of mammals with the cervical-located, carotid bodies appearing to be the most physiologically relevant for the initiation of cardiorespiratory reflexes in response to hypoxia. These organs have a characteristic morphology and receive an arterial blood supply in excess of their metabolic requirements, which may be important for their function, and they receive an afferent and efferent innervation. Type 1 cells are believed to contain the necessary transducing elements of these chemoreceptors and are pre-synaptic to afferent nerve terminals. Type 1 cells respond to falls in the partial pressure, but possibly also the O_2 content of blood, by inactivating species-dependent K channels to induce cell depolarisation, voltage-gated Ca^{2+} entry, neurotransmission and augmented afferent nerve discharge frequency. The identity of the protein sensor responsible for detecting hypoxia is not known with certainty but a number of candidates, including the enzymes AMPK and HO-2, have recently been proposed. In addition, these organs sense many other blood-borne, natural stimuli and are therefore most probably acting as polymodal receptors.

1 Introduction

A number of tissues are now known to have the ability to alter aspects of their normal function when faced with a reduced oxygen supply. In most cases, these alterations act to preserve the integrity of the affected tissue, principally by a reduction in energy expenditure. Mammalian peripheral chemoreceptors appear unique, therefore, not so much in their ability to sense, quickly, relatively modest falls in arterial oxygen levels but in the coupling of this sensory function with an active, energy-consuming response that leads to the initiation of appropriate, and corrective,

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systemic cardiorespiratory reflexes aimed at an increased delivery of oxygen rather than its conservation. They perform this critical function acutely in the absence of any alteration in protein expression, although a longer-term alteration of function is a characteristic feature of these organs that must occur via stimulus-induced changes in transcription. The carotid body, found, in mammals, at relatively discrete sites at the rostral end of the common carotid artery and close to the branch of the internal and external carotid artery, was the first peripheral chemoreceptor to be identified morphologically and also the first to be correctly identified as a sensory organ in the late 1920s (de Castro 1926) with a role for it in cardiorespiratory homeostasis being subsequently confirmed in a series of cross-perfusion experiments (Heymans and Heymans 1927). Functionally, the carotid bodies are the most important mammalian peripheral chemoreceptors and this, plus their particularly accessible anatomy, has led to a considerably greater literature regarding this organ than any other peripheral chemoreceptor; but chemoreceptor tissue of similar embryological origin and histology is also found in the thorax located along the arch of the aorta and pulmonary vessels and in abdominal chemoreceptor tissue found along branches of the abdominal vagal nerves.

What the separate roles of these various peripheral chemoreceptor tissues are precisely and whether they act in a co-ordinated fashion are questions that remain to be answered but presently there is little information on this topic. Instead, since the early, pioneering experiments were performed in the first half of the twentieth century, attention has focussed principally on two primary areas: (a) what is/are the transduction mechanism(s) involved in chemosensing and (b) do peripheral chemoreceptors have any role in the aetiology of human disease? This review will aim to provide some answers to the former question by examining the evidence gained at whole-animal and sub-cellular levels of organisation. Much of the evidence derives from carotid body studies or from cells derived from these organs, and this bias necessarily will be reflected in the focus of this chapter. Most recently, it has been appreciated that the duration, pattern and/or intensity of stimulation can have markedly different effects at the cellular response level and thus at the level of reflexes; and a complete answer to the second question awaits a more complete development of these animal models of human disease. It may not be too long before a full appreciation of mammalian peripheral chemoreceptor transduction is achieved, but there may be a longer wait before its functional significance is understood, as the emphasis of research in this area shifts back towards the integrative.

2 Characteristics of Peripheral Chemoreceptor Tissue

2.1 Anatomical Distribution of Chemoreceptor Tissue

An understanding of the transduction mechanism of the carotid body and other peripheral chemoreceptor tissue necessarily involves some understanding of its anatomy, its embryological origin and its evolution. It is not within the scope of this

review to provide a full account here, but a brief overview of the main points pertaining to the functionality of these tissues will be given in this section. In a recent paper, Milsom and Burleson (2007) argued convincingly that, during the course of evolution, peripheral chemoreceptor tissue became consolidated, as seen in modern-day birds and mammals, into at least two major sites, principally in the aorta and at the bifurcation of the external and internal carotid arteries. They suggest that this process was driven by a transition from aquatic to air-breathing and the consequent loss of intra-cardiac shunts, and speculate that current evidence may suggest different adequate stimuli and reflexogenic roles for these two, anatomically separate, organs. Certainly the ability to sense and thus respond to environmental, rather than arterial, levels of O_2 seems to have been lost from mammalian species for whom environmental changes in O_2 are relatively unlikely, at least in the short term, and in whom the need to respond quickly and appropriately to metabolic demand appears to be paramount. Peripheral chemoreceptor tissue is identified histologically as being comprised of two major cell types (Type 1 and Type 2), which lie within a matrix of connective tissue and are surrounded by a dense capillary network that together gives the tissue a characteristic, "lobular" appearance. These tissues, in the cat, dog, rat and mouse carotid bodies, lie most often together within single, dense connective tissue capsules, located bilaterally and usually separate from the vessels from which they derive their blood supply. In the rabbit and guinea pig these tissues are, in contrast, often found enveloping the arteries providing the cerebral blood supply (Clarke and de Burgh Daly 1981).

Additionally, in all mammalian species, varying amounts of chemoreceptor-like tissue are also found more diffusely arranged in regions adjacent to the main sensory organs in the neck, which may reflect its embryological derivation from the neural crest that it shares with the closely located sympathetic neurones. Such clustering of the cell types into a single organ is not so evident in the case of the extra-cervical, peripheral chemoreceptor tissues, where these cells are more likely to be found isolated in numerous, small groups within connective tissue, if at all. In the cat and dog, four groups of cells within the mediastinum have been identified on the basis of their topographical distributions along the aorta, pulmonary trunk and subclavian arteries, their blood supply and their innervation (Coleridge et al. 1967), but only so-called group 3 and 4 bodies receive a systemic arterial blood supply in the adult, and it is these that are commonly called "aortic" bodies. Innervation is largely afferent with fibres in the aortic nerve but, as in the carotid body, there is also an efferent innervation from sympathetic fibres and possibly also from parasympathetic fibres (Neil and O'Regan 1971a, b). These aortic body chemoreceptors have been ascribed a sensory function in cats (Lahiri et al. 1981b), but only a sparse number of such cells have been identified in the aortic arch region of rats (Easton and Howe 1983), and may be entirely absent, either morphologically or functionally, from rabbit and mouse (Chalmers et al. 1967), thus calling into question their importance in the larger scheme of cardiorespiratory control.

In the abdomen, chemoreceptor tissue is associated with the origin of the coeliac artery as it branches from the abdominal aorta and runs with branches of the abdominal vagus nerves in the rat. This tissue is responsive to arterial hypoxia and to

locally applied cyanide (Andrews et al. 1972; Howe et al. 1981), and appears to be the most caudal location of peripheral chemoreceptor tissues in mammals. The most complete description of abdominal chemoreceptors exists for the rat (Howe and Pack 1984). Their functional contribution to the reflex responses to cardiorespiratory disturbances appears quite minimal in the presence of the other peripheral chemoreceptor tissues, and as such they may simply represent a consequence of the natural migratory nature of neural crest cells.

2.2 Cellular Constituents of Chemoreceptor Tissue

The present consensus assumes that the thousands of Type 1 cells (also commonly termed "glomus" cells) of the carotid body act individually as primary transducer elements of this organ, as the isolated Type 1 cell can mount a robust response to hypoxia and all appear to form synaptic contacts with afferent nerve endings and with each other (McDonald 1981). Typically, in the rat, Type 1 cells are generally around 10 μm in diameter, with a large spherical nucleus and a narrow outer shell of cytoplasm containing numerous typical cell organelles such as mitochondria and endoplasmic reticulum, as well as a number of Golgi apparatus-associated vesicles with either clear or electron-dense cores. Many chemical substances are stored in these vesicles but the dense-core vesicles appear principally to contain catecholamines (Lever et al. 1959) and give the Type 1 cell the appearance of chromaffin cells, as found in the adrenal medulla. Each Type 1 cell receives afferent innervation from more than one afferent nerve and each afferent nerve makes synaptic contact with more than one Type 1 cell. In addition, some of the Type 1–Type 1 contacts (Baron and Eyzaguirre 1977; Eyzaguirre and Abudara 1995) and even some Type 1–nerve ending contacts appear to be gap junctions (Kondo 2002), as evidenced by dye transfer occurring through these routes between cells and by direct measure of electrical coupling. It appears that connexin 43 is the main, or even sole, protein involved in these structures (Abudara et al. 1999), and the function of such coupling seems to be aimed at facilitating the transduction process by enabling electrotonic spread of currents. Models of chemotransduction presently appear focussed upon single Type 1 cells acting in isolation, but more research needs to be aimed at establishing a physiological role for the interaction between Type 1 cells and between Type 1 cells and nerve endings, in order to fully understand whether the anatomical arrangements in the carotid body might produce a form of basic signal processing, via membrane potential summation and/or action potential collision, prior to information being received by the CNS.

The Type 2 cell appears more or less unexcitable and, although associated with nerve endings, it also wraps its many processes around groups of 3–5 Type 1 cells, suggesting that it may play a more supportative, glial-like role in the transduction process, although what precisely that role may be is far from certain. Type 2 cells lack the dense-core vesicles found in Type 1 cells and have slightly more flattened nuclei but are otherwise unremarkable in their appearance. The proportion of

Type 1 and Type 2 cells in rat and cat are similar, but Type 2 cells of the cat appear to occupy twice the volume of the carotid body than they do in the rat (Blakeman and Pallot 1982). To date, no function has been ascribed to this difference. The Type 2 cell may, however, have a greater role to play than presently presumed, and this will depend in large part on the development of appropriate models that enable a closer examination of its role in the chemotransduction process. As a "taster" of what may be to come, it has been confirmed that the Type 2 cell is relatively unexcited by membrane depolarisation (Xu et al. 2003), and it has been proposed that P2Y receptors localised on its plasma membrane may be a site of action for ATP released from Type 1 cells during hypoxia, thus suggesting a paracrine role for these cells. More work is needed before such a role can be confirmed.

Innervation of the carotid body is both afferent and efferent, the afferent fibres supplying information in the form of graded action potential frequencies, in both myelinated and non-myelinated fibres of the IXth cranial (glossopharyngeal) nerve, with central terminations in the nucleus tractus solitarius (NTS) sub-component of the respiratory neurones of the dorsal respiratory group in the brainstem, which is intimately involved with cardiorespiratory homeostasis (Jordan 1994). At diameters ranging from 1 to 10 μm, these afferent fibres are some of the smallest and some of the largest that are found in the carotid sinus branch of the IXth cranial nerve that also supplies the baroreceptor afferents originating from the nearby carotid sinus region. All afferent chemoreceptor fibres are believed to convey similar patterns of sensory information, but this has not been determined in any systematic manner to date and, given the heterogeneity in the responses observed in individual Type 1 cells to controlled, hypoxic stimuli (Bright et al. 1996), it may seem unlikely that this would be the case, but the consequences of this are not often, if at all, considered. The efferent supply to the carotid body is autonomic in origin, from both sympathetic and parasympathetic fibres. The sympathetic fibres most probably provide a control of the blood supply and hence the tissue stimulus intensity to the receptor cells, and evidence suggests that the parasympathetic supply provides a direct inhibition of Type 1 cells, seemingly via the release of nitric oxide (Campanucci and Nurse 2007).

2.3 Blood Flow

A characteristic of the carotid body in particular, but perhaps also of other peripheral chemoreceptor tissue (although this has never been measured), is its remarkably high blood flow relative to its size. In most species, this blood supply derives from a specific artery arising from larger, nearby arteries, but the precise origin of the blood supply appears to show some inter-individual variation as well as being quite species-dependent. Blood enters the organ to supply the tissues through an interacting network of convoluted capillaries and anastomosing sinusoids. With a mass of ca. 100 μg wet weight in the rat and ca. 2 mg in the cat, coupled with total flows of between 1,000 and 2,000 ml $100\,g^{-1}$ min^{-1} (De Burgh Daly 1954; Acker and

O'Regan 1981; Clarke et al. 1986; Barnett et al. 1988) it is apparent that this is a significant flow of more than ten times greater than that observed per unit mass in the human cerebral circulation. Much of this flow, at least at normal blood gas tensions, may, however, bypass the transducing elements of the organ entirely and simply flow through to the venous supply via the plentiful arterio-venous anastomoses. Certainly, the high venous PO_2 levels reflect this "excess" flow relative to its metabolic demand (carotid body O_2 consumption being estimated to be non-remarkable at between 1 and 2 ml $100\,g^{-1}$ min^{-1}). This high flow may function, perhaps via autonomic control, as a reserve to ensure adequate supply of O_2, even during severe hypoxia when the active organ must endure a high metabolic demand, as demonstrated by a >40% increase in glucose consumption (Obeso et al. 1989).

Clarke and colleagues (Clarke et al. 1990, 1993) used electron microscopy to examine serial sections of the carotid body in a quantitative morphological approach to compare the relative masses of the vascular and extra-vascular compartments of the carotid body during development within a single species (the cat) and across species. Their findings made from tissues obtained from fetal, newborn and adult cats showed that an increase in organ size of ca. almost 4× with full maturation, was due to matched growth in both "compartments" such that the proportion of small blood vessels supplying the Type 1 and Type 2 cells remained a fairly constant ratio of both the total vascular volume and the entire organ volume. This, indirectly, suggests that growth maintains the blood delivery to the transducing elements, and any alterations in sensitivity to particular blood-borne stimuli most probably occur as a consequence of alterations in cellular protein sensors and/or downstream transducing elements in Type 1 cells. When carotid bodies of adult primates, cats and rats were compared, the proportion of small vessel volume to total organ volume remained remarkably constant at ca. 5–6% across species, although the total volume varied more than fourfold from ca. 0.045 mm^3 in rat to 0.200 mm^3 in cat, with the monkey (of similar body weight to the cat) carotid body volume being ca. half that of the cat. Taken together, the data suggest that the particular vascular anatomy of the carotid body appears to be relatively fixed across species and during development, which may hint at an important role for this arrangement in the functioning of the organ. Such structure–function relations cannot be studied easily in vitro where much, if not all, of this anatomical integrity is lost, and further in vivo or in situ investigations are required if we are to truly understand how this organ may function in sensing blood-borne stimuli.

3 Natural Stimuli of Peripheral Chemoreceptors

Peripheral chemoreceptors have a number of natural stimuli, of which hypoxia is but one, albeit perhaps the one most often used to define them. Certainly in the absence of peripheral chemoreceptors, augmented ventilation in the face of hypoxia is lost, whilst the response to their other natural stimuli, for example hypercapnia, may remain, being mediated through other tissues. Despite this, their response to

these other, blood-borne, stimuli can be shown to be as physiologically relevant as their response to hypoxia, if not more so, and a full description of chemoreceptor transduction should attempt to account for these responses by determining, at the very least, whether there are multiple, stimulus-specific, transducing pathways, or whether these natural stimuli all converge upon one such pathway. Until then, it may be beneficial to consider these receptors as being polymodal with a number of adequate stimuli, rather than simply an oxygen sensor.

3.1 Hypoxia

The carotid body responds to hypoxia: that is, it produces a characteristic, graded chemoafferent discharge in proportion to the fall in arterial, or more correctly, tissue PO_2. This response is rapid in onset, non-adapting and, over the physiological range, takes the form of a single exponential or hyperbola with offset (Pepper et al. 1995). Discharge rises from values of PO_2 little below arterial normoxia (ca. 90 mmHg), and peak discharge of between 10 and 20 Hz in single fibres occurs at arterial PO_2 values between ca. 20 and 30 mmHg, after which it may fall, concomitantly, in vivo, with the blood pressure. There appears to be no absolute threshold to this response with basal discharge rates of around 1 Hz being observed during arterial hyperoxia — ca. half to a third of that observed during normoxia. This discharge is aperiodic, the interspike intervals being randomly distributed with respect to each other, but an underlying rhythm related to the respiratory frequency can be observed if discharge is summed over a number of respiratory cycles (Kumar et al. 1988). In recent studies (Pearson and Kumar, unpublished data) we have shown that this basal discharge can be considerably reduced by application of adenosine receptor antagonists and take this to suggest that a steady, albeit small, release of transmitter substances, presumably adenosine but also perhaps ATP, occurs even in the absence of any hypoxic drive. The possibility that this basal drive might originate from the normal PCO_2 is something that needs to be considered and, if important, points to the necessity for in vitro experiments to be performed in bicarbonate-buffered solutions rather than HEPES-buffered solutions wherever possible. Is this small, absolute decrease in discharge between normoxia and hyperoxia of physiological importance? Whilst, on the face of it, it might appear to be of little consequence, at least relative to the considerably larger differences that occur when PO_2 is reduced, there is some evidence to show that when PO_2 is increased from normoxia to hyperoxia this decreases chemoreflex-mediated sympathetic tone in humans (Seals et al. 1991).

The high total blood flow, small arterial-venous PO_2 difference and rich efferent innervation of the carotid body raise the intriguing question as to the precise stimulus to which the organ is responding and to the nature of any sensor(s). Tissue PO_2 in healthy animals is a variable that depends upon a number of factors, including: (1) the inspired level of O_2 that naturally, for mammals, should depend solely on the altitude, as well as (2) the O_2 capacity of blood, which is largely determined

by the haemoglobin concentration, (3) the specific vascular structure of the particular tissue in question and its blood flow, (4) local diffusion concentrations, and finally (5) the rate of cellular O_2 consumption. In more chronic situations, of hours to days of systemic hypoxia, which may be induced by sojourns to altitude or by the adverse consequences of cardiopulmonary disease, variations in arterial blood PO_2, vascular re-arrangement, and/or O_2 transport capacity may also influence the tissue PO_2. Thus, at least in the acute time-frame of seconds in which the carotid body responds to a change in arterial PO_2, the tissue PO_2 is determined almost exclusively by a balance between local vasodilator and constrictor influences and the O_2 consumption. If, for example, tissue PO_2 is maintained relatively high during acute systemic hypoxia, then any chemosensing apparatus needs to be particularly sensitive to moderately small falls in tissue PO_2. If, however, the tissue PO_2 is held relatively low compared to other tissues, then these sensors need perhaps express no such exquisite sensitivity, but their cells should perhaps be able to demonstrate an ability to maintain an increased metabolism despite reduced O_2 delivery. In fact, measurements of carotid body microvascular PO_2, made using a non-invasive optical method, point to a relatively normal to high tissue PO_2 of ca. 50 mmHg (ca. 6.5 kPa) when the organ is perfused with blood (Rumsey et al. 1991). This suggests that the carotid body does not appear to have any particular specialisation that sets its tissue PO_2 differently from other tissues of comparable metabolic rate, and also, therefore, that any carotid body sensor of hypoxia must have a low affinity for O_2. The maintenance of tissue PO_2 at these high to normal levels may be necessary to account for the finding that the carotid body increases its metabolism by $>40\%$ during hypoxia as determined either by rate of glucose consumption (Obeso et al. 1986, 1993) or by O_2 disappearance (Rumsey et al. 1991). Rumsey et al. (1991) claimed that the organ would be forced towards anaerobic metabolism when tissue PO_2 fell below 10 mmHg, and it has been described how steady state chemoreceptor afferent discharge in vivo, although maintained for long periods at moderately severe levels of hypoxia, will ultimately fail to be sustained when that hypoxia becomes more severe (Biscoe et al. 1970b). The organ simply cannot operate normally in anoxia, and towards these extremely low levels of O_2, responses that are not part of the physiological transduction process may be initiated. The importance of relating any response to estimations or actual measurements of *tissue* PO_2 should therefore not be underestimated when making comparisons or establishing potential transduction mechanisms, whether what is actually being routinely measured is inspired, arterial or particularly superfusate PO_2 in vitro (Kumar 2007b).

3.1.1 Oxygen Tension or Content?

The assumption exists that the carotid body responds to falls in arterial PO_2 rather than falls in the arterial content of O_2. Thus, despite the fact that both must fall together during, so-called, hypoxic hypoxia, carotid chemoreceptor discharge frequency remains unchanged by the challenge of anaemia induced either by haemodilution (Biscoe et al. 1970b) or by carbon monoxide poisoning of blood

haemoglobin (Duke et al. 1952), although this latter response is not a universal finding in all fibres (Lahiri et al. 1981b). In significant contrast, the intrathoracic, aortic chemoreceptors are deemed to be particularly specialised for the sensing of arterial O_2 content, as they increased their afferent discharge somewhat exponentially and considerably more than carotid body chemoafferents during an experimental protocol of graded increases in blood HbCO induced by inhalation of sub-lethal doses of carbon monoxide (Hatcher et al. 1978; Lahiri et al. 1981b). Interestingly, the elevated discharge from aortic chemoreceptors was not associated with a ventilatory chemoreflex response, but instead led to variation in cardiovascular parameters, most notably blood volume and red blood cell count as well as systemic vascular resistance (Daly and Ungar 1966). In addition, aortic chemoreceptors seemed also to be sensitive to hypotension of just a few mmHg from normal, in contrast to their carotid body counterparts which do not show any response to a falls in arterial blood pressure until this is less than 60 mmHg, at which point the tissue may be hypoxic (Biscoe et al. 1970a). Taken together, these findings led Lahiri and coworkers in the early 1980s to suggest that O_2 delivery in aortic bodies must be more limiting than in carotid bodies, making them particularly sensitive to O_2 transport capacity. Blood flow through aortic chemoreceptors has, unfortunately, not been measured, but the anatomical and anecdotal evidence seems to indicate that it is probably not much different from the excess to metabolic requirement observed in carotid body blood flow, with blood being supplied by adjacent large arteries to a lobular, highly vascular tissue, and with the colour of the venous blood draining the aortic bodies of cats being described as "arterial" by Howe in his 1957 PhD thesis, as described by Daly (Daly 1997). It is noteworthy, however, that hyperoxia could reduce aortic chemoreceptor discharge induced by HbCO and this, as described by Lahiri et al. (1981b), indicates that, ultimately, the stimulus for both aortic and carotid chemoreceptors is most likely a reduced tissue PO_2. The possibility remains, however, that these two organs represent different evolutionary systems, with their anatomical positions determining their precise cardiovascular or ventilatory function (Milsom and Burleson 2007). However, a note of caution with regard to species variation is required, as the mammalian recordings of aortic chemoafferent discharge in response to variations in O_2 capacity were all performed on cats. In rats, which do not have any noticeable aortic tissue (Easton and Howe 1983), the *ventilatory* reflex response to HbCO was not significantly different from the response to hypoxic hypoxia and this suggests that rat carotid bodies may sense arterial O_2 content. How this may occur, in the absence of any noticeable limitation in O_2 delivery, is not known. Experiments in vitro, in the absence of blood perfusion, are most commonly utilised with rat carotid bodies, but these would not appear to be able to resolve this issue and, instead, exploiting the recording of rat carotid body chemoafferents in vivo, which has only been reported once (Vidruk et al. 2001), seems to offer the only way forward if a final, definitive answer is to be found regarding this key point. It is worth remembering at this point that, irrespective of whether tension or partial pressures are being sensed, what is detected a *lack* of oxygen.

3.2 Hypercapnia and Acidosis

The sensing of hypoxia, at least in vivo, must occur in the presence of other, putative, blood stimuli that may, or may not, be changing concomitantly. Thus, an observed chemoresponse to falling levels of blood PO_2 may represent instead a response to some other stimuli that is significantly modulated by oxygen or its lack. A disentangling of this conundrum will be difficult but is not of pedantic interest: it rather could inform future studies regarding chemoreceptor function. This interaction between stimuli is particularly apparent for CO_2. Unlike hypoxia, stimulation by CO_2 does have an absolute threshold in the carotid body. Discharge is abolished at PCO_2 values lower than around 18–25 mmHg (Eyzaguirre and Lewin 1961) and increases linearly from that point to provide around 30–40% of the total hypercapnic drive (the remainder provided by central chemoreceptors located in the brainstem). Discharge cannot be maintained in very high PCO_2 and often flattens off at values above 100 mmHg (Lahiri and Forster 2003). Also, in contrast to the response to hypoxia, the carotid body response to hypercapnia, when applied at a sufficiently abrupt rate of change, shows an over/undershooting response with adaptation to increases and decreases in stimulus intensity. As blood gas tensions are known to vary with a respiratory periodicity, this dynamic response to CO_2 provides a potential, oscillating signal in chemodischarge that is independent of the mean level of blood gas tensions, and thus could provide feedforward information to the CNS regarding, for example, exercise intensity (Band and Wolff 1978; Kumar et al. 1988).

The mechanism underlying CO_2 transduction has been much less studied than that for O_2, but it is now generally believed that hydrogen ions, rather than CO_2 per se, act as the principal stimulus to Type 1 cells during the respiratory acidosis of arterial hypercapnia. Carbonic anhydrase is localised within Type 1 cells (Ridderstrale and Hanson 1984) and, as the carotid body response to PCO_2 was reduced by membrane-permeant, but not by membrane-impermeant, isoforms of carbonic anhydrase inhibitors (Hanson et al. 1978), it was suggested that the concentration of intracellular rather than extracellular H^+ was more probably the transduced signal. Subsequent, direct measurements of the intracellular pH (pHi), made using fluorometric techniques (Buckler et al. 1991), confirmed that intracellular pH would only fall if extracellular pH (pHo) also fell and that the rate of change of pHi was greater if pHo was altered with change in PCO_2 rather than without. More interestingly, these authors also showed a remarkable dependency of pHi upon pHo in Type 1 cells that was of two to three times greater magnitude than observed in other cell types and which could not be explained by any specialisation in either transmembrane acid (or acid equivalent) transport mechanisms or in intrinsic buffering power. Thus, the carotid body response to CO_2 appears to be subsequent to its intracellular acidification, and the relatively large fall in pHi could confer the particular sensitivity to CO_2 observable in this organ. It is worth noting, however, that despite these convincing findings at the single cell level, isohydric hypercapnia (i.e., an increase in CO_2 without sustained change in pH) was shown to have positive, demonstrable effects upon carotid body dopamine release and afferent discharge (Rigual et al. 1984; Putnam et al. 2004), and it may be that single cell

studies do not provide the entire picture with regard to transduction mechanisms of this composite organ.

Metabolic acidosis is also stimulatory to the carotid body, generating chemoafferent response curves to variations in pH that are similar to the response to CO_2 (Gray 1968; Biscoe et al. 1970b; Black et al. 1971) with linear responses to pH down to ca. 6.9 (Lahiri and Forster 2003). This may provide a unique signal for respiratory drive during exercise (Whipp and Wasserman 1980) but most probably acts similarly to variations in pH induced by changing PCO_2.

3.3 Stimulus Interaction

When hypoxia is applied together with hypercapnia to provide an "asphyxial" stimulus, the resulting chemoafferent discharge frequency is greater than the sum of the two responses when applied independently (Lahiri and DeLaney 1975; Pepper et al. 1995). Thus, hypoxia sharpens the chemoresponse by increasing the carotid body sensitivity to CO_2. Of course, it may be equally stated that CO_2 sharpens the sensitivity to hypoxia and, correspondingly, that normoxia/hyperoxia damp down the sensitivity to CO_2, thus establishing a low basal rate at these levels of O_2. What is potentially revealing, however, is that, although the threshold to CO_2 is decreased by increasing hypoxia (Lahiri and DeLaney 1975), there always remains a threshold, and this implies that the hypoxia-sensing mechanism has an absolute requirement for CO_2/acidosis. What this requirement may be is not known, but the outcome is that a "fan" of CO_2 stimulus–response curves can be derived with slopes that increase with hypoxia, and that all cross the abscissa at positive values of PCO_2. This multiplicative interaction is a characteristic feature of carotid body function and can be observable also at the level of ventilation (Nielsen and Smith 1952), although here it may have some additional input from further multiplicative interaction with central chemoreceptors (Robbins 1988).

It is interesting to note that this multiplicative interaction in carotid body chemoreceptor discharge is not observable in newborn animals from birth until around the time of weaning (Pepper et al. 1995) and can be prevented from maturing naturally by resisting the natural rise in arterial PO_2 that occurs post-natally with the onset of air-breathing (Landauer et al. 1995). This postnatal increase in multiplicative interaction could underlie the established phenomenon of chemoreceptor resetting, whereby, with increasing gestational and postnatal age, hypoxia sensitivity is increased in chemoafferents of both the carotid (Blanco et al. 1984; Kholwadwala and Donnelly 1992; Pepper et al. 1995) and aortic bodies (Kumar and Hanson 1989), as demonstrated by an upward and rightward shift in the PO_2 stimulus–response curves that is not unlike the effect of additional CO_2 seen in the adult response. This is seen in both in vivo and in vitro preparations and thus suggests resetting to be an O_2-dependent process inherent to chemoreceptor tissue. There is little evidence to suggest morphological changes in this organ over this time period (Clarke et al. 1990; Moore et al. 1991). Resetting of chemoreceptor afferents is translated into

observable effects at the level of reflex ventilatory responses to hypoxia in animals (Hanson et al. 1989) and humans (Williams et al. 1991; Calder et al. 1994a) and it has been suggested that a failure to reset adequately may predispose infants to cardiorespiratory disturbances that could lead to increased morbidity or even mortality (Calder et al. 1994b). Mechanisms proposed for hypoxia chemotransduction should be able to account both for its interaction with CO_2 and for this postnatal maturation. To date, the focus has been on the former, and for this there is some evidence to show that interaction most likely occurs in carotid body Type 1 cells at the level of neurotransmitter release (Peers 2004), but evidence also exists to show correlative changes in ionic conductances in Type 1 cells with postnatal age (Hatton et al. 1997; Wasicko et al. 1999, 2006).

The aortic chemoreceptors, of the cat, like the carotid chemoreceptors, are also able to transduce an hypercapnic stimulus, but, as noted (Lahiri et al. 1981a) when simultaneous afferent nerve recordings were made from both receptor groups in vivo, the response of the aortics is considerably less sensitive than that of carotid chemoreceptors, and they suggested that this alone may account for the reduced sensitivity to PO_2 and the reduced multiplicative interaction of aortic chemoreceptors. The reason for this reduced sensitivity is not known, but it may perhaps lie with a lower dependency of pHi upon pHo in this organ when compared to the carotid body. This has yet to be confirmed.

3.4 Other Non-Hypoxic Stimuli: Is the Carotid Body a Glucosensor?

In addition to hypoxia and hypercapnia, the carotid body can also transduce a number of non-blood gas- and pH-dependent stimuli. These have been described more fully elsewhere (Kumar and Bin-Jaliah 2007) and only a brief description will be given here. Thus, elevations in arterial K^+ concentration, elevations in circulating blood temperature, decreased arterial osmolarity, and decreased plasma glucose concentrations, as well as a number of circulating chemicals and hormones, including adrenaline, vasopressin and adenosine, have all been shown to augment chemoafferent discharge at the carotid body and, importantly, at physiological levels of stimulus intensity.

Of these stimuli, most recent interest has focussed upon a potential role for the carotid body as a glucosensor, sensing falls in plasma levels of this substrate and acting to initiate corrective, reflex neuroendocrine responses. A consensus does not yet exist, but, if found to be correct, this role could potentially be of greater physiological importance than its role as an O_2 sensor, given the more natural and frequent variations that occur in plasma glucose concentration, relative to variations in blood PO_2. This putative role as part of the neuroendocrine axis was initially suggested by experiments in which intracarotid glucose infusion was found to reduce carotid body activity, and carotid body stimulation by cyanide was shown to increase hepatic glucose output via an adrenal gland-dependent mechanism and also

increased brain glucose retention (Alvarez-Buylla and de Alvarez-Buylla 1988). This same group subsequently demonstrated that the increase in plasma glucose following chemostimulation was mediated via activation of hepatic arginine vasopressin V1a receptors (Montero et al. 2006). These findings were confirmed in conscious, carotid body chemo-denervated dogs that, when challenged with insulin, were shown to mount a less effective counter-regulatory response than their sham controls (Koyama et al. 2000; Silveira et al. 2003) through an impaired ability to raise plasma glucagon and cortisol appropriately. The presumed adequate stimulus in these experiments was hypoglycaemia, although a role for adrenaline cannot be excluded (Maskell et al. 2006). Adrenaline may act to increase carotid body and ventilatory CO_2 sensitivity, as had been demonstrated to occur with insulin-induced hypoglycaemia in rats (Bin-Jaliah et al. 2005); the latter suggested that, in vivo, the reflex response was appropriate for the elevated metabolism induced by insulin infusion. In these experiments, direct recording of single fibre chemoafferents in vitro showed no elevation and often a decrease in discharge frequency with reduced glucose concentration. This is in agreement with other reports of where reduced glucose levels had no effect on chemoreceptor discharge or transmitter release (Almaraz et al. 1984; Delpiano and Acker 1985; Conde et al. 2007). It has been noted that in these experiments a relatively high PO_2 may have prevented the full response to glucopenia (Kumar 2007a; Zhang et al. 2007), as indeed a positive interaction between hypoxia and glucopenia has been described (Pardal and Lopez-Barneo 2002). However, this would suggest an absolute requirement for at least some degree of hypoxia to be present for a response to glucose to occur, which itself may question whether low glucose is acting as an adequate stimulus in its own right or simply facilitating an hypoxic sensitivity. In this regard, studies measuring ventilatory responses in humans who had been made hypoglycaemic for 30 minutes showed that the expected elevation in ventilation occurred without change in arterial PCO_2, and was thus unlike the reflex response to hypoxia. This suggests, again, an appropriate hyperpneic response to elevated metabolism rather than a response to hypoglycaemia per se that may be mediated through an action of counter-regulatory hormones acting at the carotid body (Ward et al. 2007). That said, there is a growing literature regarding carotid body sensitivity, and this organ, when examined in the reduced states of slice preparations (Pardal and Lopez-Barneo 2002), co-cultures (Nurse 2005; Zhang et al. 2007) or isolated cells (Garcia-Fernandez et al. 2007), does show a brisk, reproducible and reversible response to falls in bath concentration of glucose to 2 mM or lower. On examining the mechanism of glucose-sensing in these preparations, it appears that, although the transduction process shares some common downstream elements, such as raised intracellular Ca^{2+} concentration and neurotransmitter release, these are triggered through a quite different membrane-delimited process than that currently ascribed for hypoxia sensing (see below). Thus, low glucose induces a Na^+ conductance through a reduction in membrane resistance (Zhang et al. 2007) due to activation of Type 1 cell transient receptor potential (trp) C channels (Garcia-Fernandez et al. 2007) that had previously been described in this organ (Buniel et al. 2003) but ascribed no function. How the disparate findings with regard to glucose sensitivity in this organ are to be reconciled is not yet known, but

the answer may lie in the preparations being utilised and/or their metabolic status (Kumar 2007a).

4 Mechanism of Hypoxia Chemotransduction

Since the late 1980s and the establishment of Type 1 cell isolation procedures, experiments in carotid body physiology have largely been aimed at an understanding of the cellular and molecular basis of hypoxia transduction. Presently, our view of this process is that it has an absolute requirement for Ca^{2+}-dependent neurotransmission between Type 1 cells and afferent nerve endings, with the elevated Type 1 cell intracellular Ca^{2+} deriving almost exclusively from extracellular sources (Buckler and Vaughan-Jones 1994) and entering the cell through voltage-gated Ca^{2+} channels. Neurotransmission is complex in the carotid body with a large number of putative inhibitory and excitatory transmitter substances localised in Type 1 cells and a variety of receptor subtypes on Type 1 cells, as well as on afferent nerve endings, of which acetylcholine, catecholamines and ATP appear to play key roles. A full description of these, their co-transmission, species-dependency and regulation is beyond the scope of this article but other recent reviews provide a thorough account (Iturriaga and Alcayaga 2004; Shirahata et al. 2007; Zapata 2007).

4.1 O2 Sensitive K Channels

The voltage-gated Ca^{2+} channels are activated subsequent to the plasma membrane depolarisation induced by inactivation of a variety of O_2-gated K channels. Comprehensive reviews of these channels and their role(s) in O_2 sensing have been published recently (Buckler 2007; Lopez-Lopez and Perez-Garcia 2007; Peers and Wyatt 2007; Varas et al. 2007). In brief, these include a number of voltage-gated K channels, including the maxi K (also called BK, or calcium activated K) channels in rats, the Kv3 channel in mice, and the Kv4 channel and HERG-like channel in rabbits, as well as the voltage-independent TASK-like, leak channels, originally described in rats. This latter sub-type is particularly interesting as it may contribute more to the resting membrane potential during normoxia than the other sub-types, being activated at potentials as low as -90 mV, and thus play a crucial, initial role in initiating the membrane depolarisation during hypoxia. It has an appropriate PO_2 sensitivity with a $K_{1/2}$ of 12–13 mmHg. The debate regarding the role of each of these channel types continues, but to date the evidence provided shows only a relatively small depolarisation of ca. 10 mV, induced by hypoxia in Type 1 cells. This could appear insufficient to precipitate (principally L-type) voltage-gated Ca^{2+} entry. Similarly, the effect of K channel blockers upon carotid body chemoafferent discharge, which by prediction should mimic hypoxia, has not been convincing (Donnelly 1997, 1999), although there is presently a poor pharmacology for the

TASK channels and, once again, the specific preparation utilised may influence the results (Pardal et al. 2000). Presently, all testable hypotheses of chemotransduction centre upon K channel inhibition and a key role for this inhibition in chemotransduction is widely accepted, and much evidence in reduced preparations supports the finding that it is an essential requirement for cell depolarisation and subsequent voltage-gated Ca^{2+} entry.

4.2 Protein Sensors

Although there has been some indication that K channels of the carotid body may have intrinsic O_2 sensitivity, it is more commonly believed that this sensitivity is conferred to the channel by an associated protein O_2 sensor that may or may not be coupled directly to the channel. As pointed out recently (Ward 2008), it may not be necessary for any downstream effectors coupled to these sensors to have an O_2 sensitivity that correlates with the P_{50} of its sensor protein, as coupling between the two is likely to be non-linear. That this protein sensor may contain a haem group is a long-held supposition in this field, borne out initially by the close correlation between the PO_2 dependency of red blood cell haemoglobin saturation and the carotid body afferent discharge. Presently, a few such sensors appear to have the potential to act in this capacity in the carotid body, and not all contain haem groups. The full PO_2 stimulus response curve has a complex shape and may require several sensors working over different ranges (Prabhakar 2006), and perhaps even a degree of redundancy might necessarily be present to ensure the functioning of the receptor throughout a lifetime when sensitivity can change. Their precise O_2 affinities are not yet known with absolute confidence, but there is no reason to believe that neither these sensors, nor indeed any "downstream" effectors such as ion channels or transmitters, need be mutually exclusive, and a degree of species difference is known to exist. What is presumed, however, is that these sensors have a low O_2 affinity that enables them to sense falls in PO_2 from levels that are too high to affect the flow of electrons through the mitochondrial protein transport chain. Thus, despite the long-recognised finding that all blockers of the mitochondrial electron chain and all uncouplers of oxidative phosphorylation are potent chemoexcitants (Gonzalez et al. 1994; Lahiri et al. 2006), it is not clear how protein complexes in these organelles might act as O_2 sensors unless they, or a sub-group of them in the Type 1 cell, possess a particularly low O_2 affinity. That this may actually be the case is suggested by measurements of mitochondrial membrane potential and NAD(P)H autofluorescence that showed a particular sensitivity to Po_2 which was not apparent in other chromaffin and non-chromaffin tissues and was presumed to be conferred through a lowering of the O_2 affinity of mitochondrial cytochrome c oxidase (Complex IV) (Duchen and Biscoe 1992a, b). Depolarisation of the mitochondrial membrane potential would, presumabl,y elevate intracellular Ca^{2+} by reducing its buffering. This hypothesis is intriguing but requires further experimentation and the finding that, at the onset of hypoxia, mitochondrial membrane depolarisation occurs after

cell membrane depolarisation (Buckler and Vaughan-Jones 1998) would need to be considered in any refinement of the hypothesis.

4.2.1 AMPK

A consequence of hypoxia, however, is that mitochondrial function will be impaired and the cellular ADP/ATP ratio rises. The action of adenylate kinase brings about the conversion of two molecules of ADP to produce a molecule each of AMP and ATP, and thus elevates the AMP/ATP ratio as a square function of the ADP/ATP (Hardie and Hawley 2001). Cell function depends upon a ready supply of ATP, and cytosolic ATP levels are defended by the action of an energy sensor protein termed AMP-activated protein kinase (AMPK) that is activated by increases in the cellular AMP/ATP ratio, however this may be brought about (Hardie et al. 2006). AMP binds to AMPK and its activation initiates a number of energy-conserving mechanisms. AMPK is ubiquitously expressed in eukaryotic cells, including Type 1 cells of the carotid body (Evans et al. 2005), where it is localised to within just a few μm of the plasmalemmal membrane, where (it has been hypothesised) it acts to phosphorylate both maxi-K and TASK-like K channels to induce inactivation and cell depolarisation (Wyatt et al. 2007). These effects upon these specific O_2-sensitive ion channel conductances were observable with the AMPK activator, AICAR, as were effects both upon Type 1 cell intracellular Ca^{2+} concentration and, importantly, chemoafferent discharge. In all cases, the effects are reversible. The AMPK antagonist, compound C, attenuates the effects of both AICAR and hypoxia, the full effect in the whole organ taking some time to occur and often being irreversible. The attenuation in hypoxia is not complete, however, in the short term and this suggests that other hypoxia-sensing mechanisms may also be operating. Notwithstanding these points, AMPK appears to provide a unification of both mitochondrial and membrane hypotheses of chemotransduction, and future experiments should be aimed at demonstrating how it may be involved in establishing cell specific O_2 sensitivity as well as its role in carotid body plasticity.

4.2.2 Haem Oxygenase

Attractive as the AMPK hypothesis is, it is not the only hypothesis that is presently receiving attention. An alternative, non-mitochondrial hypothesis for hypoxia chemotransduction centres around a role for the gas transmitter CO and its activation of BK channels in rat carotid body Type I cells (Williams et al. 2004; Kemp 2005). This proposal arose from a proteomic approach that found a close, physical association between the constitutively expressed form of the O_2-dependent enzyme, haem-oxygensase-2 (HO-2), and the maxi K channel protein, and suggests that the product of HO-2, carbon monoxide (CO), is essential for maxi K channel activation. Apparently contradicting this, is the finding that transgenic mice lacking HO-2 did not have any alteration in their hypoxia sensitivity (Ortega-Saenz et al. 2006) and,

although a defence has been put forward based on the finding that maxi K channels may not be functional in mice, this may be a strain difference effect as up to 70% of the O_2-sensitive K current in some strains of mice may be iberiotoxin, and therefore maxi-K-dependent (He et al. 2005). Additionally, the available biochemical evidence does not support an appropriate P_{50} for HO-2 to operate as a tissue sensor of hypoxia (Ward 2008). Nevertheless, previous studies had already shown that CO, produced by HO-2 in the presence of its substrates, O_2, haem and NADPH, could be an inhibitory modulator of chemoreceptor excitability (Prabhakar et al. 1995) and even earlier studies had shown that CO could activate both Kv and maxi K channels (Lopez-Lopez and Gonzalez 1992; Riesco-Fagundo et al. 2001). With a lack of O_2, CO production would decrease and maxi K channels would inactivate, inducing depolarisation. CO is a remarkably inert molecule, reacting principally with metallo-proteins commonly found in haem groups. Gonzalez and colleagues did not therefore propose that CO had any direct action of the K channel, as they could not discern any motifs present on the ion channels for CO binding, and instead proposed that CO acted upon an independent sensor protein which then interacted with regulatory subunits of the K channel, or indeed that HO-2 may have acted via an antioxidant action. Kemp and co-workers have, however, subsequently demonstrated that rather than binding to histidine residues as previously supposed, CO binds instead to a structural motif in the C terminus of the maxi-K channel where it acts to gate the channel in a redox-independent manner (Williams et al. 2008). Gonzalez and colleagues (Gonzalez et al. 2007) have raised the question of why, if a binding site for CO exists on K channels, might not there be one simply for O_2, thus removing the need for HO-2 and CO completely in O_2 sensing? This challenging question has yet to be answered fully.

5 Conclusions

The past 20 years have seen an explosion of information regarding mammalian peripheral chemoreceptor physiology in general, and carotid body physiology in particular. These studies have informed numerous other studies regarding O_2 sensing in many other tissue types, including the pulmonary artery smooth muscle cells and central neurones, although an integrated mechanism that links all of these tissue types through a common transduction pathway appears unlikely at present. We do appear, however, to have now a greater understanding of the variety of stimuli that can be sensed by peripheral chemoreceptors and a detailed explanation of how this sensing may occur, at least for hypoxia. Although some aspects of the transduction mechanism appear to be generally accepted, it is worth remembering that many discrepancies still exist that cannot simply be due to species and/or strain differences. Some of these appear to be due to differences in the various models currently being utilised to study chemoreception — with a wide range of expression systems, single-cell, multiple-cell and whole-organ studies in vitro lying alongside studies performed in vivo. For a tissue whose metabolism appears key to its function, this is

perhaps not surprising, and it will be of interest to determine the basis of these differences as they may help shed light on the important transduction processes. It could be that a number of mechanisms are essential to generate the full response over the wide range of stimulus intensities that this receptor operates at, or it may be that a degree of redundancy is to be expected for such a vital player in cardiorespiratory homeostasis. Whatever the answer, it is hoped that the development of less reduced models of chemoreception, including more integrative approaches, will generate the answers to the key questions that should now be asked.

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Central Chemosensitivity in Mammals

L.K. Hartzler and R.W. Putnam

Abstract More than a century has passed since the beginning of direct experimentation on control of ventilation, and the ensuing years have brought considerable insight into the mechanisms of this control. Much of what we know about cellular chemosensitivity in mammals comes from a limited number of species; yet, given the diversity of circumstances in which mammals exist, their potential has been greatly underused. Here we review some of the environmental situations for plasticity of mammalian central chemosensitivity and function of chemosensors. "Normal" breathing patterns change during sleep, hibernation, and exercise, and central chemosensitivity must be altered during acclimation or adaptation to altitude, burrowing, or disease states. Where central chemosensitive cells are located, and what qualifies a cell as chemosensitive, is currently debated. The chemosensitivity of these cells changes over development, and the signaling mechanisms of these cells vary between chemosensitive regions, probably accounting for plasticity in response to environmental perturbations.

1 Introduction

More than a century has passed since the beginning of direct experimentation on the control of ventilation, and the ensuing years have brought considerable insight into the mechanisms of this control. In this chapter we highlight a few of these ideas and present directions in which central chemosensitivity in mammals are going. Haldane's experiments at the turn of the century demonstrated that, in humans, the ventilatory control center is exquisitely sensitive to changes in $[CO_2]$ while a relatively larger change in $[O_2]$ is required to get an equivalent increase in ventilation

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(Haldane and Priestley 1905). This led to investigations into peripheral and central chemosensitivity (where and how blood gases are sensed) and how breathing is altered accordingly. Here we focus on central chemosensitivity. While central chemosensitivity has long been associated with terrestrial animals, its presence in aquatic air-breathers suggests that it is an ancient ventilatory control mechanism (Remmers et al. 2001; Wilson et al. 2000).

There are many similarities in control of breathing between all of the vertebrate classes, but mammals have a few unique characteristics. During normal metabolic states, most mammals have a regular breathing pattern (no periods of apnea) at rest, alter their ventilation with changes in $[CO_2]$ and not $[O_2]$ (until oxygen levels are quite depressed), and keep blood gas concentrations within a narrow range compared with ectothermic vertebrates (for further information, see Chaps. 3 and 5). A number of conditions observed in mammals result in altered breathing patterns, possibly due in part to changes in central chemosensitivity. For example: some groups of mammals undergo frequent bouts of torpor, and, more extensively, periods of hibernation; fossorial life histories result in altered CO_2 sensitivity; some mammals dive for extended periods of time, accumulating CO_2, thus altering central CO_2 sensitivity; acclimatization or adaptation to high altitude leads to changes in blood pH and O_2 sensitivity; and, in humans at least, a number of disease states result in or from altered central chemosensitivity. Additionally, central control of breathing rapidly responds to changes in metabolic state (e.g., sleep vs wakefulness, onset of exercise, anesthesia). In the following sections of this chapter, we discuss how the organismal response of central chemosensitivity is altered during these situations, where and how the chemical signals are sensed, and some of the ways in which this information is being put to use. Due to space limitations and given our broad approach, not all of these topics can be explored in detail, so a number of excellent reviews will be recommended where appropriate.

2 Organismal Chemosensitive Responses to Environmental or Metabolic Stimuli

Being endothermic, most mammals maintain constant, high body temperatures, but, during periods of environmental stresses, some mammals accommodate by entering periods of torpor or, more extensively, hibernation. The molecular bases for entrance into hibernation and subsequent arousals are not yet known (Carey et al. 2003), but it is a regulated metabolic depression that protects hibernators from cell death (Boutilier 2001). During hibernation, the rate of ventilation slows and becomes episodic (Nicol and Andersen 2003), and is accompanied by a decrease in the respiratory exchange rate, possibly by a retention of carbon dioxide in body fluids (Malatesta et al. 2007). Even during euthermia, the low metabolism of monotremes is matched by lower and variable rates of ventilation (Nicol and Andersen 2003).

Mammals that undergo hypometabolic states (torpor) switch from eupnea to intermittent breathing (Malan 1982; Milsom 1992). Torpid bats switch between

these two breathing strategies to optimize ventilatory requirements to meet metabolic demand (Szewczak 1997). Intermittent breathing has the consequence of high fluctuations in arterial pH [up to 0.24 pH unit in *Eptesicus fuscus* (Szewczak and Jackson 1992b)] during the ventilatory cycle. Relatively large changes in pH and periods of apnea are characteristic of ectothermic vertebrates, so it appears that when mammals undergo periods of torpor, even transient periods, they express "ectothermic breathing patterns" rather than maintaining eupnea (Bickler 1984). During torpor, the cost of ventilation is about 5–10% of all metabolic costs, so optimizing the ventilatory strategy becomes important (Szewczak 1997). With such large fluctuations in arterial pH and Pco_2, it appears that chemosensitivity is decreased, yet *E. fuscus* (Szewczak and Jackson 1992a) and *Paragnathus longimembris* (Withers 1977) consistently respond to hypercapnia and hypoxia.

While there is variability between species in breathing pattern during hibernation, the same chemical signals contribute to changes in those patterns. A strong hypoxic ventilatory response and a blunted hypercapnic ventilatory response during euthermia suggest that arterial Po_2 predominates over arterial Pco_2 and pH as a ventilatory control mechanism in echidnas (Parer and Hodson 1974), ground squirrels (McArthur and Milsom 1991), and bats (Szewczak and Jackson 1992b). However, during hibernation this hypoxic ventilatory response is greatly reduced, and the hypercapnic ventilatory response is elevated (McArthur and Milsom 1991; Nicol and Andersen 2003; Szewczak and Jackson 1992b). While changes in arterial Po_2, Pco_2, and pH all contribute to changes in ventilation during entrance into hibernation in *Spermophilus spp.* (McArthur and Milsom 1991), arterial Pco_2 and pH are primarily responsible for changes in ventilation during hibernation in ground squirrels (McArthur and Milsom 1991) and hedgehogs (Tahti 1975; Tahti and Soivio 1975). Since many hibernators are also fossorial, they typically exhibit a reduced hypercapnic ventilatory response (Milsom et al. 1993). This reduced hypercapnic ventilatory response in burrowing mammals (Boggs et al. 1984) is a probable adaptation to the chronic hypercapnic (and hypoxic) environments of burrows (Milsom 1998). Ventilatory and acid–base adaptations vary widely among fossorial mammals (Birchard et al. 1984; Lechner 1976; Walker et al. 1985).

While hypothermia is normal in animals that regularly undergo periods of torpor, for those animals that cannot enter a torpid state, progressive hypothermia eventually results in death, as opposed to the long apneas typical during hibernation (Rosenhain and Penrod 1951). Neonatal animals retain a greater tolerance to hypothermia than do adults (Mortola 2001). This loss of ventilatory control during hypothermia appears to occur at the network level rather than within the chemosensitive neurons (Mellen et al. 2002) and has interesting implications for sleep-disordered breathing in humans. Even in hibernators, hypothermia affects neural connections (von der Ohe et al. 2007) by dissociating synaptic proteins from synapses. Only very recently have the unique characteristics of control of ventilation during hibernation begun to be examined at the neuronal level (Drew et al. 2007; Ruediger et al. 2007).

One characteristic shared by many hibernators is a fossorial lifestyle (Milsom 1992). This life history trait can lead to burrowing mammals being periodically

exposed to high CO_2 environments without a depressed metabolic state. These mammals retreat to their burrows to escape predation, raise their young, or minimize fluctuations in environmental temperature. While some burrows are well-ventilated (Vogel et al. 1973), others have relatively high CO_2 levels. As inspired CO_2 triggers changes in ventilation, the properties of chemosensitive neurons in fossorial mammals should give us valuable insight into adaptation to high CO_2 in neuronal areas (Ar et al. 1977; Arieli and Ar 1979; Boggs et al. 1998; Chapman and Bennett 1975; Darden 1972). Under fossorial conditions they are no longer as CO_2-sensitive as non-fossorial mammals, suggesting a change in either cellular chemosensitive signaling pathways and/or chemosensitive neuronal networks.

Increases in CO_2 production during exercise are exquisitely matched by concomitant increases in ventilation (see Chap. 20 for extensive discussion of exercise). There are examples, however, of high metabolic demand without the usual capacity for normal gas exchange to accommodate this high demand, such as the extended dive times in several marine mammal species (Kohin et al. 1999; Parkos and Wahrenbrock 1987). No external gas exchange during dives is a source of periodically elevated arterial Pco_2 levels. Weddell seals, elephant seals, and other amphibious species actively forage for long periods of time: a metabolically expensive activity resulting in relatively large swings in blood oxygen and carbon dioxide levels (for example, venous Po_2 as low as 2 Torr has been reported in elephant seals (Meir et al. 2008)). Deep-ocean dwelling cetaceans must experience similar swings in blood gases as well as the additional factor of experiencing hyperbaric pressures. Even in humans, extreme breath-hold divers show a blunted hypercapnic ventilatory response (Eynan et al. 2005; Ferretti 2001; Masuda et al. 1982). Interestingly, some animals' ventilatory mechanics are coordinated with their locomotion (Boggs 2002), and the input from chemical stimulation to breathe is not strong enough to alter ventilation during locomotion (Gillespie et al. 1991). It is clear that there is a complex interplay among metabolism, temperature regulation, and environmental conditions which alters ventilatory control, and these interplays are poorly understood.

3 Altered Chemosensitivity in Disease

Humans are one of the mammalian species that keep blood gases within a very narrow range; however, there are a number of circumstances under which this is not the case. Diseases such as chronic obstructive pulmonary disease, sleep apnea, idiopathic hyperventilation, and chronic heart failure all result in blood gas values (especially CO_2) at a new set point (Dunroy et al. 2003; Jack et al. 2004; Kara et al. 2003). Additionally, individuals who are mechanically ventilated after respiratory failure are often chronically hypercapnic (as a result of low tidal volumes to prevent over-inflation of the lung), which may increase time to wean from a ventilator (Georgopoulos 2006; Laffey and Kavanagh 2006). While humans normally do not experience periods of torpor, artificially induced hypothermia during open thoracic surgery is becoming a common practice; however, whether to keep arterial blood

at pH 7.4 and allow Pco_2 to rise, or to maintain arterial Pco_2 at 40 Torr and allow pH to rise, has not been determined (Kofstad 1996). Further complications during surgery include the effects of anesthetic agents on ventilatory control (Dahan and Teppema 2003). Interestingly, a number of respiratory control disorders appear to be related to sleep states, so this may be a critical time during which the regions of chemosensitive neurons associated with the sleep state swamp the input from other regions (supporting the distributed chemosensitive regions model discussed below). Once again, understanding the relationships between ventilatory control during sleep states, changes in metabolic state (such as torpor), and temperature are important.

4 Development of the Organismal Chemosensitive Response

At birth the respiratory system of mammals must respond to very different ambient gases. Having developed in a relatively hypoxic environment in utero, neonates are essentially exposed to an initially hyperoxic environment. Because there is such a range of developmental stages at which birth occurs (e.g., highly altricial marsupials and precocial ungulates), there is great potential to examine the characteristics of ventilatory control centers and central chemosensitivity during the transition from the uterus (or egg in the case of monotremes) to ambient air (Bavis et al. 2006). The presence of fetal breathing movements in utero increases over the gestational period (Berger et al. 1986), and these movements also increase in response to hypercapnia and decrease in response to hypocapnia (Jansen et al. 1982; Van Weering et al. 1979), indicating fetal chemosensitivity to blood gas levels. The mechanisms by which irregular fetal breathing movements transition to well-regulated breathing at birth remain unknown (Mortola 2001). It is clear that neural control of ventilation is not as mature in pre-term infants as it is in those full-term (Darnall et al. 2006), and CO_2 chemosensitivity continues to change during the developmental period (Carroll 2003; Davis et al. 2006; Stunden et al. 2001).

5 Central Chemosensitivity

Much of what we know about cellular chemosensitivity in mammals comes from a limited number of species; yet, given the diversity of circumstances in which mammals exist, their potential has been greatly underused. Both peripheral (see Chap. 18) and central chemoreceptors are sensitive to changes in CO_2; with peripheral chemoreceptors removed, the ventilatory responses to CO_2 are reduced, indicating the influence of peripheral chemoreceptors on central integration of blood gas stimuli. As central CO_2 sensitivity returns after the removal of peripheral chemoreceptor input, it is clear that there is plasticity in the central chemoreceptors

themselves (Dahan et al. 2007). Where blood gases are sensed centrally is not clear, although there are a number of putative chemosensitive regions (Feldman et al. 2003; Hodges et al. 2004; Nattie and Li 2006, 2008); the contributions of numerous sites is currently under debate (Guyenet et al. 2008; Nattie and Li 2008). Criteria for areas of central chemosensitivity include an increase in ventilation during focal acidification, a change in ventilation with the inhibition or lesion of neurons, CO_2-responsive neurons in in vitro preparations, and/or c-fos expression after exposure to CO_2 (Putnam et al. 2004). Using a combination of these criteria, regions of putative central chemosensitivity have been identified within the ventral–lateral medullary surface (Akilesh et al. 1997; Biancardi et al. 2007; Guyenet et al. 2005; Leiter et al. 2003; Mulkey et al. 2004, 2007; Paterson et al. 2006; Ritucci et al. 2005b), locus coeruleus (A6 neurons) (Ballantyne et al. 2004; Biancardi et al. 2007; Filosa et al. 2002; Hartzler et al. 2008a; Li and Nattie 2006; Nichols et al. 2008; Ritucci et al. 1998), solitary tract nucleus (Dean et al. 1990a, 1997; Kline et al. 2002; Mulkey et al. 2003; Nichols et al. 2008; Ritucci et al. 1998), medullary raphé (Bernard et al. 1996; Bouyer et al. 2004; Bradley et al. 2002; Cao and Song 2006; Hodges et al. 2004; Paterson et al. 2006; Richerson et al. 2001), preBötzinger complex (Feldman et al. 2003; Neubauer and Sunderram 2004), and the fastigal nucleus (Martino et al. 2007).

Considerable progress has been made in the study of central chemosensitivity over the past 20 years and several recent reviews highlight various aspects of the subject (Feldman et al. 2003; Nattie and Li 2006; Putnam et al. 2004). Despite the progress, several significant questions remain which are major foci of research. We will highlight four questions. One important question is why there are so many brainstem sites that are chemosensitive and what might be the role of each of these areas in chemoreception. In addition, it is not clear what the developmental pattern of central chemoreception is in altricial species such as humans and rats, and whether there might be a "neonatal" form of chemoreception that is distinct from "adult" chemoreception. New data are also suggesting a re-interpretation of the role of central vs peripheral chemoreception in the ventilatory response to hypercapnia. Finally, the cellular mechanism(s) involved in transducing a change in CO_2/H^+ into altered neuronal firing rate are not fully elucidated.

6 Multiple Chemosensitive Regions in the Brainstem?

Currently, there are two main theories for the basis for central chemosensitivity in mammals: the distributed chemoreception theory and the specialized chemoreception theory (Guyenet et al. 2008; Nattie and Li 2008). For years, the sensing of elevated CO_2 was believed to reside in a few specialized areas in the ventral medulla (Loeschcke 1982; Mitchell et al. 1963). This view came to be challenged by two different approaches that suggested that CO_2 sensing is more distributed, residing in multiple brainstem sites. The first approach was studying individual chemosensitive neurons in a dorsal medullary site, the nucleus tractus solitarius (NTS) (Dean et al.

1989; Miles 1983). In these studies, between a third and two-thirds of the neurons within the NTS were found to respond reversibly, with an increased firing rate in response to acute exposures to hypercapnia. It was further shown that this neuronal response was intrinsic, because the increased firing rate in response to hypercapnia was still seen in synaptic block medium (Dean et al. 1990b). Significantly, the hypercapnia-activated response of NTS neurons occurred even in brainstem slices in which the ventral medulla had been removed, eliminating any possibility that the response resulted from activation of the ventral medulla (Dean et al. 1990b). These findings suggested that sites other than the ventral medullary surface could be involved in central chemosensitivity.

The other approach that supported the suggestion of multiple sites of central chemosensitivity was the use of focal acidosis in restricted brainstem sites within the intact animal. Coates et al. (1993) found that the output of the phrenic nerve, the major nerve supplying the diaphragm, increased when various brainstem sites, including the retrotrapezoid nucleus (RTN), the caudal NTS, and the locus coeruleus (LC), were exposed to focal acidosis. Since this initial work, additional sites have been reported that increase ventilation (or phrenic nerve activity) when focally acidified, including the medullary raphé (Bernard et al. 1996; Hodges et al. 2004; Nattie and Li 2001), the pre-Bötzinger complex (PBC) (Solomon et al. 2000) and the fastigial nucleus of the cerebellum (Xu et al. 2001). Interestingly, focal acidosis of each of these areas raised ventilation by only a fraction (usually 10–30%) of the increase in ventilation induced by the animal breathing hypercapnia, strongly indicating that no single area was sufficient to give a full central chemoreceptive response to increased inspired CO_2 (Nattie 2001). It is also noteworthy that most of the areas that result in increased ventilation when focally acidified contain neurons that are reversibly activated by elevated CO_2/H^+, including in the RTN (Mulkey et al. 2004; Ritucci et al. 2005b), the NTS (Dean et al. 1989, 1990a; Miles 1983), the LC (Filosa et al. 2002; Filosa and Putnam 2003; Oyamada et al. 1998; Ritucci et al. 2005a) and the medullary raphé (Richerson et al. 2001; Wang et al. 2001). These studies have led to the theory that the sensing of CO_2 by the central nervous system arises from numerous chemosensitive regions distributed throughout the brainstem, and has thus been termed the distributed chemoreception theory (Guyenet et al. 2008; Li et al. 2008; Nattie and Li 2006, 2008).

The distributed chemoreception theory has further been strengthened by studies of cell-specific lesions in various putative chemoreceptive regions. The concept of such studies is to eliminate specific cell types (e.g. serotonin- or catecholamine-expressing neurons) and determine the effect of this on central chemosensitivity. Many of these studies employed the approach of targeting neurons that produce specific neurotransmitters, so an added benefit is that insight has been gained into the neurotransmitters involved in central chemoreception as well. The major findings of such work have recently been briefly reviewed (Nattie and Li 2008). For instance, injection of ibotenic acid (a toxin for excitatory amino acids) into the RTN resulted in a loss of 35% of the neurons and a decrease of 39% in the CO_2/H^+-induced increase in ventilation (Akilesh et al. 1997), suggesting that glutamatergic RTN neurons are involved in central chemoreception. This is further indicated by

inhibiting RTN neurons with focal injection into the RTN of the GABA-agonist muscimol, which results in a 24% reduction of the response of ventilation to inspired CO_2 (Li et al. 2006). Another group of neurons that have been indicated in central chemoreception are serotonergic neurons of the medullary raphé. In mice in which serotonergic neurons have been knocked out the ventilatory response to inspired hypercapnia is decreased by 50% (Hodges et al. 2008). Using a different approach, medullary serotonergic neurons were killed by attaching the cytotoxic agent saporin to an antibody to the serotonin receptor. In such animals, ablation of medullary serotonergic neurons reduced the hypercapnic ventilatory response by about 17% in both awake and asleep rats (Nattie et al. 2004). Similar injections in the raphé magnus (which contains about 20% serotonergic neurons) reduced the hypercapnic ventilatory response by as much as 62% (Dias et al. 2007). Interestingly, lesions of serotonergic neurons from the caudal raphé had no direct effect on the hypercapnic ventilatory response but reduced the response of RTN neurons (Li et al. 2006), suggesting that serotonergic neurons from the rostral regions of the medulla are involved in central chemoreception, but those from the caudal regions serve to modulate the response of RTN neurons to hypercapnia (Nattie and Li 2008). Finally, catecholaminergic neurons from the pons also have been shown to play a significant role in central chemoreception. These neurons can be lesioned by complexing saporin to an antibody to an enzyme involved in catecholamine synthesis (dopamine-β-hydroxylase), which results in a reduction of the hypercapnic ventilatory response by 28% when injected into the cisterna magna (Li and Nattie 2006). The effect is even more dramatic when the lesion is focused on the LC, decreasing the hypercapnic ventilatory response by 64% (Biancardi et al. 2007).

Taken together, these ablation experiments show that no one area is completely responsible for the hypercapnic ventilatory response and support the idea that central chemoreception is a distributed phenomenon. This concept is further supported by the fact that many of these putative chemosensitive neurons contain receptors for substance P (NK1 receptors), including neurons from the LC (Chen et al. 2000), the RTN (Stornetta et al. 2006), and broadly throughout the ventral medulla (Nattie and Li 2002, 2006). Focal lesions of NK1-expressing neurons reduce the hypercapnic ventilatory response by at most 30% (Nattie and Li 2002, 2004), but broader lesions throughout the ventral medulla cause a much larger reduction in the hypercapnic ventilatory response, by as much as 65% (Nattie and Li 2006). Thus, there is considerable evidence supporting the hypothesis that central chemoreception arises from a distributed network of chemosensitive regions.

An interesting suggestion to arise from the distributed chemoreception theory is that chemoreception arose in a hierarchical fashion (Nattie 2001). Different sites involved in the control of ventilation may have arisen at various landmark stages in phylogeny. Such landmarks may include emergence from an aqueous environment to become air-breathing, the development of a high constant body temperature, the need for sleep, and the evolving of different stages of sleep. In such a hierarchical system, different chemoreceptive sites may not so much reflect duplication of an important physiological process as the accretion of new sites involved in a different aspect of ventilatory control. Such a hierarchical concept is entirely consistent

with the observation that chemoreception is clearly state-dependent, varying with states such as being awake, anesthetized or asleep. The response of chemosensitive neurons from various regions is also state-dependent. For instance, focal acidosis of the RTN increased the hypercapnic ventilatory response only in awake, but not sleeping, rats, and the effect was largely due to increased tidal volume (Nattie 2000). In contrast, focal acidosis in the medullary raphé increased ventilation only during sleep and the effect was entirely due to an increased respiratory frequency (Nattie 2000). Loss of catecholaminergic neurons reduced the hypercapnic ventilatory response during both wakefulness and sleep (Li and Nattie 2006). In fact, it is intuitively obvious that central chemoreception is state-dependent, given the number of disordered breathing states associated with sleep (e.g. congenital central hypoventilation syndrome, sleep apnea, sudden infant death syndrome) that are not apparent in the awake animal (Feldman et al. 2003). The state dependence of central chemoreception is most consistent with central chemoreception being a distributed property.

In contrast to the distributed chemoreception theory, it has very recently been argued that chemoreception for the purpose of controlling ventilation is largely (if not exclusively) the function of specialized neurons restricted to a specialized region, probably within the RTN. This theory has been termed the specialized chemoreceptor theory (Guyenet et al. 2008). It is noteworthy that this theory is similar to the original theory that chemoreception resides in the ventral medullary surface. In fact, the putative specialized chemoreceptive region within the RTN (Mulkey et al. 2004) markedly overlaps one of the originally proposed ventral chemoreceptive regions, the M region (Loeschcke 1982), although, while focusing on this region as *the* central chemoreceptor, no further comment is made about the other two classical chemosensitive regions on the ventral medulla. Evidence, as discussed above, suggests that RTN neurons play a role in central chemoreception. Further, glutamatergic neurons within a specialized region of the RTN have been shown to be highly and intrinsically responsive to elevated CO_2 (Mulkey et al. 2004). Also, in knock out mice lacking the transcription factor Phox2b, there is a marked deficit of RTN neurons and a complete loss of the ventilatory response to inspired CO_2 (Dubreuil et al. 2008). Finally, lesions of the medullary raphé and the RTN decrease the ventilatory response to inspired CO_2 by half (Li et al. 2006). Thus, there appears to be an important role for RTN neurons in central chemosensitivity.

Evidence for a significant role of RTN neurons in central chemoreception does not negate the likelihood that several brainstem regions contribute to central chemoreception. Many of the arguments against neurons from other regions playing a role in central chemoreception are arguments against being able to claim that any neuron that responds to acidification must be a central chemoreceptor (Guyenet et al. 2008). The claim is made that many neurons may not act the same in in vitro preparations as they do in vivo. While this is undoubtedly so, measurements of the responses of individual or populations of neurons in vivo must of necessity involve studies of anesthetized animals and are thus highly modified preparations in their own right. Further, the vast majority of studies on the chemosensitive response of individual neurons in reduced preparations are done studying neurons in areas that have already

been shown to be involved in central chemoreception based on focal acidosis or neuronal ablation experiments (Putnam et al. 2004). Guyenet et al. (2008) do not address this point in any substantive way other than to suggest that indwelling cannulae used for focal acidosis can alter the local circuit. However, this argument does not account for the lack of a response when control solutions are infused through the cannulae or when non-chemosensitive regions are being studied. Finally, while studies of the Phox2b knockout mice are interesting, there are Phox2b-containing neurons in many chemosensitive areas and, although the Phox2b neurons from these areas do not disappear as they do from the RTN, there are no electrophysiological studies of neurons from these regions to determine whether they are altered in knockout animals as well. Also, as pointed out by Nattie and Li (2008), there may be other neurons affected by Phox2b knockout, especially peripheral chemoreceptors, that account for some of the loss of chemoreception. In summary, while it is likely that RTN neurons play a role in some aspects of central chemoreception, it appears most reasonable to assume that central chemoreception arises from a distributed network of chemosensitive neurons within numerous regions of the brainstem (and possibly the cerebellum).

7 Development of Central Chemoreception

The development of physiological processes is often assumed to be an essentially linear process, progressing from a poorly functioning state at birth, through early development, until eventually reaching the adult state. An alternative view of development is that there are distinct physiological states, e.g., a neonatal state vs the adult state, and that physiological processes and their regulation are adapted for each state. Both of these views have been posited for the development of central chemoreception.

A linear development of chemosensitivity, especially within medullary raphé neurons, has been suggested (Richerson et al. 2001; Wang and Richerson 1999). Medullary raphé neurons in slices and especially in culture showed increased numbers of chemosensitive neurons and a larger response to high CO_2/H^+ with age (or days in culture) (Richerson et al. 2001; Wang and Richerson 1999), suggesting that central chemoreception increases with age in neonatal rats. A variation on this pattern has also been described, with the ventilatory response to inspired CO_2 remaining low from P0 to about P14, and then increasing rapidly after P15 to adult levels (Davis et al. 2006). The timing of this increase in the hypercapnic ventilatory response is interesting in that shortly before this time (around P11–P12) there are a number of transient changes in various neurotransmitter systems in neurons from many different medullary chemosensitive regions (Wong-Riley and Liu 2005). This pattern of development raises the possibility that there is a critical signal generated near the end of the second week of life in rats that causes a rapid increase in the hypercapnic ventilatory response.

A different pattern of development of chemosensitivity in neonatal rats has been suggested. Stunden et al. (2001) found that the hypercapnic ventilatory response to inspired CO_2 was fairly large shortly after birth (P0–P1) and decreased after that time to a point with virtually no ventilatory response to inspired CO_2 from about P6 to P10. After P10, the hypercapnic ventilatory response increased again to adult levels. This latter increase is similar in timing to that described by Davis et al. (2006) and shows general agreement that there is indeed an increase in the hypercapnic ventilatory response near the end of the second week of life in rats. The difference is in the large hypercapnic ventilatory response early in development which was seen by Stunden et al. (2001) but not by Davis et al. (2006). The latter authors attributed the difference to the need to express increased ventilation as a percentage change without normalizing for body weight, but this cannot account for the difference since Stunden et al. (2001) also express increased ventilation with hypercapnia as a percentage increase (Fig. 10 in Stunden et al. 2001). Further, this early increase in ventilation with inspired hypercapnia is due largely to an increase in respiratory frequency (unpublished observation). A similar response of hypercapnia-induced increase in respiratory frequency in young neonatal rats, but not older neonates, was also observed by Wickström et al. (2002). These authors concluded, like us, that the hypercapnic ventilatory response decreased during the first week of life in rats but increased again thereafter, the increase being largely due to increased tidal volume with hypercapnia.

This triphasic pattern of ventilatory response to hypercapnia (Putnam et al. 2005; Stunden et al. 2001), with a large response early in development, a loss of response near the end of the first week of life, and an increase again after about P10, has prompted us to hypothesize that in rats there are two forms of central chemoreception, a "neonatal" form and an "adult" form. Since rats are born highly altricial, without the ability for locomotion or temperature regulation, we would presume that neonatal chemoreception would be relatively simple, without the need for subtle responses such as exercise hyperpnea. Two recent findings have suggested a possible mechanism for neonatal chemoreception. In our studies of neurons from the LC, we find that these neurons are highly intrinsically chemosensitive during early development (P1–P10) and this response decreases substantially thereafter (Hartzler et al. 2007). Interestingly, a similar response has been reported for adrenal chromaffin cells (Muñoz-Cabello et al. 2005). In rats aged P1–P10, adrenal chromaffin cells respond to hypercapnia by releasing catecholamines, but lose this response to CO_2 after P10. Given that LC neurons are the major catecholaminergic brain region, we suggest that neonatal chemoreception involves hypercapnia-induced catecholamine release both systemically and within the brain. Such a response to hypercapnia would result in increased ventilation in a basic "fight or flight" response. This would represent a very simplistic but powerful and effective way to stimulate breathing in response to elevated CO_2. We further propose that this system wanes during the first week of life to be replaced by a more nuanced respiratory control system in adults. Another attractive feature of this model is that it predicts a critical window where the respiratory response to hypercapnia would be very weak during the transition from neonatal to adult chemoreception. Such a vulnerable period is consistent

with the epidemiological findings with sudden infant death syndrome, where the peak incidences occur 2–5 months after birth, not immediately after birth as one might predict for a linear developmental model of central chemoreception (Leiter and Bohm 2007).

Finally, it is not yet clear what factor(s) is/are responsible for the development of central chemoreception. This issue is complicated by the distributed nature of the central chemosensory neurons (see above). We have good evidence that, at least based on in vitro measurements, LC neurons decrease their intrinsic chemosensitivity with age during early development (Hartzler et al. 2007), while intrinsic chemosensitivity in NTS neurons is nearly fully developed at birth (Conrad et al. 2004, 2005; Putnam et al. 2005). The chemosensitive response of RTN neurons also does not appear to change throughout early development (Ritucci et al. 2005b) while medullary raphé neurons seem to increase their chemosensitivity with development. While these findings could be put together in a complex pattern to account for the triphasic pattern (or the linear pattern) of development of chemoreception, it is most likely that development of this important physiological regulatory system involves a complex pattern of development that includes the entire network and not just the development of the cellular response to CO_2 in neurons from a given chemoreceptive region of the brainstem.

8 Central Vs Peripheral Chemoreception

Classically, it has been assumed that the peripheral chemoreceptors, especially the glomus cells from the carotid bodies, serve a primary role increasing ventilation in response to hypoxia, and that the ventilatory response to CO_2 is largely mediated by central chemoreceptors (Dempsey 2005). This assumption was based on studies in which the central nervous system alone saw an acid challenge (Fencl et al. 1966; Heeringa et al. 1979) or ventral medullary neurons were ablated (Nattie et al. 1988). The findings in studies such as these suggested that the carotid bodies did not contribute, or at most contributed about 20–30% to the hypercapnic ventilatory response. However, it has been known for years that the glomus cells of the carotid body respond to hypercapnia as well as to hypoxia (Fidone and Gonzalez 1986), and thus it would stand to reason that they should sense and respond to CO_2/H^+ changes in the blood. A recent elegant study has re-investigated the role of the carotid bodies in the hypercapnic ventilatory response (Smith et al. 2006). In this study, one carotid body was denervated and the other had an isolated blood flow whose composition could be independently controlled. In such a preparation, the animal could be exposed to hypercapnia, both centrally (by increasing end tidal CO_2) and at the carotid body (by equilibrating the perfusion solution with hypercapnia), or to hypercapnia alone either centrally or at the carotid body. These studies clearly showed that only about 60% of the steady state increase in ventilation due to hypercapnia was due to central chemoreception and that the carotid bodies accounted for about 40%, a substantial fraction. More noteworthy, however, was the fact that the

ventilatory response to hypercapnia was delayed by about 11 s with central-only exposure as compared to exposure to both central and carotid bodies (Smith et al. 2006). The first finding indicates that the carotid bodies cannot be ignored when considering the effects of inspired CO_2 on ventilation, and that they can contribute a substantial proportion of an animal's ventilatory response to hypercapnia. The second finding suggests that the carotid bodies most probably play the major and determining role in rapid, breath-to-breath changes in blood CO_2/H^+ (Dempsey 2005; Nattie 2006). Such rapid and transient changes are seen in pathological conditions such as sleep apnea, where short periods of apnea (lasting several seconds) result in systemic hypoxia and hypercapnia. Under such conditions, it is the carotid body response that should be most responsible for generating the respiratory drive and it is within the carotid body that pathological changes are most likely to be observed. As proposed by Nattie (2006), this suggests that central chemoreceptors respond to changes in brain interstitial fluid CO_2/H^+ and would thus be sensitive to longer-term changes in systemic CO_2/H^+, as well as responding to changes in cerebral blood flow and/or metabolism. This would make the distributed network of central chemoreceptors a de facto system for indirectly monitoring brain oxygen levels, a concept that adds extra weight to our need to understand the precise signaling pathways in these chemosensitive neurons.

9 Cellular Chemosensitive Signaling

As discussed above, it is clear that there are numerous regions within the brainstem that, when focally acidified, result in increased ventilation. It has further been shown that in many of these areas, including the RTN (Mulkey et al. 2004; Ritucci et al. 2005b), the NTS (Dean et al. 1989, 1990a; Miles 1983), the LC (Filosa et al. 2002; Oyamada et al. 1998; Ritucci et al. 2005a), and the medullary raphé (Richerson et al. 2001; Wang et al. 2001), there are neurons whose firing rate responds reversibly to hypercapnia associated with decreased solution pH (elevated CO_2/H^+). [When an animal breathes air with elevated CO_2 or retains metabolically produced CO_2 (i.e., due to compromised lung function such as with chronic obstructive pulmonary disease), blood P_{CO2} rises with a fall of pH, and this condition is referred to as hypercapnic acidosis. It is mimicked experimentally by equilibrating artificial cerebral spinal fluid (aCSF) with high levels of CO_2, resulting in decreased pH (elevated H^+).] In this section we want to address the issue of how a neuron can respond to a change in CO_2/H^+, that is, what is/are the cellular signal(s) and target(s) that result in a change in firing rate of certain neurons in response to hypercapnia? The proposed models and the vast majority of the work has focused on neurons whose firing rate increases in response to increased CO_2/H^+, although in many regions there are a small percentage of putative chemosensitive neurons that respond with a *decrease* in firing rate in response to hypercapnic acidotic solution (Putnam et al. 2004). There are currently no good models for the cellular signaling in CO_2/H^+-inhibited neurons, but study of this process should provide interesting and novel

insights into neuronal responses to altered CO_2/H^+. We will focus our discussion on neurons whose firing rate is activated by increased CO_2/H^+.

Preparations for studying chemosensitive neurons. A number of preparations have been employed for the study of central chemosensitive neurons. In addition to in vivo studies of chemosensitive neurons (Mulkey et al. 2004), our understanding of the responses of these neurons to hypercapnic acidosis has been greatly facilitated by using reduced preparations. Four such preparations have been employed: the working heart-brainstem preparation, brain slices, organotypic cultures, and cell cultures. Each has distinct advantages and disadvantages that we will discuss here.

A recent example of in vivo preparations being used to study chemosensitive neurons from the rat RTN is the work of Mulkey et al. (2004). These rats were anesthetized with halothane and paralyzed, being artificially ventilated. The occipital plate was removed to allow a recording electrode to penetrate the medulla through the cerebellum. Recordings were made on groups of cells with extracellular electrodes. This technique allows for the study of neurons in near-physiological conditions with intact network connections and is thus presumably the closest measure to physiological conditions of any preparation. In fact, the argument has recently been made that in vivo measurements are needed before any neuron can be claimed to be involved in central chemosensitivity (Guyenet et al. 2008). However, such measurements are not without drawbacks. First, the use of anesthesia is known to alter the ventilatory response to inspired CO_2 (Akilesh et al. 1997) and it is clear that ventilatory control is state-dependent, differing during wakefulness, sleep and under anesthesia (Nattie 2000). Further, the use of halothane is particularly problematic since it is known to activate TASK channels, many of which are highly sensitive to changes in pH (Bayliss et al. 2001), and thus have been proposed to be a potential target of chemosensitive signaling (Mulkey et al. 2007). Other concerns about the use of anesthetized whole-animal preparations have been discussed (Nattie and Li 2006).

A reduced preparation that maintains many of the advantages of in vivo studies is the working heart-brainstem (Paton 1996) preparation. In this preparation, a mouse or rat is bisected at the level of the diaphragm, decerebrated, skinned, and the front limbs removed. A portion of the skull is removed to reveal the brainstem (the cerebellum is removed for dorsal exposures). The descending aorta is cannulated to enable the preparation to be perfused with warmed aCSF using its intact circulatory system. In addition to being more naturally perfused, this preparation benefits from having substantial amounts of the network intact, including peripheral chemoreception and cardiorespiratory reflexes, although input from higher brain centers is certainly lost. Another major advantage of this preparation is that no anesthesia is required. While access is limited for neurons within deeper centers, neurons near the surface have been studied with electrophysiological techniques (Paton 1996) and fluorescence imaging has been employed to study calcium transients within individual neurons (Bradley et al. 2008). As with in vivo preparations, the working heart-brainstem preparation requires considerable surgical skill and manipulation but has been used with both rats and mice.

The most widely used preparation to study neuronal chemosensitivity and chemosensitive signaling is the brainstem slice (Putnam et al. 2004). These studies involve the use of thin (300–400 μm thick) transverse or horizontal slices of various brainstem regions. Slices allow excellent access to individual neurons from either neonates (Filosa et al. 2002; Ritucci et al. 2005a, b) or adults (Dean et al. 1989, 1990a; Nichols et al. 2007) and excellent control over the extracellular solution. Further, signaling within these neurons can often be studied using fluorescence imaging microscopy (Filosa et al. 2002; Ritucci et al. 1996, 1997, 2005b), adding to our ability to study cellular chemosensitive signaling mechanisms. Individual neurons within the slice can also be voltage-clamped, allowing for the study of potential ion channel targets of chemosensitive signaling (Mulkey et al. 2007). These preparations can often be studied for hours and are not usually exposed to anesthetics. Indeed, much of what we have learned about cellular chemosensitive signaling over the past 15 years has derived from the use of brainstem slices (Putnam et al. 2004).

The slice preparation, however, has significant limitations. While the local network is maintained somewhat intact, there is a substantial loss of the overall network, including inputs from higher centers and from brainstem regions farther removed than a few hundred microns. There are also superfusion limitations, and the administration of drugs can take some time to diffuse into the tissue to the neuron being studied, potentially altering the kinetics of the response being studied (Ritucci et al. 2005a). The diffusion issue has recently introduced another concern about the slice preparation. Traditionally, because of the thickness of the slice, superfusion solutions are equilibrated with 95% O_2 to avoid hypoxia in the core of the slice. However, it has been shown recently that this results in a considerable hyperoxia compared to normal brain tissue in an animal breathing room air (P_{O_2} of 10–35 Torr) (Dean et al. 2003; Mulkey et al. 2001). This hyperoxia occurs even in the core of the slice, where P_{O_2} can be as high as 300–500 Torr, depending on superfusion conditions (Mulkey et al. 2001; Potter et al. 2004). Further, hyperoxia has been shown to activate some brainstem neurons, increasing their firing rate, and this activation occurs preferentially in CO_2-sensitive neurons within the NTS (Mulkey et al. 2003). This activation may be related to the accumulation of reactive oxygen and/or nitrogen species in response to hyperoxia, which have been shown to activate chemosensitive NTS neurons (Lipton et al. 2001). In fact, recent findings indicate that slices exposed to 95% O_2 will rapidly accumulate reactive oxygen species and show greater cell death than slices maintained at 60% O_2 (D'Agostino et al. 2007). Thus, it is likely that the typical slice preparation is a highly activated preparation due to the use of hyperoxic conditions, and the actual response of central chemosensitive neurons to hypercapnia, as studied with brain slices, will require a re-investigation of the proper level of control O_2 to be used.

A variant of the slice experiment has been used by some laboratories (Wiemann and Bingmann 2001). These studies employ organotypic cultures, where a slice (300–400 μm) is placed into organ culture for several weeks. The slices tend to flatten to only about 100–200 μm thick and individual neurons at the edge of the organotypic culture are easy to visualize, facilitating impalement and imaging of these neurons. In addition, these neurons maintain associations with other neurons

and with glia, and remain sensitive to CO_2 (Wiemann and Bingmann 2001). The major disadvantage to the use of these slices is the concern about changes in neuronal properties and local network properties over time in culture, and the same concerns about the lack of a fully intact network that were mentioned above for acute slices.

Finally, central chemosensitive neurons have been studied in cell culture. Hypercapnia-stimulated neurons have been cultured from the ventrolateral medulla (Rigatto et al. 1994), the medullary raphé (Wang et al. 1998), and recently the LC (Johnson et al. 2008). These neurons generally need to be in culture for over a week before they develop a chemosensitive response and are often studied after 2–4 weeks of being in culture. These neurons give a robust and repeatable response to hypercapnia, which is often larger than their response in slices (compare Wang et al. 1998 with Mulkey et al. 2007 and Johnson et al. 2008 with Filosa et al. 2002). The culture preparation is ideal for studying chemosensitive signaling because the environment can be readily controlled and rapidly altered, and there is easy access to the neurons for electrophysiological or imaging studies. Further, these preparations should prove useful for genetic manipulation such as siRNA knock-down experiments which will allow for direct testing of chemosensitive signaling models. As always, there are concerns about the use of cultured cells. The main concern is whether the physiology of the cell has been altered by its prolonged period in culture. For instance, cultured neurons have been shown to alter their expression of pH-regulating transporters compared to the same neurons that are freshly dissociated (Raley-Susman et al. 1993). As mentioned above, the magnitude of the firing-rate response to hypercapnia seems to be higher for neurons from the same brainstem region when in culture compared to in-the-brainstem slice. Thus, while cell culture will undoubtedly prove effective in studying the cellular basis of chemosensitive signaling, the results from such experiments will have to be confirmed in brain slices, and ultimately in vivo, before we can be assured that the described mechanism is physiological or not.

Chemosensitive signaling. The working model for chemosensitive signaling has been that hypercapnia results in an influx of CO_2 into a chemosensitive neuron, resulting in a fall of intracellular pH (pH_i) (Lassen 1990; Putnam et al. 2004). This fall in pH_i is believed to inhibit a K^+ channel, resulting in neuronal depolarization and increased firing rate. A hypercapnia-induced maintained fall of pH_i has been observed in many chemosensitive neurons (Filosa et al. 2002; Putnam et al. 2004; Ritucci et al. 1997; Wiemann and Bingmann 2001). This hypercapnia-induced fall of pH_i is correlated, both kinetically and in magnitude, with the increased firing rate in LC neurons (Filosa et al. 2002), suggesting that the change of pH_i is an important intracellular signal in chemosensitive cells. However, the correlation of the change of pH_i and increased firing rate is not perfect and is very poor upon removal of hypercapnia (Filosa et al. 2002). Further, a poor correlation between hypercapnia-induced acidification and the increased firing rate is seen in RTN neurons (Ritucci et al. 2005b). These findings suggest that the firing rate response of a chemosensitive neuron to hypercapnia is determined by more than the fall of pH_i. This was directly demonstrated in experiments in which LC neuron pH_i was clamped at a constant value during exposure to hypercapnic acidosis. Under these conditions,

hypercapnia was still able to induce an increase in firing rate of LC neurons, clearly demonstrating that a fall of pH_i is not a necessary signal for chemosensitive signaling (Hartzler et al. 2008b). Interestingly, Hartzler et al. (2008b) were able to show that when LC neurons were exposed to hypercapnia with both pH_i and external pH (pH_o) constant, firing rate did not increase. These data suggest that LC neurons are responding to changes of pH, either pH_i or pH_o.

There are several other factors that may be involved in the chemosensitive response of neurons (Putnam et al. 2004). The firing rate response of LC neurons to hypercapnic acidosis has been shown to be reduced by half by the L-type Ca channel inhibitor nifedipine (Filosa and Putnam 2003), indicating a role for Ca channels and/or intracellular Ca in chemosensitive signaling. As mentioned above, the production of reactive oxygen species can activate some chemosensitive neurons, indicating that the redox state may also serve to modulate chemosensitivity. Other factors, which undoubtedly contribute to the neuronal chemosensitive response, include neurotransmitters, glial cells and carbonic anhydrase activity (see Putnam et al. 2004).

Just as it appears that more than simply changes of pH_i are involved in chemosensitive signaling, there is unlikely to be a single chemosensitive channel that is inhibited by hypercapnia. Several pH-sensitive ion channels could be candidate targets for chemosensitive signaling (Putnam et al. 2004). There is, in fact, evidence for the involvement of several ion channels in chemosensitive signaling, especially in LC neurons. In addition to the activation of L-type Ca channels by hypercapnic acidosis (Filosa and Putnam 2003), the K channel inhibitor 4 aminopyridine (4AP) partially inhibits the increased firing rate induced by hypercapnic acidosis in LC neurons (Martino and Putnam 2007). A similar effect is seen with another K channel inhibitor, tetraethyl ammonium (TEA), which reduces hypercapnia-induced depolarization of LC neurons (Filosa and Putnam 2003). Finally, decreased pH_o also appears to be involved in the chemosensitive response of LC neurons (Filosa and Putnam 2003; Hartzler et al. 2007), suggesting the involvement of TASK channels in chemosensitive signaling (Bayliss et al. 2001). Thus, the response of LC neurons to hypercapnia appears to involve numerous channels, including L-type Ca channels, TASK channels, and 4AP- and TEA-sensitive K channels.

The plethora of signals and ion channel targets involved in chemosensitive signaling lead us to propose a new model for chemosensitive signaling in LC neurons, the multiple factors model (Putnam et al. 2004). In this model, the LC neuronal response to hypercapnic acidosis is not envisioned to be the result solely of changes of pH_i inhibiting a single K channel. Rather, multiple signals (changes of pH_i and pH_o, opening of Ca channels and perhaps increased intracellular Ca) affect multiple ion channels (Ca channels, TASK channels and various K channels), and the final firing rate of an LC neuron in response to hypercapnia is the result of all of these effects.

Finally, since central chemosensitivity is a distributed property, we must ask whether multiple ion channels are involved in the response to hypercapnia of neurons from all chemosensitive areas. While we know less about the signaling processes in neurons from other chemosensitive regions, the answer appears to be

that multiple ion channels are not involved in the response of chemosensitive neurons from all regions. For instance, a single K channel inhibitor, 4AP, is sufficient to fully block the increased firing rate response to hypercapnia in NTS neurons (Dean et al. 1990a; Martino and Putnam 2007). In mice that have had TASK-1, TASK-3 or both channels knocked out, hypercapnia no longer activates medullary raphé neurons (Mulkey et al. 2007). In these same knockout mice, however, the response of RTN neurons to hypercapnia is unaffected (Mulkey et al. 2007). Thus, in the NTS, medullary raphé and perhaps the RTN, a single ion channel seems to be responsible for chemosensitive signaling, although it appears to be a different ion channel in each area. This latter observation suggests that chemosensitivity arose independently in the neurons from each region and that the distributed network of central chemosensitivity had a polyphyletic origin.

10 Summary

Understanding of the mechanisms of central chemosensitivity has been greatly expanded (Remmers 2005), yet there are several pertinent questions that have not been answered (Gaultier and Gallego 2005). Why are there multiple chemosensitive sites in the brainstem? How do these sites interact with one another? Do any of the sites have more or less control under varying circumstances? Much of our understanding of central chemosensitivity comes from work done in typical model organisms, yet one of the tools to address these questions is the use of the Krogh principle (Krogh 1929) where for any physiological problem there is/are some animal(s) best suited to address that problem. In this chapter we have described several situations (e.g. hibernation, torpor, fossorial habitats, diving) where a number of mammalian species undergo extreme changes in their ventilation as part of their normal life history. To date, the potential for gathering information regarding central chemosensitivity in these organisms has not been exploited.

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Human Exercise Physiology

Stefanos Volianitis and Niels H. Secher

Abstract The oxygen transport system in the human body is described from atmospheric air to working skeletal muscles. It is illustrated that the pulmonary diffusion capacity becomes critical during whole body exercise as the arterial oxygen tension decreases markedly. Furthermore, the Bohr effect on the oxyhaemoglobin dissociation curve means that, with a lowering of pH to below 7.0, arterial oxygen saturation may decrease to below 90%. In addition, the ability to increase cardiac output limits the oxygen transport capacity when working with several muscle groups at the same time, and muscle blood flow is reduced by approximately 30% compared to when only a single muscle group is activated. Oxygen diffusion to the myocytes thereby becomes affected, while endurance training enhances blood volume and the number of capillaries surrounding the muscle cells as well as the size of the heart, and thus increases maximal oxygen uptake by approximately 50%.

1 Introduction

Exercise physiology describes the adaptive changes taking place in the organism in response to work or exercise. A related area is the biomechanics of human movement, e.g. during walking and running. Similarly, problems associated with labour and mechanisms involved in the development of muscle fatigue and eventual chronic pain are addressed. Exercise physiology evaluates the responses to training vs detraining, the adaptations to special environments such as high altitude, or eventually zero gravity during space flight vs the increased pressure during diving. Finally, it is increasingly realised that regular exercise is beneficial towards weight control and sustained health while competitive sport may be associated with problems related to doping.

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2 Skeletal Muscles

Physical work is carried out by the skeletal muscles and two main muscle fibre types have been identified. While fast twitch muscle fibres (FT) are able to generate force quickly they are, in general, unable to maintain a contraction. On the other hand, slow twitch fibres (ST) possess low myosin ATPase (Fig. 1), and can maintain the developed force for longer periods since they are designed for aerobic metabolism with many mitochondria and a large amount of myoglobin. Among all the muscles in the body, distribution of different fibre types vary but it is, on average, around 50%/50%, while endurance trained athletes appear to be selected among people with a percentage of ST fibres that is about 70% or even larger. The endurance capacity of the ST fibres is reflected not only in the high number of capillaries and mitochondria but also in the enzymes that they encompass, succinate dehydrogenase, etc., that enable them for high aerobic metabolism.

Although it is the skeletal muscles that perform the work, it is the brain that decides to initiate and terminate work. In this context, it may be that the end of exercise is defined by an inability of the brain to access the motor neurons. Such "central fatigue" manifests when exercise becomes strenuous or difficult, such as the

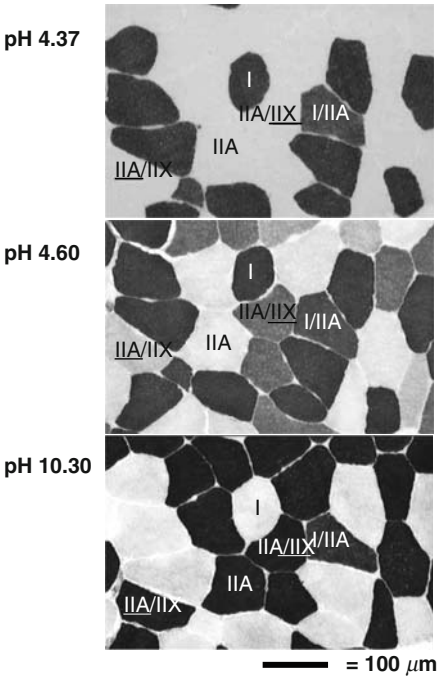

Fig. 1 Cross-sectional view of skeletal muscle with identification of the two main fibre types (I slow twitch; IIA, fast twitch trained; IIX, fast twitch untrained) by ATPase staining after incubation at pH as indicated (Jesper Løvig Andersen, Copenhagen Muscle Research Center)

first time on a bicycle, and appears, paradoxically, to be associated with difficulty in recruiting ST fibres. Thus, a fundamental aim of training is to automatise the motor pattern required for a given movement.

3 Metabolic Demand

Metabolism is divided into an aerobic and anaerobic pathway for ATP generation. At the onset of exercise it takes some time before aerobic metabolism is fully activated to cover the energy requirements of the work performed (Fig. 2). During these initial seconds of exercise, metabolism is covered, in addition to the creatine phosphate and the oxygen bound to haemoglobin and myoglobin, by the production of lactate. The required energy that is not accounted for by the pulmonary uptake of oxygen (VO_2) is termed the oxygen deficit and can be divided into a lactic and an alactic component. Conversely, it takes some time before VO_2 returns to the resting value when exercise is terminated. This additional metabolic component, termed "the oxygen debt", is related not only to the work carried out per se but also to the increase in metabolism associated with an elevated body temperature and is likely to be twice as large as the oxygen deficit.

The relative contribution of the aerobic and anaerobic components to the work carried out varies with intensity and distance covered. The aerobic and anaerobic contributions to maximal exercise are approximately equal when the event lasts ~2 min (Fig. 3). VO_2max represents the metabolic rate at the pace of an event that lasts approximately 10 min, while during longer events only a fraction of the VO_2max can be utilised. Thus, a successful marathon runner is described not only

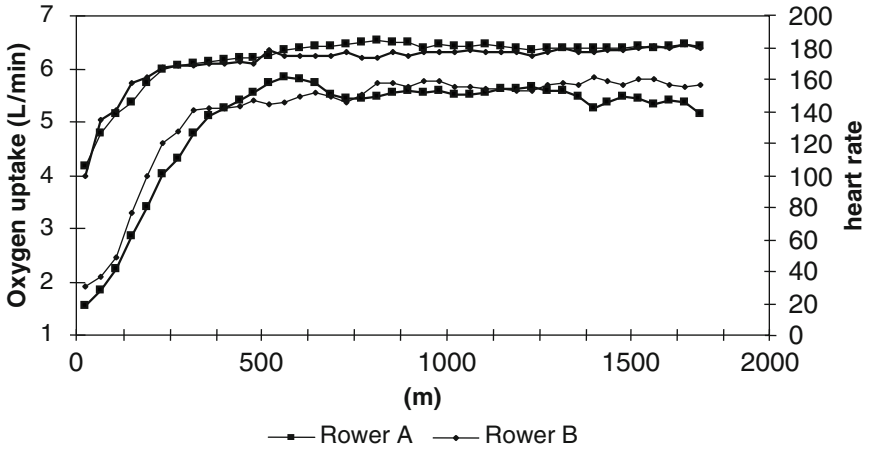

Fig. 2 Oxygen consumption (VO_2) and heart rate during a 2,000-m simulated rowing race in two rowers. The energy not accounted for by the VO_2 at the beginning of the exercise is termed oxygen deficit

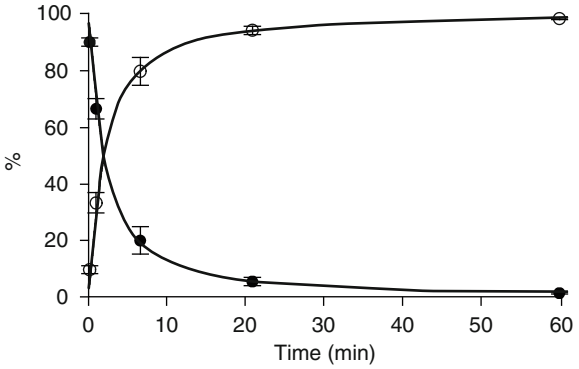

Fig. 3 The aerobic (*circle*) and anaerobic (*shaded circle*) contributions to maximal exercise. Courtesy of K. Jensen, University of Southern Denmark

by his VO_2max but also by his ability to maintain metabolism at a high level around VO_2max, e.g. 90% rather than 70% for a less qualified runner, and a high percentage of ST fibres is likely to contribute to that ability. In general, the work intensity and the time it can be maintained are related by a reverse hyperbolic function.

However, the aerobic and anaerobic capacities are not a full description of an athlete's ability to excel. Much work is performed, for example during running, to support the body in each step and to swing the legs (and the arms). Thus, the characteristics of a successful runner are long light legs and a low body weight. Conversely, in rowing, where the crew is carried by the boat, it is an advantage to be tall and heavy with a relatively long upper body and arms. For comparison of small and large individuals, the VO_2max can be expressed relative to body weight to $^3/_4$ power (Fig. 4), reflecting the dependence of running performance to VO_2/kg, while for rowing the absolute value ($L\ min^{-1}$) is most relevant.

3.1 Metabolic Rate

At rest and during exercise, aerobic metabolism, or the sum of mitochondrial respiration rates in various organs, is represented by the rate of VO_2. The relative contribution of these organs changes with physiological state, such that internal organs such as the liver, kidneys and brain account for most of the whole-body VO_2 at rest, while locomotor muscles account for >90% of VO_2max during maximal aerobic exercise. VO_2max, which reflects the maximal metabolic rate, is elicited by the energy needs of locomotor activity, and it increases with the volume of muscle mass involved in exercise. In contrast, the basal metabolic rate (BMR) reflects the lowest need of energy at rest that is used for all sorts of housekeeping functions, such as maintaining cell potentials, driving the heart, and maintenance of body temperature, an indirect measure of plasma thyroxin. BMR and VO_2max thus define the span over which the aerobic metabolic rate of the organism can vary. Whereas

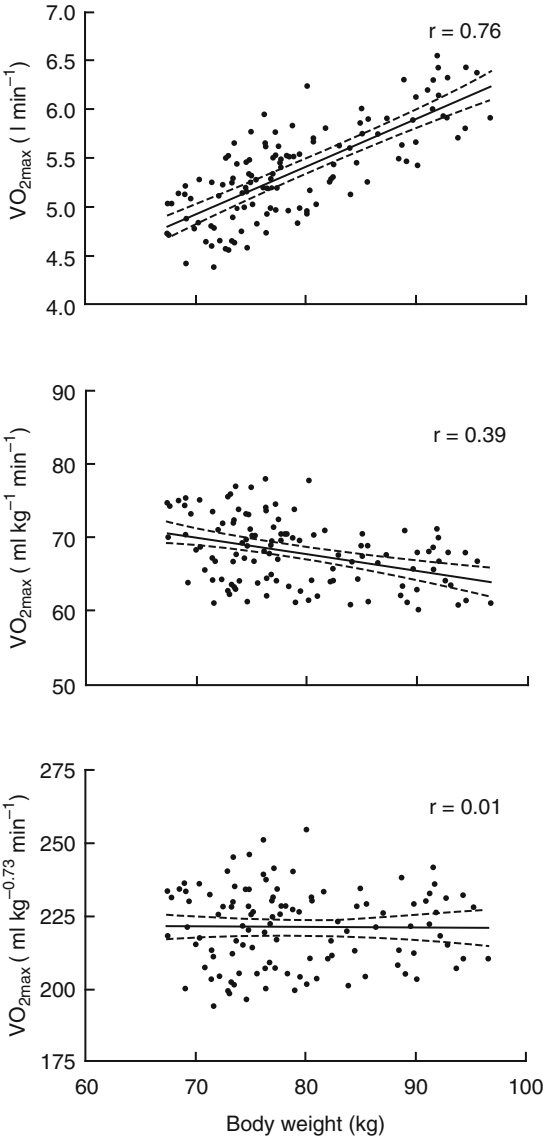

Fig. 4 Relationship between VO_2max and body mass. Regression lines with 95% confidence intervals and correlation coefficients (r) are shown. (A) The VO_2max increases with body mass, but when it is expressed relative to body mass (B), those subjects with the largest body mass show the smallest values, when trained for the same sport. When scaling VO_2max with body mass to the 0.73 power (i.e. ml $kg^{-0.73}$ min^{-1}; C), VO_2max becomes independent of body mass

BMR appears to depend on body mass to the $^3/_4$ power, VO_2max shows large inter-individual and inter-species variability, related to the degree of work or exercise capacity. It is typically about tenfold higher than BMR even though well-trained athletes can achieve a VO_2max up to 20 times higher than their BMR, and even greater variation is found in animals.

4 Static and Dynamic Work

During exercise, work is performed by muscle fibres shortening and developing tension against gravity, against the mass that is moved, and through the generation of heat, depending on the intensity of the exercise. The relationship of movement to tension varies depending on the type of work. With static work, which is quasi-isometric and can be of explosive intensity, the movement is small in relation to the tension and thus it differs from dynamic work (quasi-isotonic) in which the movement is relatively large. Dynamic work is mostly produced by repetitive contractions involving primarily ST muscle fibres and the energy cost is mainly met by aerobic metabolism. Static work entails relatively sustained muscular contraction that involves FT muscle fibres which produce lactate, and the energy cost is mainly met by anaerobic metabolism. Most exercise activities or sports entail a mixture of static and dynamic work components and thus, the physiological responses to a given exercise activity are relatively unique. For example, power weightlifting has a large static component that limits its duration to relatively short periods.

5 Energy Requirements and Cardiorespiratory Limitations

There are many different physiological and anthropometric parameters that can be used alone or in combination with other parameters to predict the competitive success of endurance athletes. It appears, however, that one of the most important physiological determinants of endurance performance is VO_2max. Therefore, obtaining high aerobic power values is of importance for the successful endurance athlete, and training is characterized by large amounts of low intensity training. Anaerobic energy systems are used mainly during the start phase of an endurance race (for approximately 60–80 s), as it takes a minute or so for VO_2 to reach its maximal level (Fig. 2), and to a lesser extent also during the finish of an endurance race.

VO_2max integrates the ability of the lungs to provide O_2 to the blood, the O_2-binding capacity of the blood, maximum cardiac output, the muscle type, and the number of capillaries surrounding the muscle fibres. The schematic view of the main organ systems that are involved in the development of VO_2max and their trainability is presented in Fig. 5. During exercise no single factor limits the O_2 transport cascade expressed as the sum of the resistances presented by each step. Yet, in order to preserve arterial pressure during whole-body exercise, blood flow to working muscles and internal organs, including the brain, is restrained, with adverse outcomes for metabolism and fatigue.

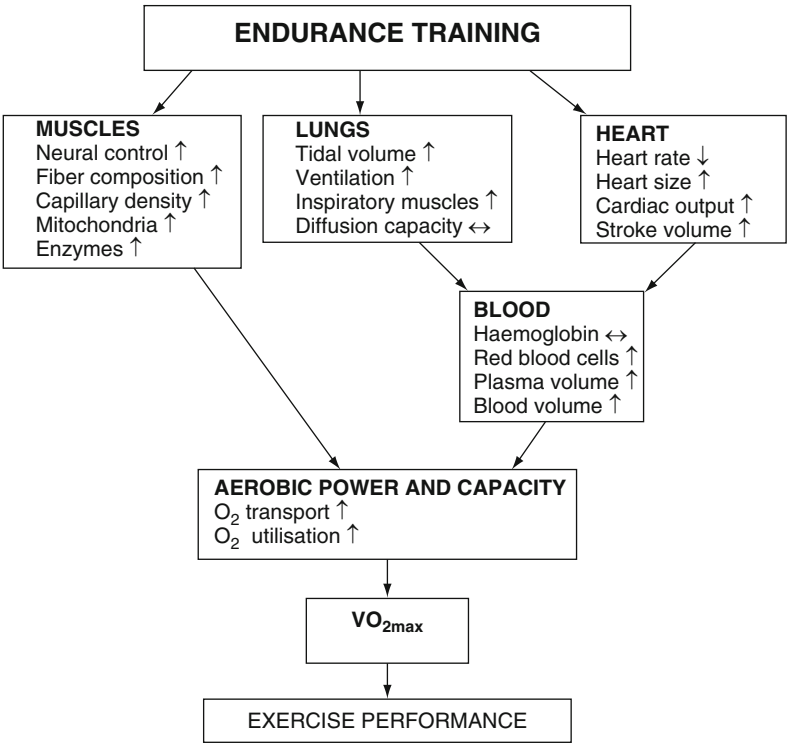

Fig. 5 Organ systems and their trainability to impact maximal oxygen consumption (VO_2 max) and exercise performance

5.1 Aerobic Metabolism

Over the course of an event lasting 5–7 min, elite athletes exercise with the majority of work performed between 95 and 98% of VO_2max. For example, male elite level heavyweight rowers have a VO_2 of up to 6.51 min^{-1}, while female heavyweights have about 4.41 min^{-1} (Table 1). There is a strong linear relationship ($r = 0.99$) established between placement in a championship and the crew's average VO_2max (Fig. 6). The highest VO_2max reported is 7.41 min^{-1} for an elite Finnish cross-country skier.

Seasonal changes in VO_2max have been described with increases of 10 mL min^{-1} kg^{-1} or ~10% during the competitive season, even though these increases in relative values of VO_2max may be highly dependent on decreases in body mass. VO_2max increases with age, training distance per year, and with sporting experience. Usually, VO_2max levels off at about the age of 18 but increases can be seen as a consequence of endurance training until around the age of 24 (Fig. 7). In parallel, increases can be seen in other endurance parameters.

Table 1 Anthropometrical and physiological data of rowers compared to sedentary people

Category	Height (cm)	Weight (kg)	VO_{2max} (l min^{-1})	VE (l min^{-1})	SV (ml)	CO (l min^{-1})
Sedentary female	168	65	2.5	100	71	15
Sedentary male	178	75	3.3	135	100	20
Junior female	177	72	4.0	150	145	28
Junior male	190	85	5.1	170	160	31
Lightweight female	170	57	4.0	150	145	31
Lightweight male	184	70	5.1	170	160	35
Elite female	182	80	4.5	170	180	35
Elite male	195	95	6.5	>200	200	40

VO_{2max}, maximal oxygen consumption; V_E, minute ventilation; SV, heart stroke volume; CO, cardiac output

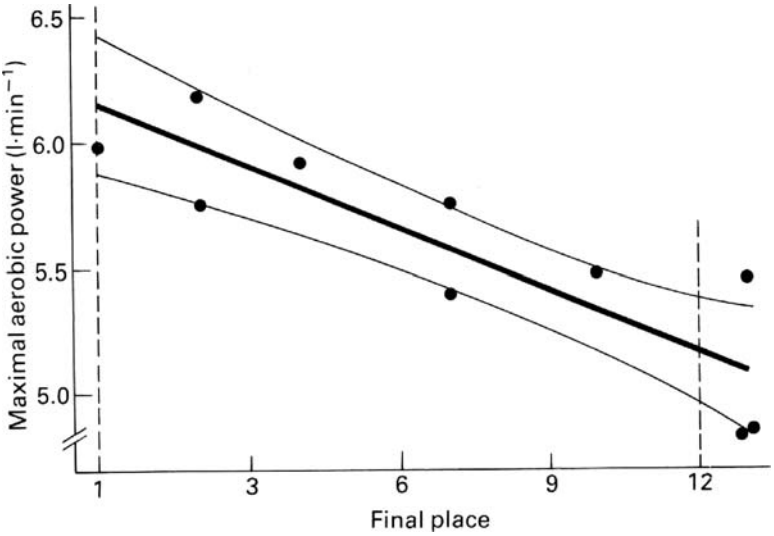

Fig. 6 Regression line between average VO_2max of a crew and placement in a rowing championship. The 95% confidence limits of the regression are also shown

A plateau in VO_2max has been observed in highly trained endurance athletes despite increased training volumes. Even with the attainment of a plateau in VO_2max, endurance performance can still be improved. This can be achieved by increasing the endurance capacity — the ability to maintain higher VO_2 over the distance — and increased economy, defined as a larger work performed for a given VO_2. Since endurance capacity appears to be important in performance prediction when athletes of similar VO_2max values are compared, alternative parameters for the measurement of endurance capacity have been proposed. For example, it is suggested that the power which elicits a blood lactate level of 4.0 mM is the most predictive parameter for competition performance.

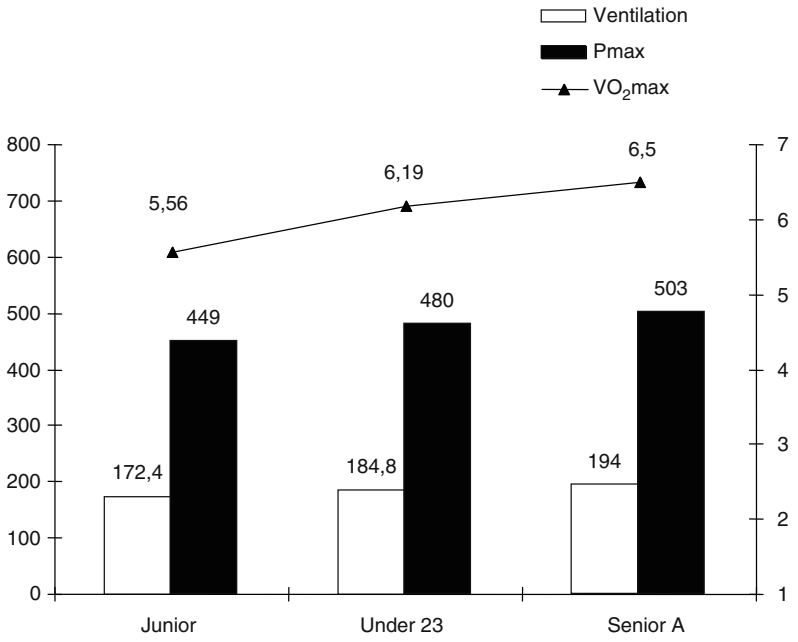

Fig. 7 The development of maximal oxygen consumption (VO_2max), ventilation and maximal aerobic power (P_{max}) of elite rowers from junior to senior level

5.2 Anaerobic Metabolism

Anaerobic metabolism is indicated by a high peak blood lactate concentration, which increases exponentially with work rate, the muscle mass involved in exercise and the motivation of the athlete. Values of 11 mM have been reported after treadmill running, while values of 15–17 mM are commonly observed after rowing competitions. Accordingly, the buffering system of the blood (bicarbonate) may be eliminated from the blood and the pH of the blood decreases from its normal value of 7.4–7.1. The record low measured pH value is 6.74 and corresponds to a blood lactate level of 32 mM. However, these values give little indication of the quantity of anaerobic metabolism. For that purpose the "oxygen deficit" can be calculated (Fig. 2). For example, in rowers the oxygen deficit has been reported to be 90 ml kg^{-1}, or substantially larger than reported in runners.

6 Endocrine and Metabolic Responses

In response to mental and physical stress, circulating eosinophils decrease from 110 to 20 mm^{-3} before an endurance race, and even to 3 mm^{-3} after exhaustive exercise, while plasma ACTH, adrenal cortical hormone and adrenaline increase. After

maximal ergometer exercise, involving a large muscle mass, plasma adrenaline and noradrenaline concentrations increase from the resting values of 0.9 and 2.3 nM, respectively, to extremely high values of 19 and 74 nM. These values are about twice as large as those noted during running, suggesting a role for muscle mass in the catecholamine response to maximal exercise. Also, pancreatic polypeptide, a hormone under vagal control, increases 2.5-fold after maximal exercise, suggesting that vagal activity may contribute to the feeling of fatigue including gastrointestinal symptoms experienced at exhaustion.

Protein turnover is larger, while the protein synthesis is not increased, in trained athletes compared with controls, supporting the theory that intense exercise does not increase basal protein turnover rate. Yet, anabolism, and hence muscle build up, may be aided when a protein rich meal is provided soon after exercise. Serum androgen and growth hormone increase from 700 ng ml^{-1} and 5 μU ml^{-1} respectively at rest, to 850 ng ml^{-1} and 50 μU ml^{-1} respectively after maximal running, but do not increase after submaximal exercise. These changes occur despite a constant serum-luteinizing hormone (60 ng ml^{-1}). It may be speculated that the raised level of androgen during exercise acts in association with growth hormone to increase the pubertal growth velocity and contributes to muscular development associated with exercise. Administration of testosterone, however, does not increase muscle glycogen build-up after maximal dynamic exercise. Serum concentration of myoglobin and creatinine kinase (a cellular enzyme) is elevated after exercise lasting 30–40 min and remains elevated 1.5 h after the training bout, indicating skeletal muscle cellular damage which could help explain the subsequent muscle soreness. Consequently, creatine supplementation enhances performance.

6.1 Blood Glucose

Blood glucose is essential for brain metabolism and supplements muscle metabolism during exercise. With the levels of plasma adrenaline observed during maximal exercise, glucolysis in the liver is stimulated and contributes significantly to the maintenance of blood glucose. Thus, intense exercise is associated with optimal blood glucose concentrations, but the liver glucose may become depleted during prolonged exercise lasting ∼12 h. Thus, during cross-country skiing and mountaineering a readily available carbohydrate source, in the form of e.g. chocolate, is essential. In these circumstances, when the blood glucose level decreases brain metabolism is compromised, resulting in an inability to maintain movement, and thus body temperature decreases, with often grave consequences.

7 Ventilation

The lung is the organ for exchange of gases between air and blood. Carbon dioxide produced from metabolism is unloaded to the alveoli while O_2 diffuses into the blood where it binds to haemoglobin. The O_2 transport is passive, as the driving

force is based on the gas pressure gradients between air and blood, and is limited by the diffusion capacity of the lungs.

At rest, ventilation (V_E) involves activation of the diaphragm muscle, which operates as a motor piston to generate negative pressure within the thorax whereby external air passively enters the lung alveoli. During exercise the diaphragm operates with increased force, while the internal intercostal muscles help to expand the ribcage and further decrease the intrathoracic pressure. Thus, increased V_E is generated by increased breathing frequency and increased depth of each breath. Very large V_E levels, typically greater than 200 l min^{-1} and sometimes as high as 260 l min^{-1}, are developed in large athletes during competition. During a 6 min all-out effort, V_E rises exponentially until the third minute, but nevertheless it continues to increase until the end of the effort. Since breathing frequency does not differ between trained and untrained, performance is more favourable for athletes with large total lung and vital capacities (VC). Hence, the lungs of athletes reflect their large bodies, and a VC of 9 L has been recorded in an elite rower. This observation reflects selection as VC is the only link of the transport O_2 chain that does not improve with training once adult stature has been attained. Thus, a correlation between endurance performance and VC is reported regularly, and successful athletes, e.g. rowers, typically demonstrate VC values of 7 l, compared to 5.5 l as expected for their body size.

A large V_E could be considered to be a disadvantage as it requires a higher activation of ventilatory muscles which compete with the locomotor muscles for their share of cardiac output. In fact, unloading of breathing by mechanical assistance increases blood flow to skeletal muscles and specific training of the inspiratory (but not the expiratory) muscles may enhance endurance performance.

7.1 Breathing Pattern

During exercise breathing is entrained to the locomotor rhythm, meaning that expiration and inspiration are performed in phase with the limb movements. The V_E and the pattern of breathing during exercise are the result of respiratory muscle contraction acting on the mechanical properties of the respiratory system. The tidal volume is determined by the size of the lungs, and both inspiratory and expiratory times are influenced by maximum airflow. Inspiratory flow rate is limited primarily by the ability to generate inspiratory muscle pressure, while expiratory flow rate is limited by airway mechanics, rather than the ability to generate expiratory pressure. Peak expiratory flow rates can reach 15 l s^{-1}, but in some of these individuals a plateau in expiratory flow rates suggestive of airway collapse is also observed.

Entrainment, or synchronisation between limb movements and breathing pattern, is a common occurrence during exercise and several factors are associated with it. Since contraction of the thoracic musculature is required both for respiration and stabilisation of the trunk during exercise, it is important that breathing and limb movements are synchronised so that one does not interfere with the other. The physiological benefits from such coupling are an improvement in the efficiency of

the respiratory muscles and gas exchange, prevention of diaphragmatic fatigue, and reduction of breathing effort sensation. Hence, limb movements influence respiratory efficiency and, at the same time, respiratory muscles modulate the locomotor rhythm.

During incremental exercise, ventilatory tidal volume progressively increases up to the point where each breath encroaches upon the flat portion of the pulmonary compliance curve. Hence, a greater portion of the negative intrapleural pressure is used to overcome pulmonary elastic work. To circumvent this mechanical limitation of lung distension during inspiration, there is a shift in the breath/stroke ratio, for example from a 1:1 to a 2:1 during rowing, where the respiratory system is confronted with a different mechanical constraint, that of expiratory flow limitation, due to the limited time of each expiratory phase.

7.2 Diffusing Capacity

Oxygen enters the body through the lungs that have an enormous gas diffusion capacity with an alveolar area of 50–100 m^2. Increased V_E is coupled intimately to exercise but the limiting role of the lungs to O_2 transport remains unknown for two reasons. First, lung function is described as a capacity rather than as the actual contribution to transport of O_2 during exercise. Furthermore, evaluation of the lungs is combined with the capacity of haemoglobin in pulmonary capillaries to take up O_2, expressed as the *pulmonary diffusion capacity* (D_LCO) using carbon monoxide (CO) as test gas. The more relevant value of diffusion capacity for O_2 is 23% larger than D_LCO and the value for CO_2 is 25 times D_LCO. D_LCO is a non-invasive measure of lung function using CO as test gas because its affinity to haemoglobin is 200 times that of O_2. When ~1% CO is added to inspired air, CO is binding with haemoglobin over the entire length of the pulmonary capillary (Fig. 8) and under these conditions D_LCO represents the gas transport capacity.

Conversely, D_LCO is dominated by acute changes of the pulmonary capillary blood volume. For example, D_LCO decreases by 15% from the supine to the seated position because pulmonary capillary blood volume is reduced while the pulmonary membrane diffusion is enhanced by 30%. During exercise, there is a doubling of D_LCO (25–50 ml $mmHg^{-1}$ min^{-1}), reflecting a support of the muscle pump to the pulmonary capillaries blood volume with recruitment of pulmonary capillaries in the apical parts of the lungs (Fig. 9).

7.3 Transport of O_2

In normoxia equilibrium between alveolar and capillary O_2 tensions is established within a fraction of the capillary blood transit time (Fig. 8). During exercise, as the velocity of red cells in pulmonary capillaries increases, equilibrium takes longer

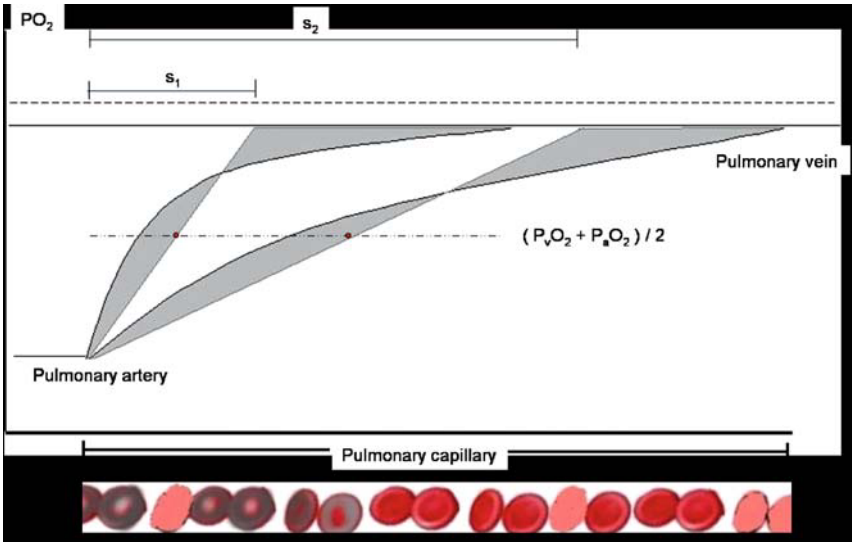

Fig. 8 Diagram of oxygen tension of blood (PcO_2) during its course through a pulmonary capillary at two flow rates. Also shown is a cartoon of red cells passing a pulmonary capillary with carbon monoxide (CO) binding to haemoglobin (*bright red*). The apparent pulmonary diffusion for oxygen (DmO_2) expresses the effective area for diffusion rather than the diffusion capacity established when the whole capillary is engaged in oxygen transfer. Because there is little or no transport of O_2 in the venous end of the capillary, the effective capillary diffusion area (s_1 or s_2) is approximated by the insertion of a line integrating the considered increase in PcO_2. When it takes longer for the alveolar to capillary O_2 equilibrium to be established because of doubled flow during exercise or because of low inspired O_2 tension, the venous end of the capillary becomes increasingly important and an enhanced effective diffusion area is expressed as an elevated DmO_2. Discrepancy between a doubling of the diffusion capacity for CO and a 8-fold increase in DmO_2 during exercise indicates that at rest in normoxia, an equilibrium is established between the alveolar O_2 tension (PAO_2) and PcO_2 within one fourth of the length of the pulmonary capillary. During hypoxemia, however, an equilibrium is not reached and the entire capillary contributes to diffusion expressed as a maximal DmO_2. Any venous admixture to arterial blood is reported as a change in DmO_2 representing an integrated measure of pulmonary function rather than of diffusion per se. To validate the absolute value pulmonary venous O_2 tension needs to replace the arterial O_2 tension (PaO_2) in the calculation of DmO_2 since blood serving the pulmonary tissue is drained in the left atrium of the heart

to be established and, ultimately, as the length of the capillary is involved in the diffusion of O_2, the diffusion capacity is utilised.

As the length of the capillary involved in diffusion increases during exercise, it becomes difficult to maintain alveolar O_2 tension (PAO_2), and an increase in V_E is required to compensate for O_2 taken up by capillary blood. During exercise, V_E follows two distinct patterns. Low-intensity exercise is associated with little drive to V_E, representing the influence from the central nervous system (*central command*). Relative hypoventilation is expressed as a $\sim$4 mmHg elevation of the arterial CO_2 tension ($PaCO_2$), which is the dominant stimulus for V_E at rest. As workload progresses, V_E increases exponentially, influenced by the decreased blood pH.

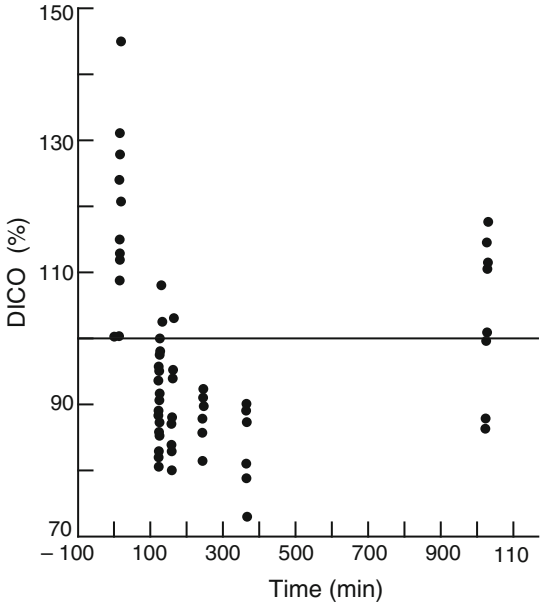

Fig. 9 Pulmonary diffusion capacity following 6 min all-out maximal exercise

The important effect of blood pH on driving V_E is demonstrated by the reduced V_E ($\sim$151 min^{-1}) following normalisation of pH with bicarbonate administration despite the 7.5 mmHg increase of $PaCO_2$. During intense exercise the marked hyperventilation decreases $PaCO_2$ below the resting value and PAO_2 increases to high levels (130 mmHg).

Even though during light to moderate exercise the lungs facilitate O_2 transport by recruiting alveoli and enhancing the within-capillary diffusion area, a diffusion capacity of $\sim$80 ml mmHg^{-1} min^{-1} (an eightfold increase from rest) is insufficient to account for the often more than 15-fold increase in VO_2 during maximal exercise in athletes. Further, during intense exercise VO_2 depends on a widening of the alveolar-capillary O_2 tension difference because gas diffusion is impeded by high pulmonary artery pressure that provokes accumulation of fluid in the alveolar-capillary space. Thus PaO_2, expressed as a physiologic shunt or exercise-induced hypoxaemia, decreases (e.g. from 90 to 75 mmHg) although P_AO_2 increases.

During intense exercise the elevated V_E, as demonstrated by the extraordinary large rates observed, increases P_AO_2 and, consequently, PaO_2 is maintained at the highest possible level. Such mechanism may explain the enhanced exercise performance following specific respiratory muscle training that supports a large V_E and PaO_2, as indicated by the elevated end-tidal O_2 tension during exercise. In contrast, pulmonary diffusion capacity does not respond to training and the large reported values for D_LCO represent selection of gifted individuals. D_LCO varies from 15 to 45 ml mmHg^{-1} min^{-1} and this range reflects differences in body size and central blood volume in addition to variation in pulmonary membrane diffusion

capacity. Pulmonary membrane diffusion capacity is larger than D_LCO because it does not include the resistance to diffusion of O_2 in plasma, or the binding of CO to haemoglobin.

7.4 Haemoglobin

Oxygen is dissolved in blood and it also binds to haemoglobin, whereby the O_2-carrying capacity is markedly increased. Dissolved O_2, described by the PaO_2 (normal value 100 mmHg), determines, together with the acidity of the blood (normal blood pH is 7.43), the level of haemoglobin saturation with O_2 (normal value 97–99%). The transport of O_2 in blood by haemoglobin is described by the oxyhaemoglobin dissociation curve (Fig. 10) reserving ~2% of the transport to O_2 dissolved in plasma during maximal exercise.

At rest and during moderate exercise, pH has little influence on the amount of O_2 transported. During maximal exercise where, despite the increased drive to breath, PaO_2 is reduced to a level similar to that seen at rest in high altitude, a right-shift (Bohr effect) of the oxyhaemoglobin dissociation curve demonstrates that any deviation in pH affects the ability of haemoglobin to transport O_2 and, consequently, VO_2 max is affected in proportion to the reduction in haemoglobin O_2 saturation. During maximal exercise the Bohr effect can reduce arterial haemoglobin O_2 saturation to ~90% and impose a 5–10% restraint on VO_2 max. By breathing O_2-enriched air, where the inspired O_2 fraction is increased from 0.21 to 0.30, PaO_2 is increased

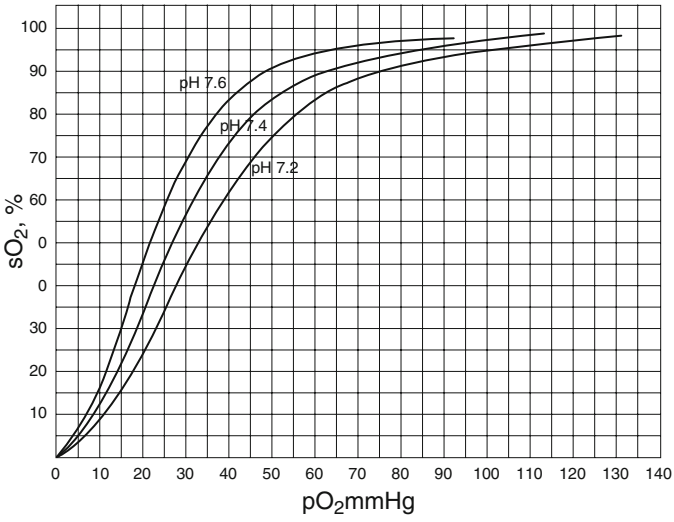

Fig. 10 The oxyhaemoglobin dissociation curve. With a decrease in pH and increase in temperature, the curve is shifted to the right

to above normal values and the influence of a low pH on haemoglobin O_2 binding is attenuated, resulting in the restoration of arterial haemoglobin O_2 saturation. The V_E is not affected, but, with a higher O_2 pressure gradient and high haemoglobin O_2 saturation, blood O_2-carrying capacity increases, which induces a higher O_2 uptake whereby exercise performance is enhanced by 2%. Arterial haemoglobin O_2 saturation is also restored in response to pH normalisation, following bicarbonate administration, and VO_2 max also increases. On the other hand, O_2 delivery is enhanced both by the effect of exercise-induced body temperature elevation and the Bohr effect, augmented by the lower pH of venous compared to arterial blood (e.g. 7.0 vs 7.2) as CO_2 carbon dioxide is exhaled (PCO_2 reduced from 90 to 40 mmHg; venous vs arterial).

The impact of the lung in limiting exercise performance is demonstrated as even a V_E of more than 200 l is not enough to maintain the PaO_2 during maximal rowing, and even higher V_E may offer some protection against the reduction in PaO_2. Therefore, V_E may contribute to limitations of O_2 transport and it is also a factor that may discriminate between winners and losers.

8 Blood Lactate

The Bohr effect on the oxyhaemoglobin dissociation curve illustrates the delicate balance between aerobic and anaerobic metabolism during maximal exercise. Appreciating the various tactics endurance athletes apply during a race, it is likely that the degree of O_2 transport is affected by acidity. This varies between individuals depending on PaO_2, which is influenced by the balance between hyperventilation and pulmonary membrane diffusion capacity. Middle-distance races start at high speed to accelerate the increase in VO_2 because the total aerobic metabolism is represented by the accumulated VO_2 during the race rather than the highest level VO_2 reaches.

More is known about exercise at high altitude. When the inspired O_2 tension is low, it is disadvantageous to work at an intensity that provokes lactate acidosis, because any anaerobic contribution to metabolism attenuates the more important O_2-carrying capacity of blood. While it is possible to work at an intensity that provokes lactate acidosis, exercise in hypoxia is usually associated with a small deviation in pH, a condition that has been defined as the high-altitude *lactate paradox*. Following high-altitude acclimatization, where both ventilation and PaO_2 increase, plasma lactate during maximal exercise is comparable with the sea-level values. The unproven, but probably beneficial, effect of moderate altitude training ($\sim$2,000 m) for sea-level performance may be explained by the adaptation of the respiratory muscles to the large ventilation in hypoxia. Upon return to sea level, it feels easier to maintain high ventilation and elevate PaO_2 during competition, and thus enhance the anaerobic contribution to exercise without affecting the oxygen transport capacity of haemoglobin.

8.1 Lactate Threshold

Interest has also focused on the lactate threshold or the work rate that elicits a blood lactate concentration of 4 mM. This work rate increases with training, and it seems to depend on the muscle fibre composition. Athletes with many slow-twitch fibres are able to exercise at a high intensity with a blood lactate value of no more than 4 mM.

The Bohr effect on the oxyhaemoglobin dissociation curve explains why the work rate, at a given blood lactate level, is a sensitive predictor of endurance performance. Blood lactate increases exponentially with workload but their relationship is right-shifted following training, i.e. blood lactate increases with relative workload. The workload that elicits a given lactate level (often set at 4 mM) is, thereby, an indirect measure of VO_2 max that, in itself, is a predictor of performance. Blood lactate is a more precise performance predictor because blood lactate reflects not only VO_2 max but also the ability to work without affecting the oxyhaemoglobin dissociation curve.

For a given workload less lactate is produced, with the recruitment of ST rather than FT muscle fibres, as illustrated when ST fibres are prevented from contracting with curare-induced (South American arrow poison) partial neuromuscular blockade. The workload that elicits a given blood lactate level reflects the work capacity of ST muscle fibres. The composition of muscles depends not only on the percent of ST vs FT fibres but also on their relative size. For example, weightlifters develop large FT fibres in adaptation to rapid lifts, while rowers are characterised by large ST fibres reflecting the relatively slow movements involved in rowing (Fig. 3). Furthermore, considering that central fatigue inhibits ST muscle fibre recruitment, in high exercise intensity which requires increased central command a smaller contribution to work from ST muscle fibres necessitates that work has to be carried out with a larger contribution from FT muscle fibres, which results in elevated lactate production. In other words, evaluation of blood lactate during submaximal exercise reflects the mental preparation and automatisation of the movement that determines the central command requirements.

Alongside these considerations it should also be noted that although it is lactate that is measured in blood, it is the deviation in pH that influences oxygen transport. Lactate is a substrate for tissues including muscle, liver, kidney and brain but its exponential accumulation in the blood, as the work rate increases, is a manifestation of attenuated elimination by liver and kidneys. Blood lactate is, thereby, also an indicator of how well organ blood flow is preserved during various exercise intensities.

9 The Heart Rate Response

The heart rate response to exercise is of particular interest because the almost linear relationship between heart rate and work load (or VO_2) is applied widely for evaluation of VO_2 max in population studies. Furthermore, heart rate is also a determinant

of cardiac output that supports blood pressure, which in turn affects perfusion of the working muscles and the brain. Hence, a brief presentation follows of the factors that affect heart rate, cardiac output, blood pressure and blood flow to vascular beds critical for endurance exercise performance.

9.1 Blood Volume and Cardiac Preload

The heart of quadrupedal animals is on the same level as the main portion of blood within the body, but upright humans face a circulatory challenge as the indifference point for volume is at the level of the pelvis and about 80% of the blood volume is positioned below the heart. Thus, in response to reduced central blood volume, cardiovascular reflexes including sympathetic activation and the veno-arterial reflex are important for maintaining the upright position. Yet, it is not possible to remain upright without the muscle pump preventing the accumulation of blood in dependent parts of the body, as is the case in soldiers standing still in line who faint, with a concomitant decrease in heart rate and blood pressure. This reflex, defined as *vasovagal syncope*, is elicited when central blood volume is reduced by 30% due to gravitational pooling in the upright posture, and the associated reduction in blood pressure is attributed to a *Bezold–Jarish-like reflex* that induces vasodilatation in skeletal muscles at the expense of flow to the brain.

9.2 Starling's Law of the Heart

The influence of central blood volume, or cardiac preload, on the function of the heart is described by Starling's law of the heart (Fig. 11). In this context, *normovolaemia*, defined by the absence of further increase in stroke volume or cardiac output when central blood volume increases, is achieved in the supine posture. During head-down tilt that increases diastolic filling of the heart, there is no further stroke volume increase, indicating that the upper flat part of the Starling curve has been reached. Conversely, when upright, central blood volume is reduced and the heart operates on the ascending part of the Starling curve where cardiac output depends on preload.

During exercise, central blood volume and the ~10% increase in working muscles blood volume are supported by the muscle pump, promoting venous return and the redistribution of blood volume by vasoconstriction in the splanchnic area. However, with increasing blood flow requirements in working muscles and the skin, as body temperature increases, splanchnic vasoconstriction does not recruit enough blood to maintain the central blood volume level established at seated or supine rest. Central blood volume contributes to the level of sympathoexcitation established, as illustrated by the heart rate response. For example, at supine rest, heart rate may be 60 bpm and increase to 80 bpm when standing but decrease to 70 bpm during

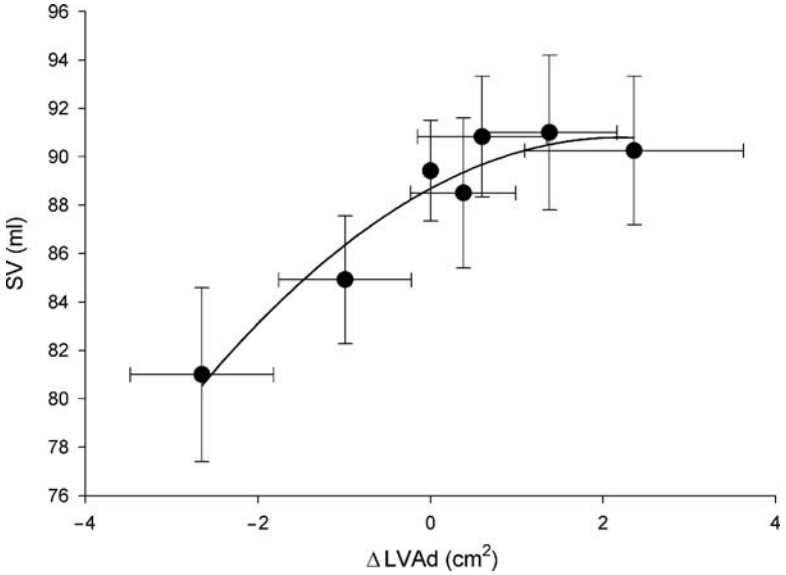

Fig. 11 Starling curve for the human heart as evaluated during head-up and head-down tilt. During supine rest, the upper flat part of the curve is reached

contraction of the legs. Muscle contractions increase sympathetic activity but the concomitant enhancement of central blood volume and central venous pressure by the muscle pump elicits a "paradoxical" reduction in sympathetic activity. Similarly, during running a reduced central blood volume attenuates central venous pressure and distension of the atria, as reflected by the plasma level of atrial natriuretic peptide (ANP).

Consequently, heart rate is higher during running than during rowing at a given exercise VO_2. Furthermore, the lowest heart rate response to exercise is observed in the supine posture. This gravitational influence on heart rate is sustained during maximal exercise, as indicated by the lower value during rowing than during running, despite the larger VO_2max established by the larger active muscle mass during rowing. With the enhanced central blood volume following rowing training, heart rate at a given work load, including maximal effort, decreases.

9.3 Cardiac Output

The cardiac output depends on the volume of blood that the heart receives, or its preload. As the total capacitance of the vasculature is larger than the total blood volume, the distribution of blood volume is critical for maintenance of blood pressure and regional flow. Blood volume encompasses both volumes of red cells and plasma that change rapidly in response to exercise. For example, plasma volume is elevated

by 20% following short-term training and it decreases during bed rest or during space flight.The enlargement of plasma volume following training, and the reduction in plasma volume when central blood volume remains elevated, demonstrate that central blood volume rather than total blood volume is the regulated variable. During exercise body weight is lost by sweating, but even after weight is restored by drinking, central blood volume remains reduced for many hours following exercise and plasma volume is expanded by further drinking as thirst is maintained. Central blood volume is reduced following exercise due to muscle oedema provoked by the combined effects of elevated perfusion pressure and muscle vasodilatation associated with exercise. Also, cutaneous vasodilatation induced by the elevated body temperature contributes to the attenuation of central blood volume during and after exercise. Even though body temperature normalises and muscle oedema is cleared rapidly post-exercise, muscle blood volume is maintained elevated for almost a day and that volume is recruited from the central circulation.

The reduced central blood volume following exercise is reflected in the levels of hormones that regulate fluid balance, including plasma vasopressin (alternatively named anti-diuretic hormone, ADH) and plasma ANP. Plasma vasopressin remains elevated while plasma ANP is low following exercise, and both these hormonal changes reduce urine production, resulting in positive fluid balance. It is less clear why the red cell volume increases in response to training. Bone marrow is stimulated by erythropoietin (EPO) released mainly from kidneys to produce haemoglobin. Exposure to high-altitude hypoxia increases haemoglobin production, but the acute increase in haematocrit reflects the loss of plasma volume. Intense exercise, in addition to the exercise-induced hypoxaemia stimulus for EPO production, may stimulate haemoglobin production via the sympathetically induced reduction in kidney blood flow, a mechanism similar to the low EPO production and associated anaemia observed in kidney diseases.

The increase in total haemoglobin is an important adaptation to training because VO_2 max is related to red cell volume rather than to the haemoglobin concentration. In a seeming paradox, despite the increased red cell volume, elite endurance athletes often present low haemoglobin concentrations (or haematocrit), due to the training-induced enlarged plasma volume. Elite endurance athletes, e.g. rowers, may have a plasma and red cell volume of 4.8 and 3.1 l, respectively, compared to control reference values of 3.2 and 2.4 l, respectively, while, on an average, their haemoglobin concentration is larger than the normal healthy population (Table 2).

Table 2 Haemoglobin, haematocrit, red blood cells, plasma volume and blood volume in male sedentary population and in elite male rowers

	Sedentary	Elite rower
Haemoglobin ($mM\ l^{-1}$)	9.5	9.9
Haematocrit (%)	43	48
Red blood cells (l)	2.4	3.1
Plasma volume (l)	3.2	4.8
Blood volume (l)	5.0	7.2

9.4 The Heart

As known from cardiac diseases, the heart adapts to the load it is exposed to, and that adaptation applies also to training. With endurance training the internal diameters of the heart enlarge with the highest values observed in professional bicyclists at 55 ml, vs 50 ml for untrained subjects. The heart of weightlifters is different because they develop high blood pressure during each maximal effort by a concomitantly performed Valsalva-like manoeuvre that stabilises the spine. To overcome the high blood pressure, the wall thickness of the heart's septum separating the left and right ventricle increases to 10 mm, while the internal diameters of the heart remain unchanged.

In some activities, such as rowing and kayaking, there is a combined demand for a large VO_2max, cardiac output and stroke volume, in addition to the need to overcome the high blood pressure, at the beginning of each rowing stroke. As a result, both the internal dimensions and wall thickness of the heart increase, and athletes of these sports possess the largest sports heart, with values for left ventricular mass of 330 g compared to 142 g for divers (Fig. 12).

About 7% of elite rowers have both a left ventricular wall thickness over the normal limit of 13 mm and an enlarged left ventricular cavity. In elite rowers the heart is so enlarged that myocardial perfusion becomes inhomogeneous, which, together with high vagal tone and low intrinsic heart rate (after combined vagal and sympathetic blockade), creates a complicated electrocardiographic presentation. As

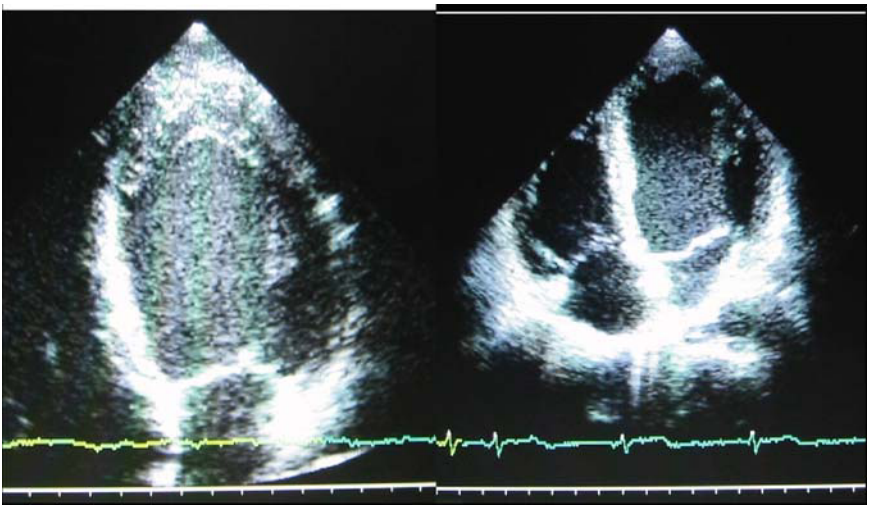

Fig. 12 Echocardiographic presentation of the heart in diastole of a World champion rower (*left*) and a control subject (*right*) of similar size. For the rower, the internal diameter of the left ventricle is 6.4 cm compared to 4.9 cm for the control subject. Similarly, the septum has a width of 1.3 and 0.8 cm, respectively and the posterior wall of the heart a width of 1.2 and 0.9 cm, respectively. Courtesy of Emma Hart

in skeletal muscles, training increases capillarization of the heart and, following detraining, the size of the heart returns to its control values.

While it is a problem to maintain an adequate preload to the heart during seated and especially upright exercise, there is little problem for the heart to pump the blood it is provided with. In contrast to the pain experienced in skeletal muscles during sustained exercise, healthy people do not complain of chest pain during exercise, indicating that myocardial oxygen demand does not limit cardiac output. Furthermore, it is energy-efficient for the heart to provide a large cardiac output.

Energy requirements of the heart depend on its rate and (systolic) pressure expressed as the rate–pressure product. Following endurance training the enlarged blood volume ensures filling of the heart and reduces sympathetic activity that attenuates heart rate by the same mechanism observed during supine and seated exercise. Furthermore, enhancement of central blood volume associated with semi- or supine posture, e.g. rowing, attenuates the pressure that arterial baroreceptors control during exercise, as exemplified by the blood pressure reduction when leg exercise is added to arm cranking. Cardiac output is elevated following endurance training with no additional strain on the heart (i.e. same rate–pressure product during maximal exercise), indicating that the enhanced blood flow to active muscles is provided by means of enhanced vascular dilatation. Yet, the muscle pump cannot provide enough blood to the heart and sometimes, at exhaustion, a restraint on cardiac preload is illustrated by decreasing central venous pressure.

9.5 Stroke Volume

The stroke volume of elite endurance athletes is impressive (e.g. in rowers 195 vs 110 m for control subjects), but it is not limited by the capacity of the heart to encompass a large volume of blood. The problem upright humans face in increasing cardiac output during exercise is different from that experienced by quadrupedal animals. In puppies, work capacity increases following pericardiectomy that allows the heart to expand. In upright humans there is not a similar restraint on stroke volume as illustrated by the filling of the heart during supine exercise and by the ability of the heart to increase stroke volume in response to a volume overload. With administration of plasma expander heart rate during maximal exercise decreases (180 vs 190 bpm) while stroke volume is enhanced by 10% (155–145 ml).

Following volume expansion the enhanced stroke volume confirms that the heart operates at the ascending part of the Starling curve (Fig. 11) when humans are upright. The size of the heart remains unchanged and it may decrease somewhat when heart rate exceeds 150 bpm, indicating that the heart propels the blood it is provided with. During exercise stroke volume increases by enhanced contractility and the systolic duration shortens to about one half, while the diastolic duration is further limited to one third of the resting value. Exercise tachycardia develops although plasma potassium increases, e.g. to 7 mM, signifying that sympathetic activation is needed not only to maintain function of the heart but also to clear plasma potassium during and after exercise.

The short diastolic interval during exercise may present a problem for the filling of the heart, as illustrated in patients with atrial fibrillation where cardiac output is compromised when heart rate exceeds 120 bpm. However, during exercise venous return is enhanced, and combined with the increased contractility of the heart, stroke volume is enhanced. Sympathetic activation is supported by a small increase in free plasma calcium released from albumin as pH decreases. Relaxation of the left ventricle may draw blood into the heart and this action is enhanced when the end-systolic volume is attenuated during vigorous exercise. Yet, the attenuated heart rate response to exercise following training is an advantageous adaptation for the filling of the heart.

9.6 Extreme Exercise

The ability of the heart to cope is less impressive during extreme exercise. Reports on horses running to death are not uncommon. It is also well-known that Pheidippides died after running the original marathon course to report to Athens the Greek victory over the Persians (battle of Marathon, 490 BC) (it may be noted that previously he had been fighting in the battle after running 2×250 km (!) in a round trip from Athens to Sparta to request support). While running to exhaustion, the heart of rats is dilated and the blood volume encompassed within the cavities is elevated by 50% and normalises only over days. That degree of exhaustion is further characterised by a state simulating thyroid insufficiency, as illustrated by rats forced to swim to exhaustion that reduce their spontaneous activity, eat more and increase body weight.

The extent that the post-exercise stress syndrome in rats relates to overtraining in humans is only a speculation, but long-distance events such as long-distance running or ultra-marathons provoke cardiac fatigue. Cardiac fatigue is presented primarily as reduced diastolic function of the heart. Both chronotropic and inotropic functions are affected by competitions lasting many hours, as illustrated by attenuated responses to sympathomimetic drugs, e.g. by beta-receptor downregulation or desensitization.

9.7 Cardiac Output

Cardiac output provides blood flow to tissues including working skeletal muscles and there is, on average, a 6:1 coupling between cardiac output and VO_2. The largest reported VO_2 max of 7.41 $l\,min^{-1}$ would thereby be expected to require a cardiac output of 44 $l\,min^{-1}$. There are, however, large inter-individual variations in cardiac output both at rest and during exercise. At rest some variation relates to body size and cardiac output is expressed as cardiac index ($3.5\,l\,min^{-1}\,m^{-2}$) in cardiology with body surface area based on height and weight ($\sim 1.7\,m^2$). During exercise

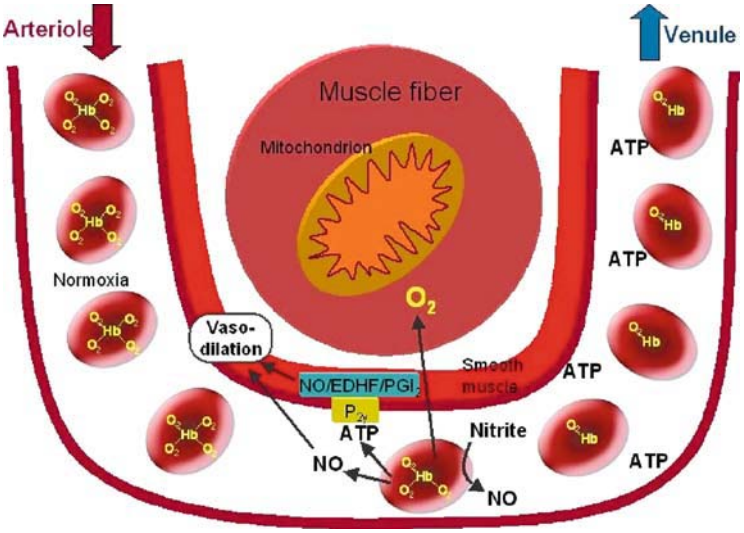

Fig. 13 Ech

cardiac output values usually range from 29 to 401 min^{-1}, with the highest values achieved by those athletes who present also large body size: height exceeding 199 cm, body mass around 90 kg, body fat percent below 9% and VO_2 max higher than 6.7 l min (Table 1). Additionally, cardiac output varies according to the variation in genes, as demonstrated by the arg16gly polymorphism of the ß$_2$-adrenergic receptor, and with haematocrit.

During exercise pulmonary VO_2 increases in relation to metabolism in exercising muscles with oxygen uptake of non-exercising tissues being 0.41 min^{-1}. Such observations underscore a tight coupling between cardiac output, regional blood flow and metabolism with some attenuation of blood flow at the highest workloads. Yet, it is not blood flow per se that is regulated but rather oxygen-carrying capacity defined as venous oxygen saturation because red cells liberate vasodilatating substances when they release O_2 (Fig. 13).

An O_2 dependent regulation of cardiac output and regional blood flow assumes that the heart provides the needed cardiac output, but that is not always the case. When the circulating blood volume is reduced, as exemplified by sweating during prolonged exercise or dehydration, cardiac output is smaller than expected according to haematocrit.

10 Blood Pressure

Arterial pressure has two roles in the regulation of blood flow to tissues. Arterial pressure is the key regulated circulatory variable, controlled beat by beat from the arterial baroreceptors that modulate peripheral resistance, as it provides perfusion

pressure to the tissues and notably to the brain. At rest, variation in blood pressure is related to the cardiac cycle, resulting in a pulse pressure of approximately 45 mmHg. During exercise, however, blood pressure also varies with locomotor rhythm, e.g. the rowing cycle, because of the Valsalva-like manoeuvre performed at the catch of each stroke, giving rise to a "pulse pressure" of more than 100 mmHg. This means that the systolic pressure may approach 200 mmHg. Regulation of arterial pressure by the arterial baroreceptors during exercise implies that their operating range is right-shifted and elevated by neural influence from central command and *the muscle pressor reflex* (Fig. 14).

Two strategies may be applied to establish the elevated pressure that the baroreceptors are reset to control during exercise. Ideally, the set pressure can be established by an increase in cardiac output to compensate for the marked decrease in total peripheral resistance induced by exercise. However, if that is not possible because of strain on cardiac output either by a restricted preload or by an inability of the heart to produce the required cardiac output, mean arterial pressure is maintained by vasoconstriction not only to internal organs but also to working muscles and to the brain.

11 Regional Blood Flow

Skeletal muscle blood flow is modulated by deoxygenation of haemoglobin adjusting flow to metabolism, and that takes place despite the enhanced sympathetic activity during exercise (Fig. 13). Such sympatholysis depends also on other factors such as the elevated muscle temperature, potassium, nitric oxide and the arterial pyruvate/lactate ratio. However, muscle blood flow is not allowed to increase at the expense of blood pressure.

Priority for blood pressure regulation over regulation of flow is demonstrated when comparison is made between flow to a muscle working in isolation and together with other muscles. As an example, flow to working legs ($\sim$101 min^{-1}) is reduced when the arms are working intensely at the same time. Equally, arm blood flow (4.61 min^{-1} in untrained vs 6.41 min^{-1} in rowers) and oxygenation are larger during arm cranking than when arm cranking is performed together with high-intensity cycling exercise (Fig. 15).

Skeletal muscles seldom receive the blood flow that their vasculature can handle. During exercise involving large muscle mass, especially, flow to the working muscles is reduced by $\sim$20–40% compared to the flow they receive during exercise involving small muscle mass, and this reduction is manifested primarily via sympathetically mediated enhanced vascular resistance. However, when leg exercise is added to arm cranking, blood pressure decreases and perfusion pressure to the arm accounts for approximately 50% of the reduction in arm blood flow.

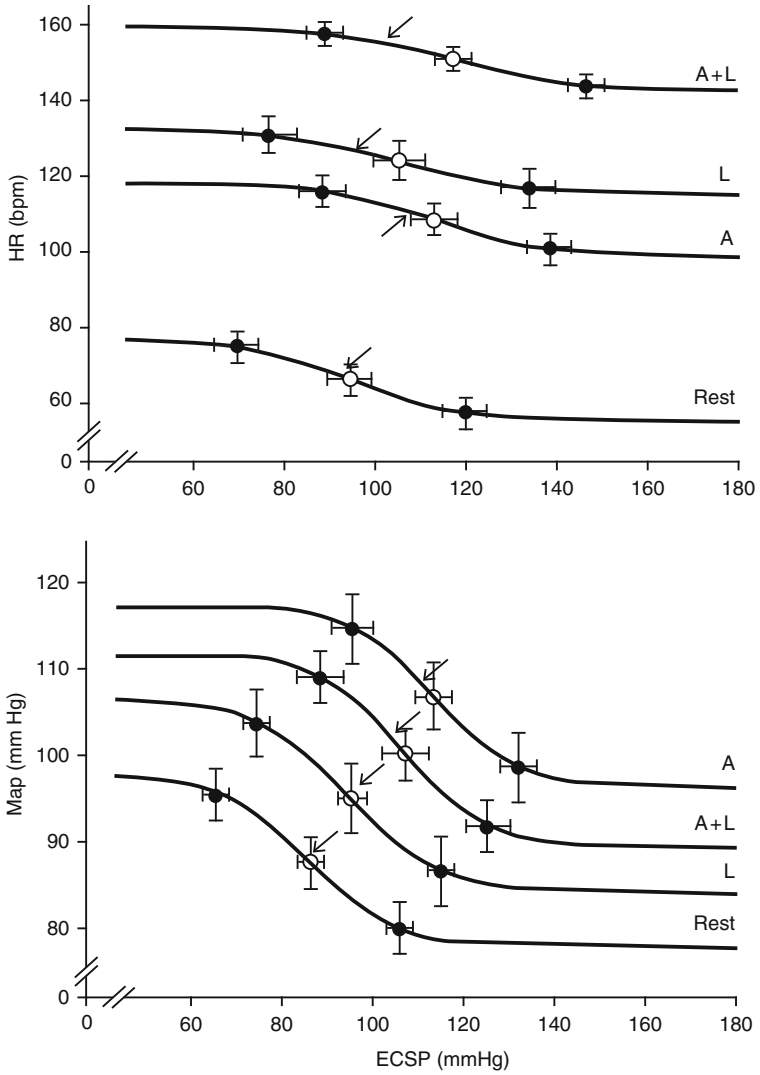

Fig. 14 The carotid baroreflex during arm (A), leg (L) and combined arm and leg exercise (A+L). At rest the actual pressure (arrow) corresponds to the maximum gain of the reflex (o), while during exercise it may be positioned at a slightly lower estimated carotid sinus pressure (ECSP) suggesting that the baroreflex detects hypotension although blood pressure is elevated. HR, heart rate; MAP, mean arterial pressure

12 Peripheral Gas Exchange

The final step in the oxygen transport chain to muscle is by diffusion. Capillaries are recruited when muscles are activated, suggesting that the capillary network is designed to provide the muscle with O_2 during exercise rather than at rest. In fact,

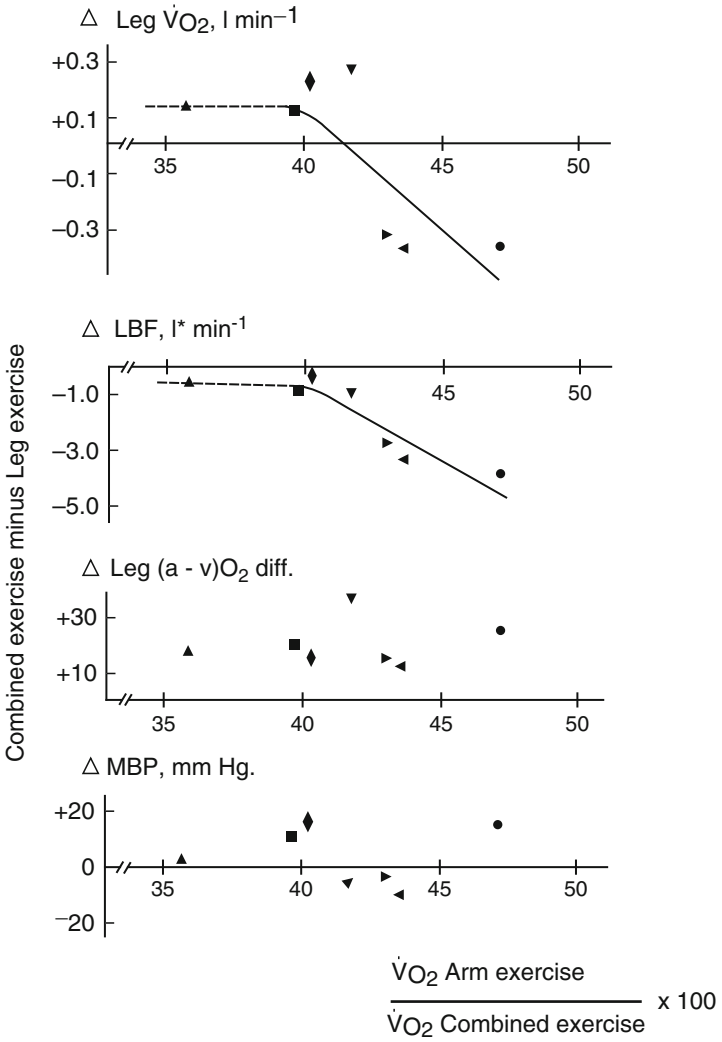

Fig. 15 Effect of adding arm exercise to leg exercise on leg oxygen uptake (VO_2), leg blood flow (LBF), the leg arterial to venous oxygen difference [(a-v)O_2 diff], and mean arterial pressure (MAP)

arteries are not gas-impermeable and a considerable amount of gas exchange (O_2 uptake and elimination of CO_2) takes place in vessels larger than the capillaries. There is a coupling between VO_2max and capillary density, and more capillaries surround ST than FT muscle fibres. Typical values for the vastus lateralis muscle are 1.8 capillaries per fibre for untrained and 2.6 capillaries per fibre for trained endurance athletes, while the highest capillarization is observed in the external intercostal muscles with six capillaries per fibre.

As with the lungs, the O_2 diffusion capacity of the muscles can be calculated. As in the lungs, the available O_2 diffusion capacity of the muscles is not always used. During moderate exercise capillary recruitment enhances the gas diffusion capacity, but during maximal exercise blood flow is limited, and thus gas diffusion is restrained. During arm-only exercise, gas diffusion values of the arm may be 20 ml min^{-1} $mmHg^{-1}$ and 50 ml min^{-1} $mmHg^{-1}$ in untrained and trained rowers, respectively, while the value decreases to 32 ml min^{-1} $mmHg^{-1}$ when legwork is added to arm-only exercise.

13 Brain

Ultimately, it is the brain that limits performance. Central fatigue was described by the Italian physiologist A. Mosso (1904). Using a finger ergograph he demonstrated that fatigue becomes pronounced after a demanding mental task such as a lecture. Since the work of Mosso central fatigue has been described in a wide range of situations and, conversely, the enhanced performance associated with so-called diverting activities may be seen as alleviating that type of fatigue. The influence of diverting activities on muscle fatigue (Setchenov's phenomenon) refers to the observation that performance is enhanced when exercise with one muscle group (e.g. with one hand) is supplemented by activity with another muscle group. Similarly, if exercise is continued until exhaustion with the eyes closed, opening of the eyes enhances strength and work can be continued for some time.

Another delineation of a limitation to recruitment of muscles is the varying strength established when contractions are performed with either one or both legs (Fig. 16). The force developed during simultaneous contraction of the legs is less than the sum of strength developed during contractions of one leg at a time, and this "leg strength paradox" is modulated by training. Walking and running are characterised by alternate use of the legs, i.e. one leg is extending while the other leg is flexing, whereas when both legs are used concomitantly the developed strength is equal to, or exceeds, the sum of the strength that can be developed with one leg, e.g. during rowing.

The ability to resist fatigue is enhanced by training and this effect is, moreover, so specific that to a large extent it must be ascribed to the enhanced ability to recruit motoneurons. Training of repeated one-legged contractions postpones, as expected, onset of fatigue during one-legged contractions whilst performance with both legs remains unchanged (Fig. 17). Conversely, training the extension of both legs benefits contractions involving both legs, whereas the performance of one leg is, surprisingly, unchanged and, under both circumstances, the electromyographic activity over the muscles decreases in parallel with force. Also, after maximal dynamic exercise, central fatigue is important, as demonstrated by electrical stimulation of the motor nerve that yields a greater power than that evoked by voluntary contractions. Finally, the consistent finding that a $\sim$25% increase in strength takes place without hypertrophy of the muscle fibres confirms that full recruitment of muscle fibres requires a central adaptation or a learning process.

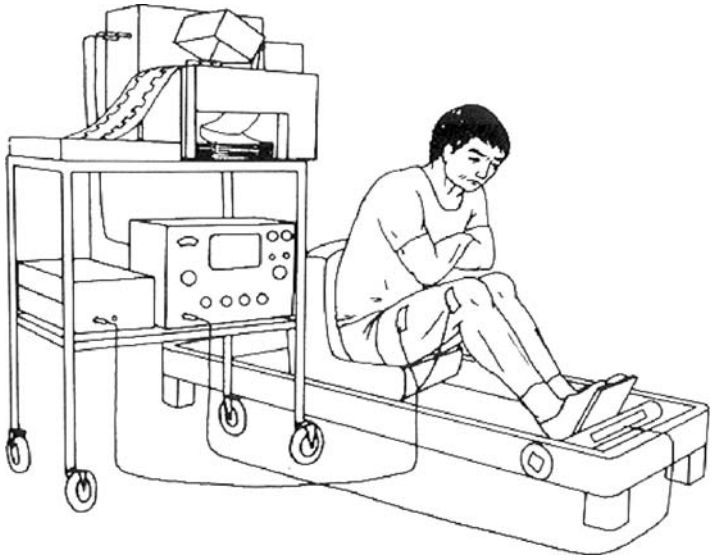

Fig. 16 Experimental setup and evaluation of leg strength

With the varying ability of the central nervous system to recruit the motoneurones, the pattern of muscle contractions developed during central fatigue is of interest. During partial neuromuscular blockade, two types of contractions can be delineated. With the use of an acetylcholine agonist drug, muscle contractions become slow but enduring. Conversely, a non-depolarising neuromuscular blocking agent provokes contractions that maintain a high rate of rise of tension, but the developed force fades off rapidly. Because the two types of neuromuscular blocking agents affect selectively fast- and slow-twitch fibres respectively, the contraction pattern manifested during partial neuromuscular blockade provides some insight into the characteristics of the two main fibre types in humans. From that perspective, it seems that central fatigue affects the recruitment of ST rather than FT muscle fibres as the contraction maintains its rate of rise of tension and, at the same time loses its endurance (Fig. 18).

The central nervous system mechanisms responsible for central fatigue remain elusive. One consideration relevant to central fatigue is that stimulation of cortical areas provokes facilitation of reflexes over fast muscles and, conversely, inhibits reflexes involving slow muscles. In the following section, the cerebral metabolic response to exercise will be addressed from the perspective that central fatigue may be provoked by a limited provision of O_2 and substrate to relevant areas of the brain.

13.1 Cerebral Blood Flow During Exercise

It has been a challenge to identify changes within the brain that correlate to hampered recruitment of the muscles. With physical activity regional cerebral blood

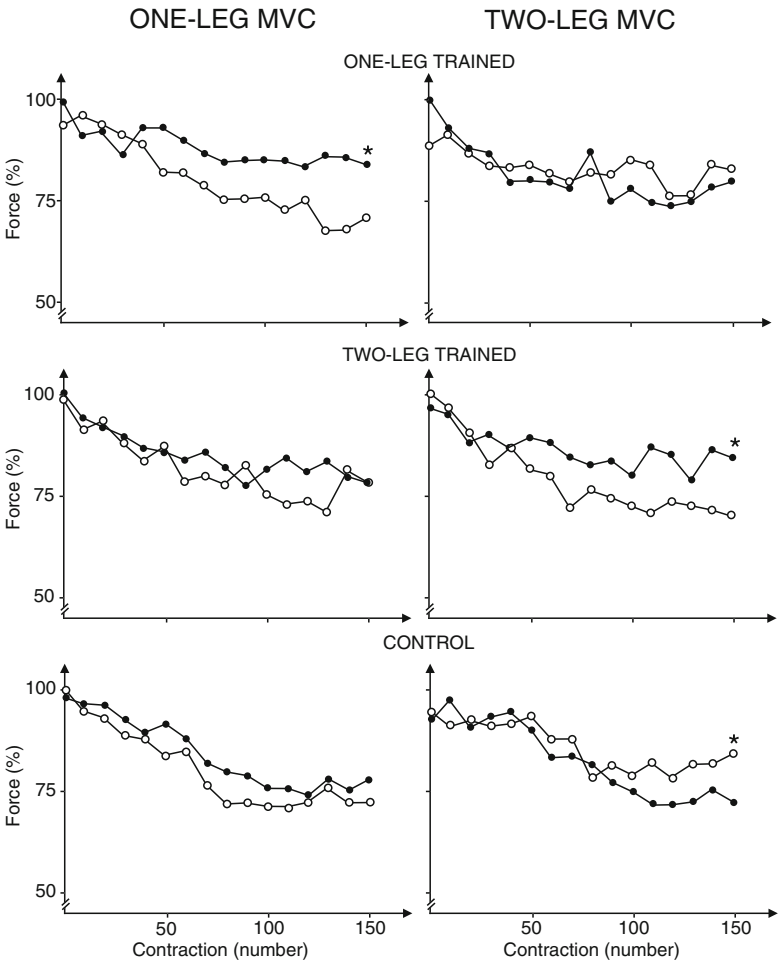

Fig. 17 Fatigure during repeated one- and two-legged contractions following one- or two-legged training

flow (CBF) increases in activated areas of the brain but, for the brain as a whole, there is not much, if any, increase in CBF. A stable global CBF during exercise may imply that increased activity in one cerebral region is compensated by down-regulation in other regions, and may explain why it is difficult to do more than one concentrated effort at a time. Furthermore, with the marked hyperventilation associated with maximal whole-body exercise, the $PaCO_2$ decreases and this reduces CBF. Together with the arterial hypoxaemia that can develop during intense whole body exercise (arterial haemoglobin O_2 saturation decreasing to 90%), the reduction in CBF means that cerebral oxygenation decreases by $\sim$10%. Such a reduction in cerebral oxygenation is significant because it approaches the magnitude seen during fainting and therefore a likely contributing factor to central fatigue.

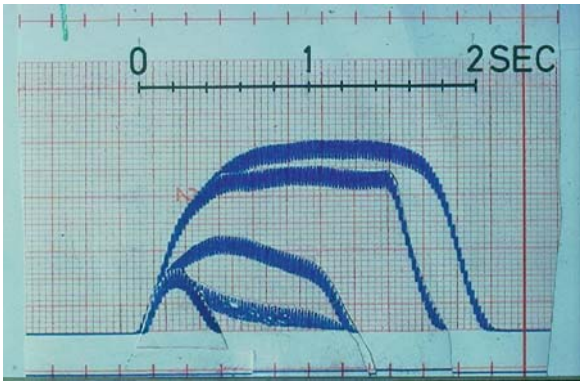

Fig. 18 Registration of force during repeated maximal voluntary handgrip contractions. As force decreases, also the ability to maintain the contraction becomes affected, while there is little reduction in the rate of rise of tension. Thus, at exhaustion, the contraction has a duration of 0.3 s, while the normal maximal contraction lasts for some 2-3 s. During partial neuromuscular blockade by tubocuarine, rate of tension is similarly maintained, while the developed force fades off after 0.15 s to continue at a much lower level after 0.3 s. Conversely, after the use of decamethonium, the rate of rise of tension is affected severely, but the developed force can be maintained for as long as desired

13.2 Cerebral Energy Metabolism

Changes in brain metabolism are expressed by the ratio of O_2 to carbohydrate uptake of the brain, known as the cerebral metabolic ratio (MR), which at rest is close to 6. Cerebral metabolism depends primarily on oxidation of carbohydrate as the brain uptake of amino acids and free fatty acids is of little quantitative importance. During brain activation, however, MR decreases and in that respect physical exercise represents an extreme. The largest reduction in MR is established during exhaustive whole-body exercise while MR increases to $\sim$6.5 during anaesthesia (Fig. 19). With intense ergometer rowing-induced activation of the brain, MR decreases to values lower than 2, suggesting that less than 30% of the carbohydrate taken up by the brain is oxidised, or that up to $\sim$10 mmol glucose-equivalents need to be accounted for.

For evaluation of MR during exercise, lactate is important. During resting wakefulness the brain releases small amounts of lactate. In contrast, when exercise provokes a substantial increase in blood lactate, there is an uptake by the brain that is proportional to the arterial concentration. Seemingly, the lactate taken up by the brain is metabolised by neurones as it is not accumulating within the cerebrospinal fluid or within the brain tissue. Breakdown of MR during exercise, however, does not depend on the lactate taken up by the brain. During prolonged exercise, there is only a modest or no increase in blood lactate, and accordingly, little lactate uptake by the brain. Yet MR decreases at exhaustion during prolonged exercise as during intense exercise of shorter duration.

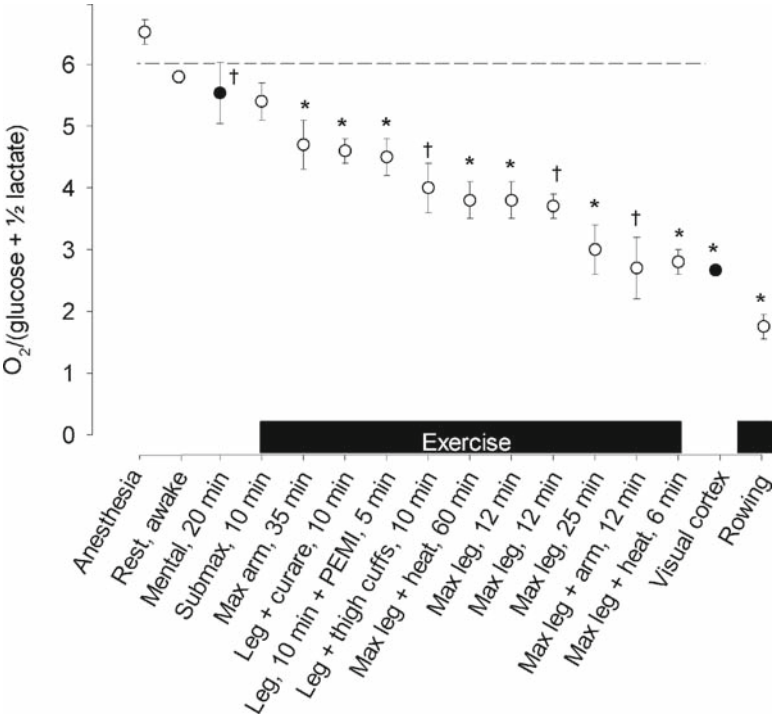

Fig. 19 The cerebral metabolic ratio (brain uptake of O_2 relative to that of carbohydrate; glucose+1/2 lactate) during general anaesthesia, at rest and during various types of brain activation including several types of exercise with ergometer rowing demonstrating the largest deviation from the resting value of 6

13.3 Brain Glycogen Metabolism

During muscle contractions part of the energy is derived from glycogen breakdown, and during sustained muscle work fatigue presents when glycogen deposits are depleted. Similarly, the brain glycogen level decreases during neuronal activity. The human brain glycogen concentration is ~6 mM (glucosyl units) in grey and white matter and as high as 13 mM in the hippocampus. Therefore the total glycogen content in the brain is estimated to ~10 mmol. In the brain glycogen is confined to the astrocytes, thus, the intracellular concentration of glycogen in the astrocytes may approach that of skeletal muscle, underpinning its potential importance as a fast accessible energy reservoir (Fig. 20).

In analogy with skeletal muscle, central fatigue could be provoked when the brain glycogen level reaches a critically low level. By provoking such a crisis during exercise in hypoxia, isometric contractions become more affected than fast contractions, suggesting that recruitment of ST fibres is more vulnerable to central fatigue than that of FT muscle fibres. Thus, central fatigue represents an exception to the

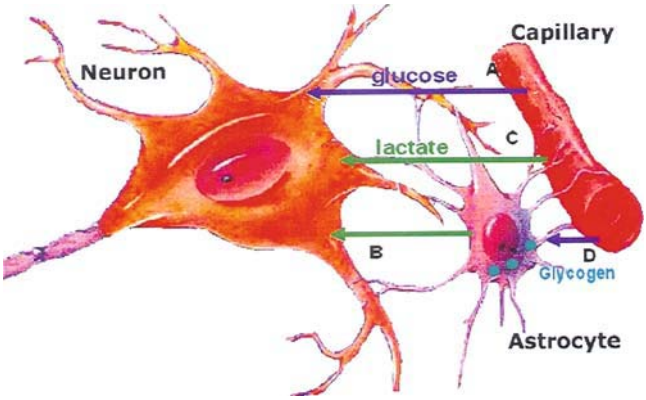

Fig. 20 Lactate from the blood and the astrocytes is taken up by activated neurons

normal "orderly recruitment" of muscle fibres where ST fibres are recruited before FT muscle fibres. Inability to maintain ST muscle fibre recruitment explains why fatigue manifests as a difficulty in preserving a smooth movement.

Brain temperature is also important, and work is stopped when its temperature approaches 40°. Conversely, proper hydration allows for evaporation of water and control of temperature while training, which may be interpreted as preparation of the cardiorespiratory system to provide O_2 and substrates to working muscles so that work can be carried out without the brain being aware of the effort. Taken together, the cardiorespiratory system has the capacity to increase VO_2max by modulating each of the different steps in the transport system, with the exception of the pulmonary system, which is the only organ for which adaptation to endurance training has not been demonstrated.

14 Diving Response

A series of cardiovascular and respiratory adaptations permit mammalian air-breathers to perform extended aquatic dives. Among them is the "diving response", consisting of selective vasoconstriction, which induces preferential shunting/blood shifts/thoracic filling, and bradycardia, which limits overall oxygen consumption and protects the heart and the brain from asphyxia. In humans, the response is induced by breath-holding while immersing the face in cold water. The magnitude of heart rate reduction is a measure of the diving response and is greater in diving than in terrestrial species. Humans, untrained in apneic diving, react with a heart rate reduction of between 20 and 30% from the resting level, during diving or apnea with face immersion in cold water. Trained human divers exhibit a bradycardia of 40–50%, which is in the range of the responses found in some semi-aquatic mammals. As the response in man reaches its maximum in water of 10°C, and considering that

most apneic diving occurs in relatively warm waters, the practical application of the diving response in humans is questioned while a beneficial effect is that it enhances cerebral perfusion.

It seems of importance for the magnitude of response whether the dive is performed during exhalation or inhalation. Diving mammals dive in the expiratory position: seals, for example, exhale before diving in an apparent attempt to accentuate the bradycardic response. In humans however, this has not been demonstrated, and the issue remains elusive as breath-holding at residual volume tends to increase rather than decrease heart rate.

Nevertheless, this mechanism delays the fall in alveolar and arterial PO_2 and, thereby, the development of hypoxia in vital organs. In conclusion, it seems that the human diving response has an oxygen-conserving effect during exercise.

15 Altitude

Ascent to high altitude entails exposure to a reduced barometric pressure and hence a reduced tension of O_2 in the inspired air that causes arterial hypoxemia, which provokes hyperventilation and leads to respiratory alkalosis. Breathlessness is conspicuous even during mild exercise. During incremental exercise the increase in exercise V_E leads to the ventilatory capacity being reached at a relatively low rate of work. Exercise is then limited mainly by the function of the lungs. Lactacidemia also occurs, but the rise in blood lactate concentration is reduced.

During exercise a number of factors contribute to the accentuation of the hypoxemia, which causes tachycardia and an increase in cardiac output relative to VO_2. The pulmonary pressure rises, thus contributing to ventilation–perfusion inequality, which in turn aggravates the hypoxemia further. Also, the conditions for O_2 transfer from the alveolar gas into the blood in the pulmonary capillaries are compromised as there is a diffusion limitation to gas transfer. Under these conditions the maximal work rate is compromised in inverse relation to the subject's pulmonary diffusion capacity.

The acute effects of exposure to altitude are ameliorated by acclimation. The respiratory alkalosis is corrected by the secretion of alcalic urine while the desaturation is corrected by increased erythropoiesis that results in polycythemia in response to enhanced production of EPO. Yet the immediate increase in haematocrit at high altitude is related to a loss of plasma volume. Also, a rise in the plasma concentration of 2–3 diphosphoglycerate partly reverses a displacement of the O_2 dissociation curve, which has been provoked by the prevailing blood gas changes. Following these adaptations submaximal exercise V_E is further increased and the hypoxemia is slightly reduced as a consequence. The cardiac output returns to its normal level.

Under normal circumstances at an altitude of 1,500 m mild exercise can be performed without acclimation but acclimation increases the exercise capacity. At 3,000 m mild exercise initially provokes an exaggerated physiological response and the ability to perform skilled tasks is impaired. In addition if the ascent is rapid acute mountain sickness pulmonary and cerebral oedema can develop.

16 Heat and Cold

During sustained heavy exercise blood flow is diverted to the skin at the expense of the active muscles as fluid is lost as sweat. These processes are rendered less effective by a hot, humid environment. As the central blood volume is reduced, heart rate relative to VO_2 is increased and VO_2max is reduced. There is an associated rise in deep body temperature, which causes reflex tachypnea and increased V_E during submaximal exercise. The maximal exercise V_E is little changed and the O_2 cost of different activities is relatively normal. Exercise tolerance, however, is limited by an inability to control brain function.

A cool environment has the converse effect of increasing the capacity for prolonged exercise. Immersion in water has the additional consequence of heat loss by conduction. In these circumstances the temperature at which thermal equilibrium can be maintained is usually relatively high, around 20°C. Exercise, instead of offsetting the heat loss, may actually augment it by increasing the water flow across the skin. The tolerance of immersion depends critically on the quantity of subcutaneous fat, with fat individuals being much better protected than those who are lean. Endurance training increases tolerance to both heat and cold, but acclimation to heat and cold appear not to affect exercise performance.

17 Genetic Influence

Human form and function are dictated by the interaction of genes with environmental stimuli. Thus variation in environmental exposures will strongly influence phenotype. However, although a core genetic inheritance is common to all humans, small functional variations in certain genes dictate that individual responses to environmental challenges will differ. Such genetic differences influence exercise performance through associated differences in anatomy and physiology and their response to training stimuli. The human gene map is growing in complexity as there are now in excess of 100 gene variants related to human performance.

However, even though there is evidence suggesting that to some extent VO_2max is heritable, identification of specific genes is less convincing. Despite the initial focus on the angiotensin converting enzyme (ACE) gene and its possible association with endurance performance, it is clear that many successful endurance athletes do not have the “endurance genotype”.

18 Health

Exercise continued throughout life attenuates the age-related deterioration of physiological functions and may favour life expectancy. Harvard University oarsmen have been reported to live three to six years longer than the American life expectancy in

the early nineteenth century. The only contradictory finding of a somewhat lower average age at death of Cambridge University crew oarsmen than in a random group of people (67.1 vs 67.4 years) from the same time period can be attributed to their engagement in the First World War. Nevertheless, having considered the interference of world affairs, the death rate from cardiovascular disease is lower in athletes than in a random control group of people, and such observations are now available from participants in a large range of activities. Thus, in perspective studies there is a direct relationship between lifespan and physical activity, including a reduced prevalence of certain types of cancer, such as colon cancer. An early observation was that the conductors of London's double-decker buses lived longer than the drivers, and similar observations show a progressive increase in the lifespan of international championship participants depending on the type of sport, ranging from power sports to ball games and endurance events. It may be, however, that such comparisons reflect evaluations of participants with different genetic make-up. In that light a specific polymorphism in the gene encoding the ACE resulting in low-range values of angiotensin in plasma is over-represented in successful competitive rowers. On the other hand, the "deletion" allele, producing relatively high levels of angiotensin, has been identified as a risk factor for the development of cardiovascular diseases.

Peak bone mass is established in both men and women around the age of 30 years and subsequently declines by $\sim$0.5% per year in men and by $\sim$1% per year in women. The decline in bone mass observed throughout life contributes to the elevated incidence of osteoporotic fractures. Physical activity, especially weight-bearing activity, is an essential requirement for maintained bone mass.

As body mass increases with age, probably due to reduced physical activity, the risks of falling, fracture and limb disability, as well as morbidity from major chronic diseases and even mortality, are increased. Regular exercise provides for the maintenance of low body mass. Obesity is linked with numerous chronic diseases including type II diabetes, hypertension and cardiac disease, and thereby to mortality. In old compared with young athletes body fat is similar to that of young sedentary men and lower than in sedentary men matched for age and body size (18% vs 23%).

Ageing is also characterised by a decrease in fat-free mass. In spite of lower fat-free mass, elderly individuals who engage in regular physical activity have a larger fat-free mass than elderly sedentary men.

Regular physical activity has also a profound effect in preventing muscle wasting as demonstrated by a larger area of leg extensor muscle compared with sedentary men (78 vs. 68 cm^2). The skeletal muscle structural changes with aging are also reflected in the arm, leg, and back strength declining at an overall rate of 8% per decade, starting in the third decade of life. The ability of the leg extensor muscles to develop power is of relevance as it enables the elderly to accomplish daily tasks. The loss of leg extension power increases the risks of falls and limb disability. The age-related decline in muscle power is not prevented by regular physical activity; leg extension power in older compared with young individuals (1675 vs. 2358 W). The parallel deterioration of skeletal muscular size and function is supported by a relationship between leg extension power and extensor muscle area.

Elderly individuals who engage in regular physical activity have a slower decline in $VO_{2\,max}$ compared with sedentary elderly. For example, elderly oarsmen have a lower $VO_{2\,max}$ compared with young oarsmen (3.0 vs 4.11 min^{-1}, Table 1) but their $VO_{2\,max}$ is larger than in sedentary men matched for age and body size (3.0 vs 2.21 min^{-1}) and similar to young sedentary men.

Elevated concentrations of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and a reduced concentration of high-density lipoprotein cholesterol (HDL-C) in plasma, are risk factors for coronary artery disease. The LDL-C is implicated in plaque formation in blood vessels, while the HDL-C is involved in plaque removal. Thus, the ratio of LDL-C to HDL-C, or that of TC to HDL-C, or the atherosclerosis index indicates, risk for coronary artery disease. Lifelong physical activity fosters attenuation in risk factors for coronary artery disease in elderly active individuals (LDL-C/HDL-C, 1.7; TC/HDL-C, 3.1) compared with both older and young sedentary men.

Leptin is a protein produced by "the obesity (ob) gene" and its secretion from adipose tissue to the circulation is considered to signal the brain regarding the size and the nutritional status of adipose tissue, a signal that seems to be lost or inhibited in people who become very fat. Physical activity continued into advanced age has a protective effect on the age-associated increase in fat mass and serum concentration of leptin, as reflected by the lower leptin values in older athletes compared with sedentary men matched for body size.

Insulin reduces glucose in the blood by facilitating its transport into the cells. There is an inverse relationship between the level of habitual physical activity and the incidence of type II diabetes. Regular physical activity benefits the insulin-stimulated whole-body glucose uptake as shown by a lower fasting plasma glucose and insulin in older athletes.

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