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.

S.F. Perry (✉)
Department of Biology, Centre for Advanced Research in Environmental Genomics, University of Ottawa, 150 Louis Pasteur, Ottawa, ON,K1N 6N5 Canada,
E-mail: sfperry@uottawa.ca

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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

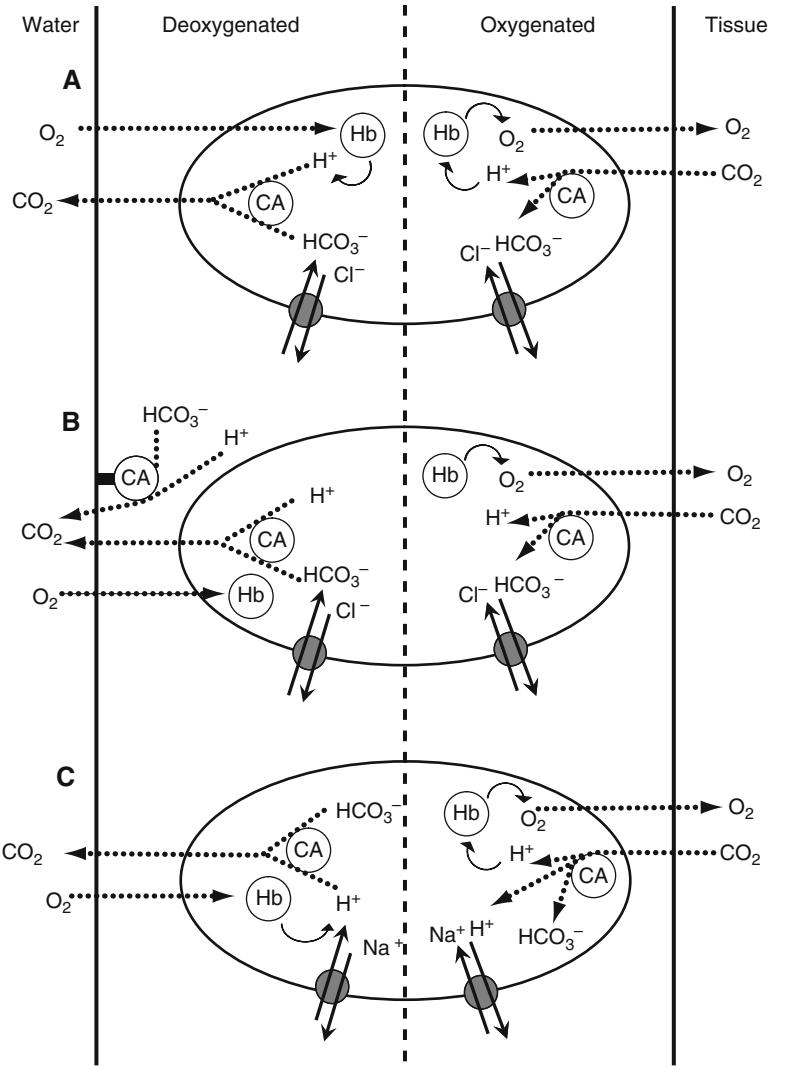

Fig. 1 A schematic model of CO_2 excretion in (**a**) teleost fish, (**b**) elasmobranch fish, and (**c**) the lamprey, an agnathan fish. Oxygen movement is from left to right, i.e. from water to blood at the gill, and then from blood to tissue, while CO_2 movement is in the opposite direction. Carbonic 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.

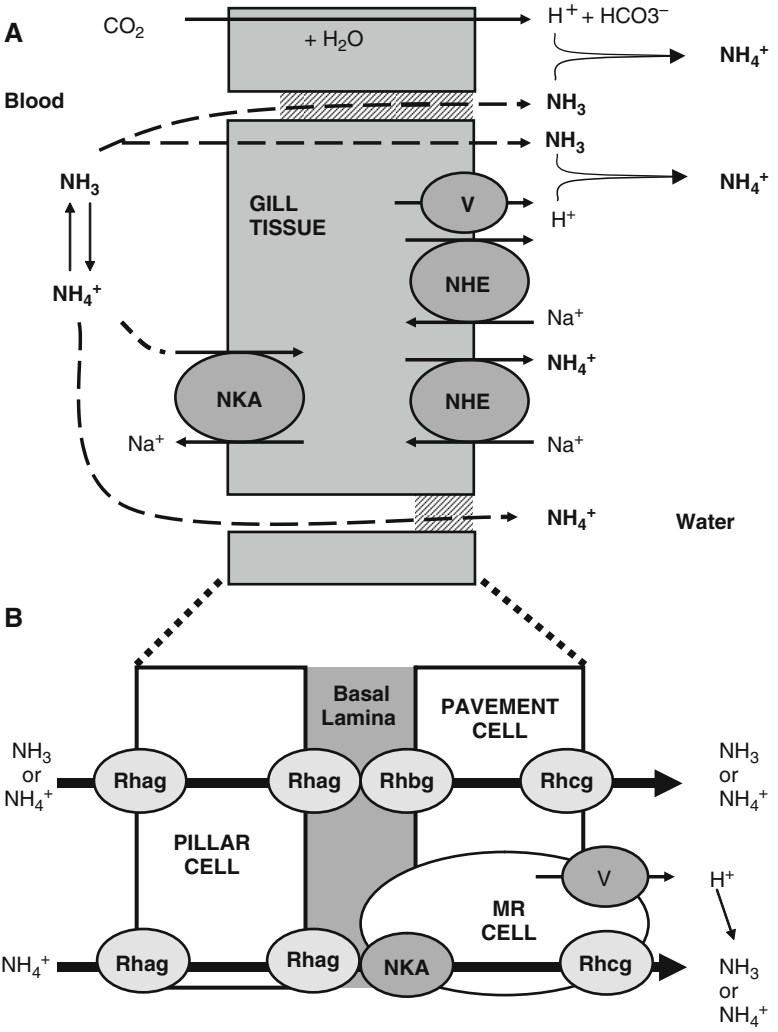

Fig. 2 **A** Schematic model of ammonia movement through fish gills. *Dashed lines* indicate diffusion, while movement through pumps and exchangers is represented with *solid lines*. NH_3 diffuses across the gills down the blood-to-water partial pressure gradient, where in many fish it is trapped as 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

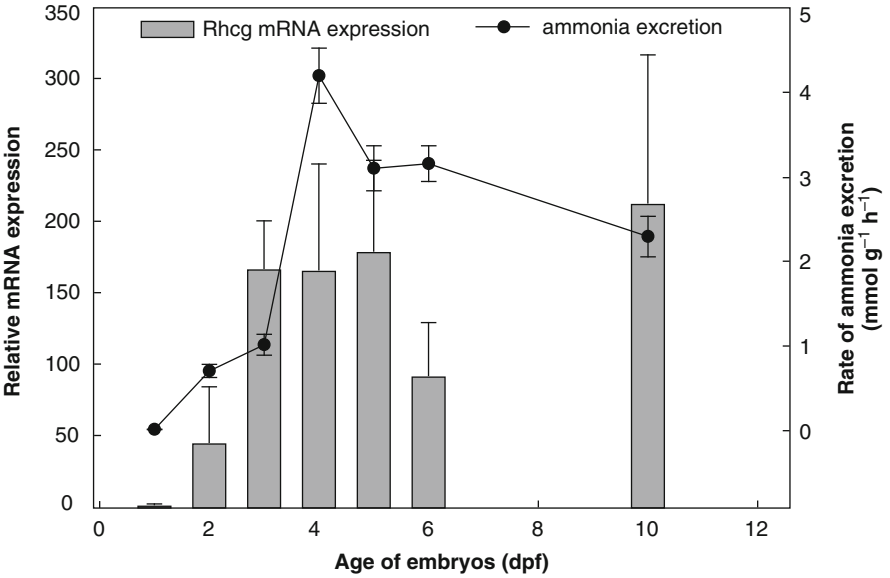

Fig. 3 A comparison of Rhcg mRNA expression and ammonia excretion during development in zebrafish (*Danio rerio*) embryos. Expression levels were measured using real-time RT-PCR and have been calculated using the delta–delta Ct method relative to the expression of Rhcg at 1 day post-fertilization (dpf). 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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