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Carlos Arturo Navas
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Aestivation

Molecular and Physiological Aspects

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Foreword

Interest in the phenomenon of aestivation dates back to at least 300 years. But it was not until about 1960 that serious and sophisticated investigations addressed the morphology, physiology, and biochemistry of organisms that appeared to be able to put life "on hold." These investigations were, initially, seemingly random and conducted in a wide variety of organisms. But, eventually, a few "favourite" organisms, vertebrate and invertebrate animals, emerged, and the studies focused somewhat and continued until the early 1980's. But remarkably, during this 20-year period, despite a plethora of data demonstrating metabolic depression, the active and close-knit group of talented comparative physiologists and biochemists involved, were refractory to the concept. The penny dropped somewhere between 1980 and 1982, and the term metabolic depression entered the comparative scientists' lexicon. It is now considered to be the defining characteristic of organisms that use some sort of dormancy as a response to environmental stress. Metabolic depression has now been reported in most of the major invertebrate phyla and in all vertebrate classes.

By its very nature, a reversible and large depression of metabolism, necessarily, comprises large changes in the rate of flux of various molecules between the organism and its environment (nutrients, gases, water, and excretory products), between organs (again nutrients, gases, and excretory products), and within cells (the biochemical pathways associated with energy metabolism, fuel and protein synthesis, oxidative stress, excretory production, DNA replication, and RNA synthesis). Associated with these changes in flux, are significant qualitative changes. Molecules that would otherwise be excreted must be stored, or replaced by, perhaps, more soluble or less noxious alternatives, and unstable molecules that are usually turned over rapidly, either must be stabilized or the organism must do without them temporarily. Also affected are some higher-order structures and structural components, that must recover from long periods of inactivity.

Aestivation and the associated depression of metabolism, therefore, represent a model which offers naturally-occurring, temporary, and major quantitative and qualitative perturbations of a variety of biological processes. This is ideal for the study of how these processes, that comprise living organisms, are regulated; and represents an opportunity to determine how flexible these processes can be and still support life. The available scope is appreciated, and researchers have assiduously applied emerging techniques to the problem. Investigations of these organisms

continues at all levels, from morphology, through gas exchange and metabolic control, to the regulation of protein synthesis and gene expression.

Many, if not most, of the mechanisms elucidated in these organisms, will be applicable across the biological spectrum. This area of research, therefore, rather than being seen as esoteric, is now recognized as an effective way of uncovering general regulatory principles. It is also proving to be a useful adjunct to the study of the causes of, the treatment of, and the recovery from, injury and disease in humans. For example, recovering from immobility is a common problem, and some cancer cells may be difficult to treat because they metabolically depress in response to hypoxia.

But last, and certainly not least, these studies show us how these organisms work. As mentioned previously, they represent a wide cross section of the biological spectrum, and inhabit many different habitats. If we are to manage, conserve, and enjoy these varied habitats, we must understand the organisms that inhabit them.

Australia

Prof. Michael Guppy
University of Western Australia

Preface

Our interest in aestivation was initially subsidiary, even if marked by deep admiration to the pioneering herpetologists of the 1960s and the founders of metabolic control theory. However, when we became interested in real problems such as the metabolic annual cycles of tegu lizards or the colonization of the Brazilian semi-arid (Caatingas) by amphibians, our readings of older literature gained a new dimension. Our research in the Caatingas had the pros and cons typical of highly underexplored areas, and began, literally, with the search for aestivating frogs digging rather randomly in huge landscapes. As our knowledge of basic natural history progressed, we perceived obvious differences among individuals captured in the middle of the dry season. Species differed in microhabitat choice, inclination for activity, characteristics of the skin, and several other behavioral and morphological aspects that, together, suggested remarkable ecological and physiological diversity. We induced that ecology should modify deeply the type and magnitude of the physiological challenge experienced by aestivating frogs in such semi-arid environment and, as a corollary, that aestivation could involve a complex array of physiological states. As our field work progressed, we confirmed the immense value of the integrative eco-physiological approach that was proposed by early researchers of anuran aestivation. In parallel, we appreciated the importance of understanding the mechanisms leading to metabolic arrest, and opened our eyes to newer approaches and problems that emerged later in the field of aestivation. It was pretty much at this state of reflection that we were contacted by the Springer Series editor regarding the possibility of editing a book on the topic of aestivation, a proposal that we soon accepted.

When we accepted to be editors of this volume, we were conscious of the difficulties to fully track the advances on aestivation physiology, given that the field focuses on questions that concern different levels of biological organization, and the dialogue between such levels is not always fluid. Moreover, information about physiological mechanisms and their control involve only a few systematic groups and even so results are rather disperse in the specialized literature. Thus, our goal was to produce a book in which authors through the world were represented, and in which chapters covered a substantial part of the approaches, levels of organization and systematic groups, and that characterize the field's state of art. Our quest to produce a truly international volume and to increase topical diversity was very

fruitful, yet we regret that this editorial policy, together with limits in the number of chapters that could be included, restrained our ability to invite all possible leading authors working in the fascinating topic of aestivation. Even within this constrains, this volume does include many of the different problems that characterize current views on aestivation, and we hope very much it appeals to a broad audience, not only researchers of aestivation but also graduate students and researchers who have collateral interests in this topic.

This book includes topics ranging from the study of the fossil register by Daniel Hembree, to control of gene expression by Kenneth Storey. In the first chapter, Phil Withers and Chris Cooper provide a historical overview of the concept of metabolic depression, a central aspect in aestivation. Next, Marlize Cravo, Alexis Welker, and Marcelo Hermes-Lima discuss the protective mechanisms against oxidative stress in aestivating animals, whereas Ip Yuen Kwong and Shif Fun Chew address nitrogen metabolism and other aspects of excretion in several aestivators. The morphological plasticity of vertebrates organ is discussed by Stephen Secor and Jean-Herve Lignot, and Rob James reviews muscular function during aestivation. Jeff Richards presents a chapter on the occurrence of aestivation in fishes, and us, together with Isabel Pereira, review amphibian aestivation. Current concepts on endotherm aestivation are analyzed by Fritz Geiser, while Stephen Loomis discusses aestivation in sponges, and Joshua Benoit presents a paper on water management in dormant insects. We are very thankful to all these authors and hope that their intense work is rewarded with a book that will become a good general reference in the area. We also appreciate the feedback of Márcio Reis Custódio, the series editor, and the Springer-Verlag staff. To the reader, we wish a pleasant journey through the world of aestivation.

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Chapter 1
Metabolic Depression: A Historical Perspective

Philip C. Withers and Christine E. Cooper

Contents

Abstract An extended period of inactivity and reduced metabolic rate of many animals and plants, as well as unicellular organisms, has long been recognized by natural historians, e.g., Aristotle and Pliny. Biologists have studied this phenomenon since the 1550s (Gessner) and 1700s (Van Leeuwenhoek, Buffon). The period of inactivity can be less than a day, a few consecutive days or weeks, an entire season, or even many years. It can involve very different physiological states in response to a variety of environmental stimuli, such as extreme temperatures or unavailability of food or water. These periods of inactivity have been described and classified according to the group of organisms in question, extent and duration of the metabolic depression, ambient and body temperatures, state of body water (frozen or hyperosmotic), or availability of oxygen. Cryptobiosis, or "hidden life," is an extreme form of inactivity, with often complete cessation of metabolism. It was first described in the 1700s, was further characterized in the 1800s, and in the 1900s physiological studies delineated the extent of metabolic depression. Molecular mechanisms for cryptobiosis have been sought since the late 1900s. Cryptobiosis includes three physiological states, anhydrobiosis (desiccation), osmobiosis (high osmotic concentration), and cryobiosis (freezing), where metabolic depression is associated with an altered physical state of cell water and

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often involves accumulation of compatible solutes, and one physiological state, anoxybiosis (anoxia), where metabolic depression occurs at the normal cellular hydration state. Dormancy (torpor) is a less extreme form of inactivity, associated with a moderate reduction in metabolic rate (hypometabolism). Although first described by Aristotle and Pliny, studies in the 1900s delineated the basic physiological changes that accompany dormancy. Dormancy allows avoidance of unfavorable short- or long-term climatic conditions and conservation of energy and water. Hibernation is long-term multiday torpor during winter, whereas aestivation is dormancy during summer. In ectotherms, the metabolic depression that accompanies dormancy is intrinsic, with metabolic rate declining to about 10 to 20% of resting metabolic rate at the same body temperature. The molecular mechanisms for intrinsic metabolic depression are poorly understood. In endotherms, torpor involves a fundamental physiological change in body temperature regulation that markedly reduces metabolic rate and water loss, often to <10% of the normothermic resting metabolic rate at the same ambient temperature. Most of this reduction in metabolic rate reflects the decreased setpoint for thermoregulation resulting in reduced metabolic heat production and a Q_{10} effect; there may be some intrinsic molecular-based metabolic depression in some hibernators. Dormancy allows species to exploit ephemeral environments and colonise habitats that would otherwise be unsuitable for growth or survival at certain times of the year. There are costs to dormancy, but for many species, the energetic and hygric advantages outweigh these costs.

1.1 Introduction

An extended period of inactivity and reduced metabolic rate is a widely recognized behavioral and physiological state in both animals and plants. Aristotle (384–322 BC), and then Pliny (23–79 AD), first described hibernation for mammals, and also erroneously for a variety of birds that disappeared seasonally (they actually migrated elsewhere during winter). More rigorous scientific observation in the 1500s (Gesser) and 1700s (Buffon) confirmed that many mammals hibernate. The invention of glass lenses and the microscope in the late 1500s/early 1600s allowed observations of various small microorganisms and animals, including seemingly lifeless invertebrates (rotifers, tardigrades, etc) that could be rehydrated and would then recommence activity. Such observations led to claims in the 1700s and 1800s of resurrection of dead animals, and spontaneous generation of life, but careful scientific study dispelled the claims of resurrection of life from dead organisms (Commission of the Biological Society of France in 1860) and spontaneous generation of life (Pasteur in 1864). Many subsequent physiological studies in the 1900s and 2000s have confirmed profound behavioural and physiological inactivity in rotifers, tardigrades, and nematodes, as well as larger animals, bacteria, fungi, and plant seeds (Keilin 1959).

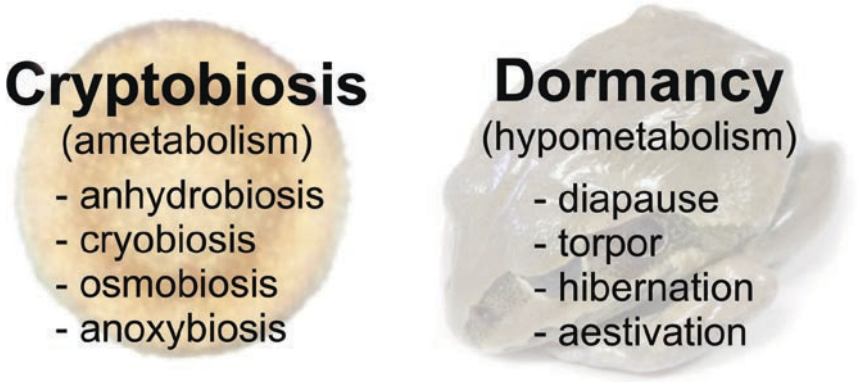

Fig. 1.1 Schematic summary of types of cryptobiosis, which is an ametabolic state (e.g., an anhydrobiotic *Artemia* cyst), and dormancy, which is a hypometabolic state (e.g., an aestivating *Cyclorana* frog). Terminology adapted from Keilin (1959)

A confusing array of different terms has been used to describe these periods of inactivity, depending on the group of animals in question, the extent and duration of the metabolic depression, the body and ambient temperatures (low or high), the physical state of body water (frozen or hyperosmotic), and the availability of oxygen. Two different states of inactivity and metabolic depression can be discerned, cryptobiosis and dormancy (Keilin 1959; Fig. 1.1). Cryptobiosis, which means "hidden life," is an extreme behavioral and physiological state, with no activity and essentially no metabolism. Dormancy describes a state of reduced (but not complete lack of) metabolism, i.e., hypometabolism. Thus, there would appear to be a continuum in the potential metabolic state of organisms, from complete absence of metabolism, through hypometabolism, to the normal resting metabolic rate.

1.2 Cryptobiosis

The study of cryptobiosis (or abiosis) started with observations by van Leeuwenhoek (1702) of certain "animalcules" (wheel animals, rotifers) that he found in dry sediment in roof gutters, in an apparently lifeless state but which resumed normal activity when rehydrated (Schmidt 1948; Keilin 1959; Tunnacliffe and Lapinski 2003). Cryptobiosis is a state of "suspended animation" associated with complete inactivity and metabolic depression; it is most commonly observed for invertebrate animals (Keilin 1959) and is often a strategy to survive seasonal cold or desiccation. The similar term anabiosis, or "return to life" (Preyer 1891), describes the resurrection of apparently completely lifeless organisms. Van Leeuwenhoek did not, however, suggest that these "lifeless" animals were completely desiccated, nor did he describe this state in terms of latent life or resurrection. His observations were followed about a half century later by descriptions of microscopic nematodes (eelworms,

Anguillulina tritici) in an apparently lifeless state; these "lifeless" white fibres in grain quickly resumed activity when rehydrated, although in their desiccated state they crumbled to powder if disturbed (Needham 1743; Baker 1753).

The extremely depressed metabolic state of cryptobiosis is often related to a dramatic change in the state of cell water through desiccation (anhydrobiosis), osmotic stress (osmobiosis), or freezing (cryobiosis), although metabolic depression due to a lack of oxygen (anoxybiosis) occurs under conditions of normal cell-water. Cryptobiosis has independently evolved several times, within bacteria and protists as well as many multicellular plants (mosses, lichens, liverworts, higher plants) and animals (nematodes, rotifers, tardigrades, crustaceans, insects; Clegg 2001; Rebecchi et al. 2007). A few remarkable organisms are able to survive all of these forms of cryptobiosis, e.g., diapaused cysts of brine shrimp (e.g., Clegg et al. 1996; Clegg 1997) and tardigrades (Nelson 2002).

Anhydrobiosis is a response to desiccation (loss of cell water). It has been observed for a variety of invertebrate animals and plants during extreme desiccating conditions (Van Leeuwenhoek 1702; Spallanzani 1776; see Keilin 1959; Rebecchi et al. 2007), but not vertebrates. Spallanzani (1776) described "resurrection" by cryptobiotic rotifers and tardigrades, which had a remarkable tolerance of high temperatures and could survive in a vacuum. The French biologists Doyère and Porchet resumed studies in the 1850s of resurrection by desiccated rotifers and tardigrades, but came to the different conclusions that these microorganisms could survive complete desiccation and cessation of life processes (Doyère) or that no organism can survive complete desiccation and be resurrected after the cessation of life processes (Porchet). A Commission of the Biological Society of France examined these counter-claims and concluded that the resistance of rotifers and tardigrades to high temperatures increased with the extent of desiccation – rotifers could be resurrected after 82 days of desiccation in a vacuum followed by exposure to 100°C for 30 min, before rehydration (Keilin 1959). Gavaret (1859) extended these observations of extreme tolerance to 50 days in a vacuum over sulphuric acid, then heating to 110°C. The best known example of cryptobiotic animals is probably the desiccated "eggs" (actually cysts of about 3,000 cells in an arrested stage of early development) of brine shrimp (*Artemia*) which can survive extended periods of complete desiccation and are also remarkably resistant to extremes of temperature. Anhydrobiotic organisms can be remarkably long-lived (e.g., 30 years for a nematode (*Tylenchis polyhypnus*; Steiner and Albin 1946), and tolerant of physical and chemical extremes e.g., very low temperatures (<−200°C) and high temperatures (>100°C) and hydrostatic pressure (>500 MPa), and irradiation (see Rebecchi et al. 2007).

Various physiological and biochemical aspects of anhydrobiosis have been reviewed by Womersley (1981) and more recently by Tunnacliffe and Lapinski (2003), Watanabe (2006) and Rebecchi et al. (2007). Many anhydrobiotic organisms must control their evaporative water loss during entry into anhydrobiosis using behavioural, physiological, or morphological adaptations. For example, tardigrades contract into a "tun" when they dehydrate, rotifers have a similar dehydrating behavior, and nematodes coil or aggregate to reduce the rate of water loss. Compatible solutes, such as trehalose, sucrose, or glycerol, are generally accumulated during anhydrobiosis.

These solutes presumably structure cell water and protect against intracellular damage, as well as retarding molecular motion for chemical reactions. Some rotifers, plants, and microorganisms, however, are desiccation tolerant in the absence of accumulated protectant solutes, so further studies are required to determine their molecular mechanisms for anhydrobiosis (Tunnacliffe and Lapinski 2003).

During anhydrobiosis, the metabolic rate is often so low as to be unmeasurable. The ametabolic state and resistance to thermal extremes can be attributed to the altered physical state of cell water. Desiccated *Artemia* cysts have a cell water content less than 0.15 ml g^{-1} of dry mass, and the remaining water is tightly bound to cell proteins (Clegg 1975). The lack of cell water as a solvent in these cysts presumably precludes molecular movement, and hence cell metabolism. In contrast, at a water content of 0.15–0.6 ml g^{-1} of dry mass, there is not only bound water but also water loosely associated with intracellular structures; this vicinal water provides limited hydration and mobility of cell metabolites, and, hence, supports limited metabolism. At higher cell water contents, the presence of bulk water allows free solute movement and maintenance of the normal metabolic rate. For example, the metabolic rate of prediapause cysts (approx. 4 µg dry mass) of 0.036 µl $O_2 h^{-1}$ is reduced to approximately 0.007 µl $O_2 h^{-1}$, then is further reduced for dormant encysted embryos to 0.0001 µl $O_2 h^{-1}$ or even less by 5 days after deposition (Clegg 1976, 1978, 1997; Glasheen and Hand 1989; Clegg et al. 1996).

Some plant seeds, particularly of annual species, are also spectacularly resistant to desiccation; they may survive with as little as 5–10% water content and remain viable despite desiccation for very long periods, e.g., 50–200 years (Becquerel 1907; Ewart 1908; Turner 1933). Indian Lotus seeds, some perhaps more than 1,000 years old retrieved from an ancient lake bed in China, can still germinate (Ohga 1923; Keilin 1959). Many desert plants survive extended periods of high temperature and low rainfall. These "resurrection" plants, such as the Rose of Jericho (*Selaginella* spp) and pincushion lilies (*Boryia* spp) can desiccate to about 5% water content during dry periods, but survive and "come back to life" after rain.

Two other forms of cryptobiosis that also involve an altered state of cell water are freezing (cryobiosis, e.g., frozen insects and frogs) and high osmotic concentration (osmobiosis, e.g., brine shrimp cysts in a salt lake). Both involve perturbation of the physical state of water in the intracellular environment, which presumably results in the extreme metabolic depression. Both freezing and high osmotic concentrations require special adaptations for survival.

Many animals (and plants) can survive freezing temperatures (see Storey and Storey 1996). Anecdotal reports of animals surviving freezing date back to Pliny (frozen fishes; see Claussen et al. 1990). Some organisms supercool or have antifreeze solutes to avoid freezing. A super-cooled state would be associated with a substantial reduction in metabolic rate due to the Q_{10} effect. However, some remarkable animals and plants tolerate actual freezing of their extracellular fluids. Henry Power, in 1663, observed that vinegar eelworms (probably the nematode *Turbatrix aceti*) survived freezing (Wharton 2002) while Réaumur (1737) first reported freeze-tolerance for an insect. The first convincing report of freeze-tolerance for a vertebrate was the European wall lizard *Podarcis muralis* (Weigmann 1929;

Claussen et al. 1990). Insects (e.g., Arctic wooly bear caterpillars, *Gynaephora groenlandica*) and the New Zealand alpine weta (*Hemideina maori*) survive freezing for up to 10 months of the year (Ring 1981; Duman et al. 1991; Zachariassen 1985). Intertidal invertebrates such as gastropods, mussels, and barnacles routinely freeze when exposed at low tide (Aarset 1982; Loomis 1987). Some nematodes, slugs, and centipedes also tolerate freezing. Amongst vertebrates, some amphibians (*Rana*, *Pseudacris*, *Hyla* spp) and reptiles (turtles, lizards and snakes) can survive freezing (Schmid 1982; Costanzo et al. 1988; Storey et al. 1988; Claussen et al. 1990; Churchill and Storey 1992; Costanzo et al. 1985; Dinkelacker et al. 2005).

Freeze-tolerant animals can only withstand freezing of their extracellular fluids as any ice formed within cells disrupts cell membranes and destroys intracellular integrity (Storey and Storey 1996). As ice forms, solutes are excluded from the ice-crystal structure and this increases the osmotic concentration of the unfrozen extracellular fluids, thereby lowering its freezing point. Intracellular fluid remains in osmotic equilibrium with the extracellular fluid, which consequently also becomes osmo-concentrated. Some freeze-tolerant animals have specific ice-nucleating agents in their extracellular fluids, to promote freezing there rather than inside cells. Extracellular freezing is promoted by ice-nucleating proteins in the tardigrade *Adorybiotus coronifer* (Wright 2001). The increased osmotic concentration of the extracellular fluids withdraws water from inside cells by osmosis, lowering its freezing point but also decreasing the cell volume. For most freeze-tolerant animals, the critical minimum cell volume is when about 65% of the total body water is frozen. Compatible cryoprotectant solutes can be used as "antifreeze" to decrease the freezing point of the body fluids and to facilitate tolerance of tissue freezing by preserving macromolecular structures within the cells (whereas high concentrations of many solutes would perturb the structure and function of the intracellular macromolecules). For example, the spring peeper frog (*Pseudacris crucifer*; Churchill and Storey 1996) and wood frog (*Rana sylvatica*; Storey and Storey 1988) release glucose from their liver in response to ice forming on its skin; the 450 times increase in blood glucose provides a compatible cryoprotectant function (it also provides locally available nutrients during thawing before the circulatory system becomes functional again). Some *Hyla* accumulate glycerol, as do many insects. Insects can also accumulate other compatible solutes such as polyols (mannitol, sorbitol) and sugars (e.g., trehalose; Storey and Storey 1989, 1990, 1991). There is likely a role of intracellular cryoprotectants (but not trehalose) in freeze-tolerance of the tardigrade *Adoribiotus coronifer*. Many plants also accumulate compatible solutes in their fluids to prevent freezing during winter, or tolerate freezing of water in their xylem and other extracellular water pools.

Metabolic rate is reduced for frozen insects and frogs; tissue ATP declines during freezing, and anaerobic end-products accumulate (Storey and Storey 1986, 1988). Freeze tolerance by hatchling turtles is associated with, but apparently is not limited by, the anoxic tolerance of their tissues (Dinkelacker et al. 2005). These freeze-tolerant animals have essentially complete metabolic depression, not just because of the low ambient temperature and its depressing Q_{10} effect, but also because of the impairment of circulatory exchange of O_2 and CO_2 as well as other nutrients and waste products, and the hyperosmotic state of the intracellular fluids.

Osmobiosis requires that animals are tolerant of a high ambient osmotic concentration (Keilin 1959). For example, the metabolic rate of hydrated *Artemia* cysts during osmobiosis (exposed to 5 mol l^{-1} NaCl) is only 0.00009 μl O_2 h^{-1} (Glasheen and Hand 1989). The obligate parasitic nematode *Steinernema carpocapsae* has similar tolerance of osmotic stress, at least for nonionic solutes such as glycerol and polyethylene glycol (Glazer and Salame 2000). Soil nematodes tolerate osmotic concentrations of up to 1 M (e.g., Van Gundy 1965). Many freshwater and terrestrial tardigrades form a resistant tun when exposed to concentrated salt solutions (Wright et al. 1992). The hyperosmotic tolerance of osmobiotic animals is similar to that of cryobiotic animals, and extracellular and intracellular compatible cryoprotectants are also important in osmobiosis.

Anoxybiosis (Keilin 1959) involves survival of a lack of oxygen, but at a normal hydration level (in contrast to the other forms of cryptobiosis). It occurs in some invertebrate and vertebrate animals. For example, cysts of brine shrimp (*Artemia*) can survive extended periods of anoxia. During anoxybiosis, the metabolic rate of hydrated *Artemia* cysts is so low as to be unmeasurable, but it returns to prediapause levels (approximately 0.007 μl O_2 h^{-1}) when the cyst is in oxygenated water (Clegg 1976, 1997; Glasheen and Hand 1989; Clegg et al. 1996). Anoxybiosis is rare amongst vertebrate animals. Hatchling turtles survive in nitrogen at 4°C for 17 (*Malaclemys terrapin*) to 50 days (*Graptemys geographica*; Dinkelacker et al. 2005). Some adult turtles are extremely tolerant of anoxia, especially at reduced temperatures, e.g., painted turtles *Chrysemys picta* survive up to 5 months of anoxia at 3°C (Jackson 1968, 2000); they have a coordinated reduction of both ATP-consuming and ATP-producing pathways involving various metabolic adaptations (Storey 1996; Hochachka and Lutz 2001).

All of these forms of cryptobiosis involve complete inactivity and a very low rate of metabolism that reflects extreme changes to the physical state of water in cells, or lack of oxygen. The ratio of depressed metabolic rate to normal resting metabolic rate (D/R) is less than one-twentieth, approaching zero for some species (Fig. 1.2; see Guppy and Withers 1999). Osmobiotic *Artemia* cysts have a low D/R, of about 0.0028, which is similar to hydrated diapaused cysts (0.0031). Hydrated prediapause cysts have a D/R of about 0.18, which is similar to that of other dormant animals (see below). Anoxic or anhydrobiotic *Artemia* cysts have an extremely low D/R, and anhydrobiotic nematodes and tardigrades presumably also have an unmeasurably low D/R. Not unexpectedly, those forms of cryptobiosis that are associated with marked perturbation of the state of intracellular water (anhydrobiosis, osmobiosis, cryobiosis) have the most extreme metabolic depression, i.e., to less than one-thousandth of normal metabolic rate. The mechanisms whereby these animals and plants survive extreme conditions at very low metabolic rates would seem to be more related to molecular tolerance of the compromised state of their cell water than molecular or physiological adaptations of the cascade of metabolic enzymes in normally hydrated tissues. In contrast, anoxybiosis is especially intriguing because it does not involve an altered state of intracellular water, but the metabolic depression is similarly extreme (like anhydro-, cryo-, and osmo-biosis) rather than moderate (as for dormancy).

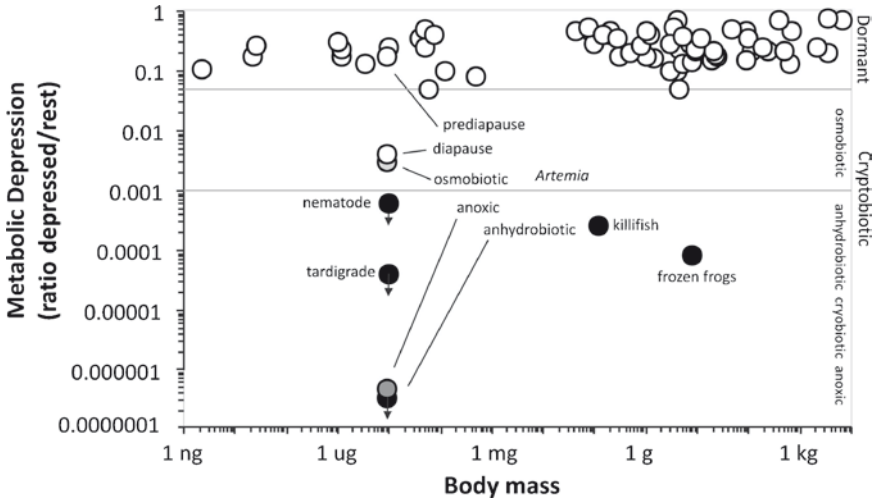

Fig. 1.2 Summary of the extent of metabolic depression (ratio of depressed to normal resting metabolic rate) for protists and animals when dormant (open symbols) or cryptobiotic (osmobiotic, light grey; anoxic, medium grey; anhydrobiotic, solid symbols); arrows indicate that lower ratios would be measured for animals in complete anhydrobiosis. Data from Guppy and Withers (1999)

1.3 Dormancy

Dormancy is a widely used term meaning inactivity or lethargy. It includes a variety of behavioural and physiological states involving inactivity and depressed metabolism. During dormancy, activity is minimal, although animals can move and respond to stimuli, and metabolic rate is reduced to a low level (hypometabolism). Dormancy can be a response to a circannual rhythm, or a variety of environmental factors, including temperature or the availability of food or water. It can be a short-term event (<24 h) or it can occur for a few consecutive days, an entire season, or even many years. Dormancy can also involve seasonal arrest (diapause) or opportunistic inactivity (quiescence) of a developmental stage.

Unlike cryptobiosis, dormancy does not involve marked changes in the physiological state of the animal (e.g., osmotic or hydration state). While cryptobiosis is a reactive response to an environmental stress (e.g., freezing at low ambient temperature; desiccation at low ambient humidity; hypoxia at low ambient pO_2), dormancy is preemptive and anticipates stressful physiological conditions to avoid dramatic changes in the internal environment. Dormant animals are inactive but can usually be aroused by disturbance without requiring any major change in their physiological state (e.g., level of hydration or oxygen level). Mechanisms for dormancy are more likely to be intrinsic metabolic controls rather than molecular adaptations to an altered state of intracellular water or oxygen levels. There are many different forms of dormancy, including developmental diapause and adult torpor.

Diapause involves the cessation of development of a subadult life stage, often for avoidance of harsh environmental conditions. It is often a programmed and obligate part of the life cycle, in response to regular and predictable environmental extremes, and synchronises the next stages of the life cycle with appropriate environmental conditions. Diapause is especially common in insects but is also observed in a variety of other invertebrate animals, as well as many plants (e.g., buds, bulbs, rhizomes, and seeds), but is rare amongst vertebrate animals. The embryonic diapause of a variety of mammals (e.g., macropod marsupials, mustelids, deer) is a reproductive strategy for delayed implantation and development of embryos rather than a strategy for metabolic depression, but presumably the diapaused embryo metabolically depresses during this period of arrested development. Diapaused eggs of annual killifish (*Astrofundulus limnaeus*) form a vitreous-like egg envelope and have a high resistance to desiccation; the embryonic tissues do not become dehydrated during diapause (Podrabsky et al. 2001). Quiescence is similar to diapause, but is a period of facultative inactivity in response to an immediate change in environmental conditions rather than a programmed and obligate response. It is terminated by the return of more favorable environmental conditions. It may be a response to harsh environmental conditions such as low or high temperature, or drought. Many invertebrates and plants (particularly seeds) become quiescent.

Torpor is inactivity and a reduction in metabolic rate below the normal resting value, often in response to extreme ambient conditions. It characteristically occurs in adults and is usually a response to daily or seasonal environmental changes (hibernation in winter or aestivation in summer). Torpor by endothermic mammals and birds involves daily or seasonal changes in their thermoregulation setpoint and use of thermogenic metabolism so it is physiologically different from ectothermic torpor, which can occur at constant ambient and body temperatures (see below).

1.4 Ectothermic Animals

Torpor by ectothermic animals is typically a seasonal or longer-term dormancy in response to environmental extremes (low temperatures and scarcity of food in winter, or high temperatures and scarcity of water in summer). Dormant animals and plants can survive long periods, e.g., over 23 years for a pulmonate snail *Oxystyla pulchella* (Baker 1934) and about 5 years for the Australian water-holding frog *Cyclorana platycephala* (Van Beurden 1980). Most dormant animals maintain normal hydration, but the earthworm *Allolobophora chlorotica* and the leech *Placobdella parasitica* can survive up to 70% loss of body water (Keilen 1959).

Early natural history observations suggested that many animals enter dormancy during unfavorable periods, and return to activity months or years later. Spencer (1896) observed aestivation of some Australian desert amphibians and Smith (1930) pioneered studies of aestivating lungfish (*Protopterus aethiopicus*). Some lizards had been inferred to hibernate over winter (e.g., Mayhew 1965) and various crocodilians

had been suggested to aestivate (Taplin 1988). Dormancy by reptiles was first considered to be more of a behavioural and thermal inactivation in comparison to the marked physiological changes that accompany dormancy in endotherms (see below). Mayhew (1965) coined the term brumation to describe hibernation and aestivation in ectothermic vertebrates, recognizing their possibly lesser physiological sequelae compared with dormant endotherms. However, it is now clear that there are physiological adjustments and intrinsic metabolic depression in many (but not all) dormant reptiles.

Physiological confirmation of metabolic depression was generally not achieved for dormant ectothermic animals until technical advances of respirometric techniques in the late 1900s. These pioneering studies of metabolic depression in aestivating invertebrates (e.g., Rakshpal 1962; Keister and Buck 1964; Coles 1968; MacMillen and Greenaway 1978; Abe and Buck 1985) and vertebrates (Moberley 1963; Seymour 1973; Delaney et al. 1974), and subsequent studies (see Guppy and Withers 1999), indicate that the extent of metabolic depression is to as little as one-fifth of the resting metabolic rate (Fig. 1.2; similar to hydrated diapaused *Artemia* cysts). The onset of inactivity can be quite immediate in response to impending environmental stress (e.g., aestivating frogs quickly seek refuge and become inactive in dry conditions), but the onset of metabolic depression requires days to weeks (e.g., Seymour 1973; Etheridge 1990; McClanahan et al. 1983; Withers 1993; Pedler et al. 1996). Consequently, daily cycles of metabolic depression are impractical for ectotherms, although temperature-induced daily torpor is energetically advantageous.

Daily thermal torpor has important metabolic savings for ectotherms since their body temperature (T_b) is essentially equal to ambient temperature (T_a), particularly in the absence of solar radiation for behavioural thermoregulation. This means that any decrease in T_a hence T_b induces an exponential decline in metabolic rate (MR) as described by the Q_{10} relationship (e.g., Withers 1992)

$$MR_{T_{b_2}} = MR_{T_{b_1}} Q_{10}^{(T_{b_2} - T_{b_1})/10}$$

where $MR_{T_{b1}}$ is metabolic rate at T_{b_1} and $MR_{T_{b_2}}$ is the metabolic rate at T_{b2}. For most physiological variables, Q_{10} is generally about 2.5 (varying from about 2 at high temperatures to 3–4 at low temperatures; Withers 1992; Guppy and Withers 1999). T_a-induced decreases in metabolic rate can thus provide substantial energy savings for an ectotherm.

Hibernation (winter dormancy) is long-term multiday torpor in response to cold and scarcity of food. The above-ground structures of plants may die during unfavorable winter conditions, but some develop protective scales around the stem tips so that the buds survive. Some plants have dormant underground bulbs, rhizomes, tubers, or corms, which are buffered from environmental extremes by the soil. For ectothermic animals, hibernation is primarily an inactive state accompanying a low T_b, with many physiological sequelae including a lowered metabolic rate. Metabolic rate is lowered during hibernation by the thermal Q_{10} effect, but some hibernating ectotherms also have an additional intrinsic metabolic depression. For example, the metabolic savings accruing to a hibernating frog from decreased

temperature of 22–2°C due to a Q_{10} effect is considerable (e.g., about 90% of the 22°C rate). This hibernation metabolic reduction from decreased T_a and a Q_{10} effect results in about 100 days survival time for even a 1-g frog, >200 days for a 10-g frog, and even longer survival times for larger frogs (Hillman et al. 2008). These periods are more than adequate for survival of a cold winter period (e.g., 3 months), so further energy reduction in energy consumption by intrinsic metabolic depression would seem unnecessary (and would result in relatively small absolute energy savings). Nevertheless, the decrease in metabolic demand for O_2 during hibernation is often augmented by further metabolic depression beyond the Q_{10} effect. For example, the metabolic rate of *Rana temporaria* declined by 40% after 3 months under water compared with prior to submersion at 3°C, and this intrinsic hypometabolism was intensified by a 75% reduction from standard metabolic rate if frogs were submerged in water made progressively hypoxic (Donohoe and Boutilier 1998; Donohoe et al. 1998). These 40 and 75% reductions in aerobic metabolism further increase survival time by 2–4 fold. Reptiles can also have an intrinsic metabolic depression during hibernation, independent of body temperature change, e.g., the horned lizard (*Phrynosoma m'calli*; Mayhew 1965) and the desert iguana (*Dipsosaurus dorsalis*; Moberley 1963).

Aestivation (summer dormancy) is long-term multiday torpor in response to hot and dry periods. It usually involves an inactive state often with a water-resistant covering since desiccation is usually a danger for aestivating animals. For example, aestivating earthworms form a mucous cocoon to resist desiccation (Laverack 1963; Righi 1972), and many insect pupae are remarkably resistant to water loss (Keister and Buck 1964; Lees 1955, 1956). Amongst vertebrates, fishes, amphibians, and reptiles enter a similar aestivation state. Eggs of annual killifish (*A. limnaeus*) enter diapause when their ephemeral ponds dry over summer; the eggs develop a vitreous-like egg envelope and have a high resistance to desiccation, so that the embryonic tissues do not become dehydrated during diapause (Podrabsky et al. 2001). Some adult fishes and amphibians aestivate (Richards, Chapter 6, this volume; Carvalho et al., Chapter 7, this volume), often in a protective cocoon. Aestivating fishes form a cocoon of dried mucus (e.g., lungfishes, Delaney et al. 1974; salamander fish, Pusey 1990) whereas many anuran amphibians and a few salamanders form a cocoon of shed skin (first described by Lee and Mercer 1967), to reduce cutaneous water loss; the cocoon covers the entire body surface except for the nostrils (e.g., Fig. 1.3). Some reptiles also aestivate (e.g., Gregory 1982; Abe 1995), but they do not need to form a cocoon to reduce evaporative water loss as their epidermis is already relatively water-impermeable.

There is an unequivocal metabolic depression for some ectotherms (e.g., snails, fishes, amphibians, and reptiles) to about 0.2 (20%) of normal metabolic rate during aestivation or hibernation. The earthworm *Glossoscolex paulistus* has a metabolic depression of about 22% of normal metabolic rate at 15°C, but metabolic depression is only about 64% of normal metabolic rate at 35°C (Abe and Buck 1985). The "side-walker" malacostracan land crab (*Holthuisana transversa*) has a metabolic depression of about 28% (MacMillen and Greenaway 1978). Metabolic depression by insects (eggs, pupae, and adults) varies from about 10 to 60% of normal metabolic rate (see Guppy and Withers 1999). Diapaused eggs of annnual killifish (*A. limnaeus*) have a low aerobic metabolic rate (10% of normal) with a minor

Fig. 1.3 Aestivating hylid frog (*Cyclorana australis*) with a thin cocoon of shed skin that reduces evaporative water loss

anaerobic contribution (Podrabsky and Hand 1999). Fish and amphibians generally have a metabolic depression of about 20% of normal metabolic rate (e.g., McClanahan 1967; Seymour 1973; Delaney et al. 1974; van Beurden 1980; Withers 1993). Evidence for metabolic depression is more equivocal for reptiles, in part, because of their less extreme changes in morphology and behaviour, but it is often about 20 to 50% of normal metabolic rate (e.g., Seidel 1978; Kennett and Christian 1994; Hailey and Loveridge 1997; Abe 1995). Moberley (1963) measured an intrinsic metabolic reduction of up to 50% of normal metabolic rate for hibernating desert iguanas (*Dipsosaurus dorsalis*). Metabolic depression to 20–50% of normal metabolic rate has been recorded for some reptiles in the field (e.g., Nagy and Medica 1986; Nagy and Shoemaker 1975; Christian et al. 1995), but these measurements would include behavioural and thermal effects as well as intrinsic depression. Christian et al. (1996) suggested that the slight decrease in metabolism of aestivating fresh-water crocodiles (*Crocodylus johnstoni*) reflected a starvation effect rather than an intrinsic metabolic depression.

Intrinsic metabolic depression in these ectotherms is independent of any obvious change in T_b or perturbation of internal homeostasis. It is essentially a preemptive metabolic depression in response to the impending environmental stress rather than being a consequence of a change in the temperature, ionic, osmotic, or any other discernable physiological aspect of internal environment. The molecular and biochemical mechanisms for this intrinsic metabolic depression are not well understood (e.g., Storey 2001; Storey and Storey, Chapter 2, this volume), although its physiological significance is a considerable extension of the hibernation/aestivation period that can be survived by conserving endogenous energy and/or water stores.

1.5 Endothermic Animals

Endothermic animals are able to maintain a constant T_b over a relatively wide range of T_as by thermoregulation achieved through proportional modulation of endogenous metabolic heat production. As such, endotherms can remain active over a wide T_a range. However, maintenance of a high and stable T_b is energetically expensive, especially, for small species with a high surface area to volume ratio, and when there is a large thermal gradient between the animal and its environment. Availability of food (or water) may, therefore, place limitations on the animals' ability to effectively thermoregulate. Thus, many endothermic species are heterothermic, and use torpor (Fig. 1.4) to reduce their energy requirements when environmental conditions are extreme or food and/or water are not available (Geiser, Chapter 5, this volume).

The characteristics used to define torpor for endotherms vary, resulting from attempts to define a phenomenon which may be more correctly viewed as a continuum of metabolic and thermoregulatory states including normothermia, facultative hypothermia, and torpor. Often, torpor is defined as a reduction in T_b to below some arbitrary level, most often to <30 or <31°C but sometimes 26–36°C (Hudson 1978; Barclay et al. 2001). However, an arbitrary T_b definition does not consider variation in normothermic body temperature between taxa, i.e., a bird with a normothermic T_b of 42°C requires a 12°C drop in T_b to be considered torpid, defined as T_b <30°C, but a marsupial with a normothermic T_b of 34°C only requires a 4°C drop in T_b. Some investigators use the more stringent definition of torpor

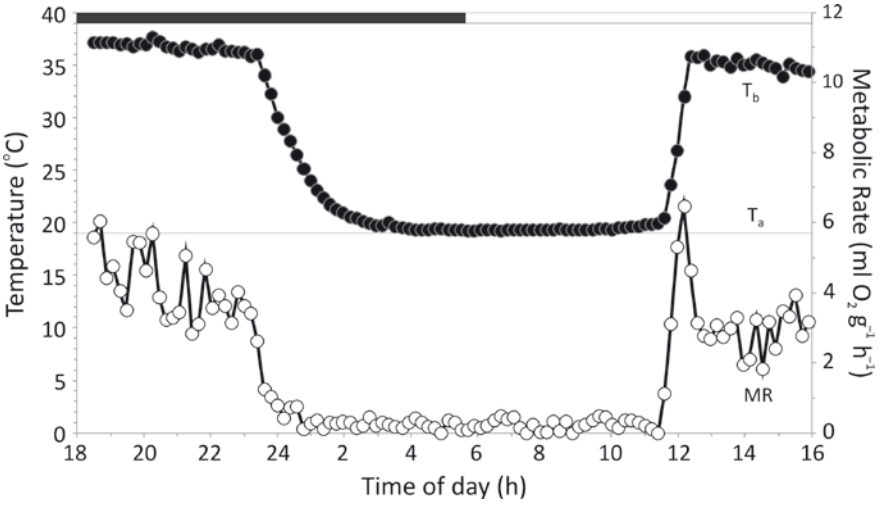

Fig. 1.4 Metabolic rate (MR) and body temperature (T_b) during a torpor cycle of a stripe-faced dunnart (*Sminthopsis macroura*) at an ambient temperature (T_a) of 18.5°C. Figure courtesy of Fritz Geiser (Zoology, University of New England)

that T_b approximates T_a, but many torpid endotherms defend a substantial T_b/T_a differential at low T_a, and at high T_a their T_b may approximate T_a even though the animals clearly are not torpid. Better definitions of torpor use a relative measure; for example, a 5°C reduction in T_b below normothermic values (e.g., Cooper and Geiser 2008) or a reduction in metabolic rate below that of basal metabolic rate (e.g., Geiser et al. 1996). Torpor may be distinguished from pathological hypothermia by the ability to arouse from torpor to normothermia using endogenous heat production.

For normothermic endotherms, metabolic rate increases as T_a decreases below the thermoneutral zone, to balance the heat lost to the environment with metabolic heat production, thus maintaining T_b constant at a species' specific set-point. Historically, heterothermic endotherms were believed to be poor thermoregulators, with torpor resulting from an inability to maintain thermal homeostasis and keep T_b at the set point when exposed to low T_as (Lyman 1978). However, by the 1970s, it was generally accepted that torpor is a controlled physiological process resulting from a readjustment of the animal's T_b set-point to a new lower level (Mrosovsky 1990). Early evidence to support this came from studies indicating that there is often a $T_b - T_a$ differential of several degrees during torpor, vasomotor activity, and muscle tone continue to have a regulatory function during torpor, and drugs known to inhibit thermoregulation in normothermic animals also reduce the $T_a - T_b$ differential in torpid animals (Mrosovsky 1971, 1990). Additionally, thermoregulatory responses to artificial hypothalamic cooling or warming (Hartner et al. 1971; Mills and South 1972), and evidence of proportional control of metabolic heat production below species-specific T_b set-points by torpid animals (e.g., Heller and Colliver 1974; Florant and Heller 1977) clearly indicated that torpor was a precisely controlled state, rather than an inability to thermoregulate. During torpor, an animal does not thermoregulate if T_a is above the torpor T_b set-point. However, if T_a falls below the torpor T_b set-point, then T_b is regulated at that setpoint by thermogenesis in the same manner as for the normothermic thermoregulatory response.

From the early 1800s, torpor was considered to be an extension of sleep (Rasmussen 1916). Subsequent experiments have indicated that entry into torpor occurs during periods of short-wave sleep (SWS), as first described by South et al. (1969). It is during SWS that an animal actively thermoregulates; thermoregulation is temporarily abandoned during periods of rapid eye movement (REM) sleep. The proportion of time spent in REM sleep decreases as the depth of torpor increases, with animals spending the majority of time in SWS during shallow torpor, and all of the time during deep torpor (Heller et al. 1978). This provides further evidence that torpor is a tightly regulated state.

The terminology used to describe patterns of heterothermy displayed by endotherms is historically variable. The term "deep hibernation" was first used by Lyman (1948) to describe the pattern of deep torpor demonstrated by the golden hamster (*Mesocricetus auratus*), whereas Hudson (1978) used the term "shallow daily torpor" to describe torpor lasting only a few hours at a relatively high T_b as occurs in dasyurid marsupials and many small rodents. Today, torpor is generally

accepted to be of two main types, multiday deep torpor or shallow daily torpor (Geiser and Ruf 1995; Geiser 2004a). Species undergoing multiday torpor may do so for several days to weeks or months. Body temperatures are usually less than 10°C, commonly between 0 and 5°C, and metabolic rates during torpor are reduced to approximately 5% of basal metabolic rate (Geiser 2004a). Species that undergo shallow daily torpor do so for periods less than 24 h and minimal body temperatures are usually >11°C. Metabolic rate during torpor usually drops to around 30% of basal. There is an overall negative linear relationship between torpor duration and minimal T_b (Geiser 1994). Hibernation is the commonly accepted term for torpor occurring in response to winter conditions (e.g., marmots, *Marmota flaviventris*; Davis 1976; Anonymous 2003), while the term aestivation applies to summer torpor (e.g., cactus mouse, *Peromyscus eremicus*, MacMillen 1965; Anonymous 2003).

Observations of torpor by mammals date back to Aristotle and Pliny, but Gessner in the 1550s and Buffon in the 1750s began the early experimental work (Rasmussen 1916; Kayser 1961). From that period to the present, a vast literature has been amassed on the subject. Much of this information is for rodents due to the prevalence of heterothermy in this group and their abundance in the temperate regions of the northern hemisphere (Kayser 1961). In particular, much knowledge has been gleaned from studies of the families Gliridae (Aristotle first wrote of hibernation for *Glis glis*), Sciruidae, and Cricetidae. Hibernation by the insectivores Tenrecidae (tenrecs) and Erinaceidae (hedgehogs) is well known and has been studied since the 1930s (Kayser 1961). Bats are famous for their propensity to enter torpor (Davis 1970). Early work on heterothermy by bats indicated that although small temperate microchiropterans regularly used both seasonal and daily torpor, large tropical megachiropterans were essentially homeothermic (Eisentraut 1934; Reeder 1949; Lyman 1970). More recently, this distinction has become blurred (Lyman 1970), and now strong phylogenetic signals in the pattern of torpor use have been identified amongst bats (Cooper and Geiser 2008). Although suspected since the early 1900s, Dausmann et al. (2004, 2005) provided the first physiological data on hibernation by a primate, the Madagascan fat-tailed dwarf lemur (*Cheirogaleus medius*), which also was the first tropical mammal shown to undergo multiday torpor. The monotreme *Tachyglossus aculeatus* has been known since the early 1900s to hibernate, and amongst marsupials torpor has been recognized since the mid 1950s. The work of Geiser and coworkers since the early 1980s has added substantially to our knowledge of heterothermy amongst marsupials.

Aestivation was described for mammals by MacMillen (1965) in his classical study of cactus mice (*P. eremicus*), which ceased activity during dry summer periods. Although torpor was historically associated with cold climates, it is now known to occur in species inhabiting a range of habitats including arid environments, e.g., marsupials, bats, and rodents (Geiser 2004b) and the tropics, e.g., primates (Dausmann et al. 2004, 2005) and marsupials (Cooper et al. 2009). Presumably, the energy and water savings associated with torpor are of adaptive advantage for surviving periods of energy and water shortage in a wide range of habitats (Geiser 2004a).

There is a strong phylogenetic pattern in the type of torpor used by marsupials and bats (Cooper and Geiser 2008). Didelphid, dasyurid, myrmecobiid, and petaurid marsupials, and small pteropodid bats use daily torpor, whereas burramyid, acrobatid and microbiotheriid marsupials, and vespertilionid bats undergo multiday torpor. The pattern of heterothermy for rodents is complex and shows no phylogenetic relationship in this speciose group, presumably being related more to environmental and biological factors than to phylogenetic history (Cooper and Geiser 2008).

Heterothermy is not as well studied in birds as in mammals. Anecdotal reports of torpid birds date back to the 1760s, but it was not until the 1950s that the use of torpor by birds was widely accepted (McAtee 1947; Lyman et al. 1982). Early reports of torpidity amongst birds referred to swifts, swallows, hummingbirds, ptarmigans, and parakeets (McAtee 1947). Hummingbirds have long been the classical avian heterotherms (e.g., Ruschi 1949), undergoing regular daily torpor which may be either spontaneous (occurring despite an abundance of food) or induced (occurring in response to limited food) to balance their considerable energy budgets during periods of nocturnal rest (Pearson 1960). Jaeger (1948, 1949) described avian hibernation in the poorwill (*Phaenoptilus nuttali*), and this species remains the only bird for which there is physiological evidence of multiday torpor. A number of birds use shallow daily torpor, and recent data suggest that torpor use may be more common amongst birds than first appreciated. Heterothermia has now been reported for the coraciiform, coliiform, apodiform, trochiliform, strigiform, columbiform, and passeriform families (McKechnie and Lovegrove 2002), although separating true torpor from the more commonly occurring nocturnal hypothermia is a definitional issue. It also appears that wild, free-living birds are more likely to undergo torpor bouts than birds studied in captivity (Cooper et al. 2008), contributing to the difficulties of describing avian torpor.

Torpor has important energetic consequences for endotherms, as it substantially reduces the energetic costs of endothermy at T_as below the thermoneutral zone (Fig. 1.4). The thermoregulatory increment above basal metabolic rate is reduced if the animal defends a smaller thermal differential between T_a and T_b, or is eliminated if the animal simply thermoconforms (if T_a>torpor T_b set point). The reduction in T_b resulting from the decrease in heat production leads to a further, exponential, decline in metabolic rate as a consequence of the Q_{10} effect. Some authors (e.g., Geiser 1988) have suggested that intrinsic metabolic depression may also play a role in reducing energy expenditure during torpor in species such as bats, based on reductions in metabolic rate further below basal values than expected by the Q_{10} effect (i.e., Q_{10}>3). However, the contribution of any intrinsic metabolic depression to the overall energy savings during torpor would be so small compared with that of the reduction of a thermoregulatory increment and the Q_{10} effect that the adaptive value to the animal is likely to also be small (Withers 1992; Guppy and Withers 1999). The overall energy saving resulting from torpor depends on the length of the torpor bout and the depth of torpor. For example, the daily energy saving for a dunnart using shallow daily torpor is about 36% for 13 h of torpor but for a hibernating ground squirrel, is about 85% over 6 months.

Torpor may also play an important role in maintaining water balance. First noted for placental mammals and birds (e.g., Carpenter 1969; Lasiewski 1964; MacMillen 1965; Buffenstein 1985) and more recently for marsupials (Cooper et al. 2005; Cooper et al. 2009), a combination of a decrease in respiratory water loss and a drop in body temperature reduces total evaporative water loss to as little as 14–50% of normothermic values. Respiratory water loss is decreased by a reduction in respiratory minute volume (due to a reduced demand for gas exchange resulting from a lower metabolic rate) and a drop in T_b reduces water vapour pressure at the lungs and skin surface. Interestingly, relative water economy, or ratio between metabolic water production and evaporative water loss, becomes less favorable during torpor, presumably because the reduction in metabolic rate, and thus water production during torpor is greater than the reduction in evaporative water loss (Cooper et al. 2005, 2009).

Torpor is distinguished from pathological hypothermia by a torpid animal's ability to arouse to normothermic T_b using endogenous metabolic heat production. This thermogenesis may occur by shivering (unsynchronized contractions of skeletal muscle) or by nonshivering thermogenesis such as the metabolism of brown adipose tissue (BAT) as occurs in some placental mammals, or more general tissue thermogenesis. Arousal from torpor is energetically expensive, and the high cost of rewarming tissues is one reason that precludes animals with large body masses from using torpor. Recent evidence (Geiser et al. 2002) has indicated that passive rewarming, using external heat sources (e.g., sun basking) may dramatically reduce the energetic costs of arousal and enhance the overall energetic benefits of torpor.

For animals undergoing long-term hibernation, periodic arousals to normothermia during the hibernation period are common. Although the reason for these periods of arousal and subsequent reentry into torpor is not clear, it is apparent that there is some physiological "need" to periodically arouse, perhaps, to reestablish homeostatic conditions perturbed by the reduction of metabolic substrates or accumulation of metabolites, to overcome temperature-dependant effects on circadian cycle, neural sensitivity, or rapid eye movement sleep, or to regain free water lost by evaporation during hibernation (Thomas and Geiser 1997). Dausmann et al. (2004, 2005) found for hibernating mouse lemurs that periodic arousals to normothermia occurred in individuals with well insulated hibernacula, which remained at low T_a. However, for individuals with poorly insulated hibernaculae, which experienced large ambient temperature cycles, passive rewarming to a T_b of 30°C was sufficient to substitute for arousal and these animals remained torpid.

Torpor, hibernation, and aestivation have important ecological consequences as they allow endotherms to avoid unfavorable environmental conditions by undertaking a period of inactivity, during which they dramatically reduce their energy expenditure. By avoiding unfavorable conditions, species can inhabit environments that would otherwise be inhospitable on a daily, intermittent, or seasonal temporal scale. The dramatic energy reductions associated with periods of dormancy obviously extend the period of time during which animals can remain inactive, but may also be an important mechanism for species to balance their daily energy budgets, and spontaneous torpor is common in many species. Despite these advantages, there also appear to be significant costs associated with torpor. Many species can not use or

survive torpor, and species physiologically capable of torpor do not necessarily use it routinely. Many physiological processes (e.g., digestion, growth, muscle function) are optimal at a relatively high and narrow range of T_bs, in part due to the Q_{10} effect on chemical processes and because of optimal temperatures for enzyme-mediated biochemical processes. Thus, maintenance of thermal homeostasis has fundamental physiological advantages over heterothermy (but this occurs at the cost of high energy expenditure). Use of torpor is associated with some physiological risk, such as having insufficient energy reserves or being at too low a T_b for arousal, or thermal death (e.g., freezing). In addition to physiological costs, there are also potential ecological drawbacks of torpor, including vulnerability to predation, competition for resources from individuals remaining active, reduced or delayed reproduction or development, and lower rates of essential activities such as cell division and digestion. The periodic arousals characteristic of multiday hibernators indicates that there is a necessity for at least periodic periods of high T_b (e.g., fat-tailed lemurs; Dausmann et al. 2004, 2005), and the use of torpor is restricted to relatively short-term periods.

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Chapter 2
Metabolic Regulation and Gene Expression During Aestivation

Kenneth B. Storey and Janet M. Storey

Contents

Abstract The biochemical regulation of aestivation, a state of aerobic hypometabolism, achieves actions including strong overall suppression of metabolic rate, reprioritization of energy use by diverse cell functions, and enhancement of defenses such as protein chaperones and antioxidants that aid long-term life extension. This is accomplished by mechanisms that include differential action of intracellular signaling cascades, reversible protein phosphorylation to alter the activity states of multiple enzymes and functional proteins, global suppression of transcription and translation, and selective gene upregulation. Recent advances in understanding the regulation of aestivation are discussed with a particular emphasis on land snail and anuran models.

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2.1 Introduction

Many organisms experience highly variable environmental conditions ranging from those that are conducive to rapid growth and development to those that are incompatible with normal life. A critical option for promoting survival under inhospitable conditions is hypometabolism. By strongly suppressing metabolic rate to low values, organisms gain an extension of the time that they can resist stressful conditions and sustain viability using their only endogenous fuel reserves. Hypometabolism is the common element of numerous animal survival strategies including aestivation, torpor, hibernation, diapause, dormancy, anaerobiosis, dauer state, and anhydrobiosis. Principles of hypometabolism include (a) an overall strong suppression of metabolic rate, typically at least a 70–80% reduction as compared with normal resting rate but ranging up to virtually 100% in anhydrobiosis, (b) differential control over the rates of various metabolic processes so that energy use is reprioritized to favor core vital cell functions (e.g., membrane potential difference) while largely shutting off "optional" activities (e.g., protein synthesis, cell division), and (c) implementation of actions that protect cells and preserve viability over what could be many months of dormancy (Storey and Storey 2004, 2007). How is this accomplished? Suppression of overall metabolic rate during hypometabolism is partly due to reduced energy use by physiological functions because breathing and heart rate are lowered, little skeletal muscle work is done, digestion is halted and some tissues may atrophy (e.g., moderate skeletal muscle atrophy can occur and intestinal mass can decrease) (Guppy and Withers 1999; Hudson and Franklin 2002; Secor 2005; Hudson et al. 2006). In aestivating anurans, for example, intestinal masses were reduced by 44% and total intestinal uptake capacities were lowered by 60% in *Ceratophrys ornata* and *Pyxicephalus adspersus* after 1 month of aestivation (Secor 2005). Other extrinsic factors that influence metabolic rate depression include the partial pressures of O_2 and CO_2, pH, and reduced temperature (Barnhart and McMahon 1988; Guppy and Withers 1999); in aestivating snails, for example, CO_2 retention resulting from apnoic breathing patterns is a key factor in metabolic depression (Barnhart and McMahon 1988). However, intrinsic regulation of cellular metabolism is very important, particularly, for achieving a coordinated suppression of ATP-producing versus ATP-consuming pathways, for reprioritizing energy use in the hypometabolic state, for changing patterns of fuel consumption (e.g., states of aestivation or hibernation rely heavily on lipid reserves whereas anaerobiosis requires a switch to carbohydrate fuels), and for implementing cell preservation mechanisms. Furthermore, it is now clear from studies on a wide variety of animal systems of hypometabolism, that most of the molecular mechanisms of metabolic rate depression are highly conserved across phylogeny so that the principles of hypometabolism are becoming well-defined (Storey and Storey 2004, 2007).

This chapter focuses on recent advances in understanding the intrinsic mechanisms that control hypometabolism as they apply to aestivation. We will focus primarily on (a) the use of reversible protein phosphorylation (RPP) to change the activity

states of enzymes and functional proteins, (b) intracellular signal transduction cascades, and (c) differential gene expression. Studies on land snails and anurans will be featured since these are the major models that have been used for molecular studies of aestivation.

2.2 Metabolic Control by Reversible Phosphorylation in Aestivation

Isolated tissues from aestivating animals (snails, frogs) of several species show stable reductions in oxygen consumption compared with tissues from active animals indicating that stable intrinsic metabolic controls are involved in metabolic rate suppression (summarized in Guppy et al. 2000; Storey 2002). Studies with multiple forms of hypometabolism have shown that the major mechanism involved in intrinsic metabolic suppression is RPP (Storey 2002; Storey and Storey 1990, 2004, 2007). Entry into hypometabolic states including aestivation, hibernation, anaerobiosis, and others involves coordinated changes in the phosphorylation states of many cellular enzymes and functional proteins. The addition of phosphate groups via protein kinases or their removal via protein phosphatases can have multiple consequences for target proteins/enzymes including major changes in activity (ranging up to complete on/off control), changes in substrate affinity and susceptibility to allosteric activators or inhibitors, changes in binding to other proteins, subcellular structures or DNA, and changes in subcellular location, to name a few. In comparative biochemistry, the first indication of a role for RPP in adjusting metabolic response to stress came from studies of anoxia tolerance in marine mollusks, initially, with the demonstration that anoxia induced the phosphorylation of pyruvate kinase (PK), thereby inhibiting the enzyme and rerouting the catabolism of phosphoenolpyruvate into anaerobic pathways (Storey and Storey 1990; Brooks and Storey 1997). A broader role for RPP was appreciated when it was shown that other enzymes of carbohydrate catabolism were similarly controlled under anoxia. Finally, the global role of RPP in metabolic rate depression was demonstrated when studies showed that (a) glycolytic rate depression was mediated by RPP in multiple states of hypometabolism including aerobic systems of snail aestivation and mammalian hibernation, and (b) that RPP regulated and coordinated many other metabolic functions when animals entered hypometabolism (Storey 2002; Storey and Storey 1990, 2004, 2007). Both of these criteria have been amply demonstrated in research on aestivation.

RPP control over enzymes of carbohydrate catabolism has been clearly demonstrated for both vertebrate (toad) and invertebrate (land snails) aestivators (reviewed by Brooks and Storey 1997; Storey 2002). Initial studies showed phosphorylation-mediated control of two regulatory enzymes of glycolysis during aestivation: PK and phosphofructokinase (PFK) (Whitwam and Storey 1990, 1991; Cowan and Storey 1999). For example, in skeletal muscle of spadefoot toads, *Scaphiopus couchii*,

high and low phosphate forms of the enzymes were separated by isoelectric focusing and showed different kinetic properties (Cowan and Storey 1999). The proportions of low phosphate PK and PFK increased during aestivation and kinetic analysis showed that these were the less active forms. Both aestivation and anoxia also suppressed maximal activities and altered kinetic properties of PK and PFK in foot muscle and mantle of land snails, *Otala lactea* (Whitwam and Storey 1990, 1991). Furthermore, *in vitro* incubations that stimulated cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), or Ca^{2+}/phospholipid-dependent protein kinase (PKC) altered the properties of PK and PFK from control snails in a manner that mimicked the effects of aestivation/anoxia but did not affect the enzymes in extracts from aestivating snails. This indicated that phosphorylation was responsible for enzyme suppression during entry into hypometabolism. Aestivation-responsive changes in PK properties were similarly found in foot and mantle of another snail, *Helix aspersa* (Fields 1992) but in both species the properties of PK in hepatopancreas (digestive gland) were differently affected during aestivation, although changes were still phosphorylation mediated (Whitwam and Storey 1990; Fields 1992). Other enzymes of carbohydrate catabolism are also regulated by RPP during aestivation in *O. lactea*. Glycogen phosphorylase activity decreased as did the amount of pyruvate dehydrogenase in the active form; activity of both of these enzymes is well known to be controlled in an on–off manner by RPP (Brooks and Storey 1990a, 1992). In addition, the concentration of fructose-2,6-bisphosphate ($F2,6P_2$), a strong activator of PFK, fell to <10% of control in foot muscle and the enzyme that produces $F2,6P_2$ was inhibited during aestivation (Brooks and Storey 1990a; Storey 2002). These changes in kinetic properties are consistent with enzyme phosphorylation as also typically occurs during starvation and carbohydrate sparing in vertebrate species.

Subsequent work has indicated a global involvement of RPP in the control of many aspects of metabolism during aestivation in *O. lactea*. In general terms, a comparison of ^{32}P-labelling patterns in active versus aestivating snails revealed aestivation-responsive changes in both the molecular masses and the subcellular distributions of phosphoproteins in *O. lactea* tissues (Brooks and Storey 1995a). Targeted studies of other enzyme systems have revealed what some of these RPP targets must be.

2.2.1 Glucose-6-Phosphate Dehydrogenase

The first demonstration of stress-responsive RPP control over glucose-6-phosphate dehydrogenase (G6PDH) in an animal system was supplied by a recent study of the *O. lactea* hepatopancreas enzyme (Ramnanan and Storey 2006a). G6PDH gates carbohydrate entry into the pentose phosphate pathway to produce sugar phosphates for anabolic purposes (e.g., nucleotide biosynthesis) and NADPH reducing power to drive many kinds of biosynthesis as well as antioxidant defense (to produce reduced forms of glutathione and thioredoxin).

Indeed, modulation of G6PDH activity (particularly in liver) has a known impact on cell growth, nutrient processing, antioxidant defense, and death (Tian et al. 1998). Therefore, regulation of G6PDH could be one way to exert general control over biosynthesis during hypometabolism. *O. lactea* hepatopancreas G6PDH showed significant stable changes in enzyme properties during aestivation: activity increased by 50%, substrate affinity improved (K_m of G6P decreased by 50%), and sensitivity to citrate activation increased (K_a of Mg.citrate decreased by 35%). Ion exchange chromatography revealed two peaks of activity that were identified as low and high phosphate forms; the low phosphate form dominated in active snails (57% of total activity) and the high phosphate form in aestivation (71%). PKG and protein phosphatase 1 (PP1) appeared to be responsible for the interconversion of the two forms. The high phosphate form of G6PDH was also less sensitive to urea inhibition and more resistant to thermolysin proteolysis; this suggested greater structural stability which could be advantageous during long-term dormancy. However, the kinetic properties of G6PDH from aestivating snails argue for a more active enzyme during dormancy. Why would this be? The prominent role of G6PDH in antioxidant defense may be the answer. Enhancement of antioxidant defenses is proving to be a universal feature of hypometabolism across phylogeny (Hermes-Lima et al. 1998; Storey and Storey 2007; Ferreira-Cravo et al. 2009). This helps to prevent/minimize oxidative damage to macromolecules during dormancy, a state in which organisms cannot afford the high ATP expenditures associated with repairing, degrading, or resynthesizing macromolecules damaged by reactive oxygen species (ROS). Since the backbone of antioxidant defense is NADPH reducing power, the key role of G6PDH in gating the pentose phosphate pathway can be appreciated. Indeed, G6PDH activity rises in response to oxidative stress in systems ranging from yeast to humans (Ursini et al. 1997) whereas inhibited and/or reduced G6PDH activity has been correlated with reduced antioxidant capacities, ROS-related cellular damage, and ROS-induced cell death (Tian et al. 1999).

2.2.2 Ion Motive ATPases

Successful entry into hypometabolism requires strong suppression of the rates of all energy-expensive metabolic processes. Ion-motive ATPases are certainly among the most expensive; indeed, the Na^+K^+ATPase ion pump alone can consume up to 40% of total ATP turnover in some cell types (Clausen 1986). Furthermore, the critical importance of ion pumps and channels to supporting a large number of cell functions that are linked to transmembrane potential difference (e.g., nerve conductance, muscle contraction, Ca^{2+} signaling, numerous transport mechanisms, etc.) means that control over their activity during entry into and arousal from hypometabolic states needs to be closely regulated and coordinated. Studies with *O. lactea* showed that RPP is critical to managing the activities of ion pumps during aestivation. Both the plasma membrane Na^+K^+ATPase and the sarco(endo)plasmic Ca^{2+}ATPase (SERCA) are regulated in this manner in foot muscle and hepatopancreas during

aestivation in *O. lactea* (Ramnanan and Storey 2006b, 2008). The same mechanism is seen in other hypometabolic systems including hibernating mammals and diapausing insects (MacDonald and Storey 1999; McMullen and Storey 2008). During aestivation in *O. lactea*, the maximum activities of both enzymes decreased by 33–50% in muscle and hepatopancreas, although Western blotting showed no change in the amounts of enzyme protein. Substrate affinities also decreased during aestivation; for example, K_m Mg.ATP values were 40% and 30% higher for Na^+K^+ATPase and SERCA (Ramnanan and Storey 2006b, 2008). Foot muscle Na^+K^+-ATPase from aestivated snails also showed reduced affinity for Na^+ (K_m for Na^+ rose by 80%) and for Mg^{2+} as an activator (K_a for Mg^{2+} increased by 60%) and K_m Ca^{2+} of foot muscle SERCA doubled in aestivating animals. In vitro incubations that manipulated the activities of endogenous protein kinases and phosphatases confirmed that the aestivation-induced changes in activities were regulated by RPP, the enzymes in aestivating snails proving to be the high phosphate forms. Thus, incubation of foot muscle extracts under conditions that stimulated protein kinases A, C, or G mimicked the changes in Na^+K^+ATPase enzyme properties that were seen during aestivation, whereas treatments that stimulated PP1 or PP2A had the opposite effect (Fig. 2.1a,b) (Ramnanan and Storey 2006b). SERCA activity was sensitive to PKG or Ca^{2+}-calmodulin protein kinase (CaMK) in foot muscle (Fig. 2.1c,d) and PKA or PKG in hepatopancreas whereas reactivation of SERCA was facilitated by protein phosphatases PP2A and PP2C in foot muscle and hepatopancreas, respectively (Ramnanan and Storey 2008). The commonality of PKG effects on both ion-motive ATPases suggest that this may be the kinase that naturally mediates aestivation-induced suppression of these enzymes in vivo. PKG has also been implicated in regulating enzymatic responses during anaerobiosis (another hypometabolic state) in marine molluscs (Brooks and Storey 1990b; Michaelidis and Storey 1990; Larade and Storey 2002) and was singled out as the kinase mediating aestivation-induced phosphorylation of PK in *O. lactea* (Brooks and Storey 1994).

2.2.3 Protein Synthesis

Another major energy expense in cells is protein turnover, both synthesis and degradation being ATP-expensive processes. Indeed, suppression of protein synthesis is one of the first responses by most cells when put under stress or nutrient/energy limitation (DeGracia et al. 2002). It is no wonder then that suppression of global rates of protein synthesis is a consistent response in all systems of hypometabolism that have been studied to date (summarized in Storey and Storey 2004). With respect to aestivation, suppression of global protein synthesis has been documented in both vertebrate and invertebrate models. Using liver slices from the desert frog *Neobatrachus centralis*, Fuery et al. (1998) found that the rate of protein synthesis was reduced by 67% in aestivation as compared with slices taken from awake frogs and that this accounted for 52% of the metabolic depression in liver and 4.9% of the

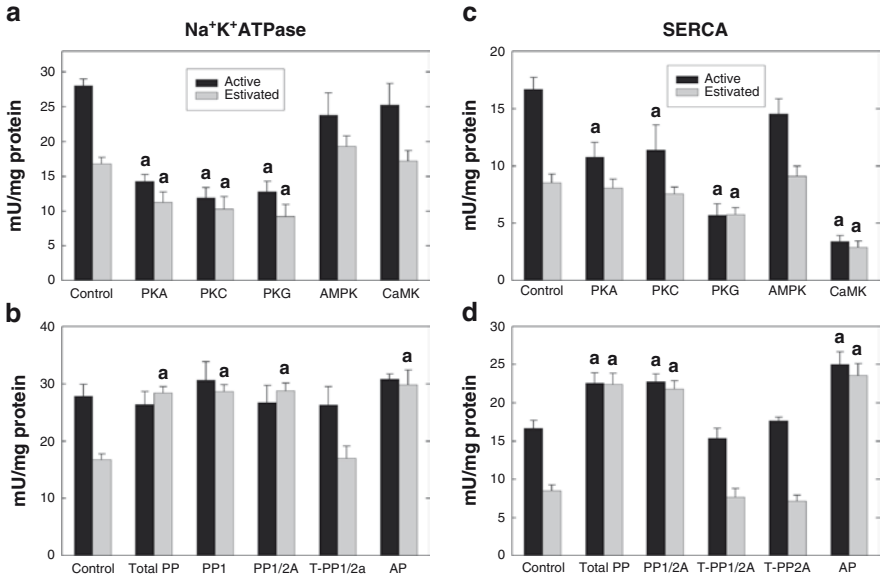

Fig. 2.1 Effect of in vitro incubations that stimulated the activities of endogenous protein kinases or protein phosphatases on the activities of Na^+K^+-ATPase or sarco(endo)plasmic Ca^{2+}-ATPase (SERCA) in foot muscle extracts from active or 10-day aestivated *O. lactea.* Incubation time was 4 h prior to assay of maximal activity at 22°C. Protein kinase incubations stimulated cAMP-dependent protein kinase (PKA), Ca^{2+}, and phospholipid-dependent protein kinase (PKC), cGMP-dependent protein kinase (PKG), AMP-activated protein kinase (AMPK), or Ca^{2+}-calmodulin protein kinase (CaMK). Appropriate combinations of stimulators and inhibitors allowed protein phosphatase incubations to promote total phosphatase activities (total PP), PP1, protein phosphatase 1 and 2A (PP1/2A), total phosphatases minus PP1/2A (T − PP1/2A), total phosphatases minus PP2A (T − PP2A). Incubations were also done with exogenous alkaline phosphatase added. Data are means ±S.E.M, $n = 4$ determinations on separate tissue extracts. (**a**) Significantly different from the corresponding control incubation using the Student's *t*-test, $P < 0.05$. Compiled from Ramnanan and Storey (2006, 2008)

decrease in whole animal metabolic rate. In the snail, *H. aspersa*, the rate of protein synthesis was reduced by 77 and 47% during aestivation in hepatopancreas and foot muscle, respectively (Pakay et al. 2002). *O. lactea* showed similar results: ^{3}H-leucine incorporation into protein in extracts of foot muscle and hepatopancreas was reduced by ~80% within 2 days when snails entered aestivation (Ramnanan et al. 2009).

One of the main mechanisms for regulating protein synthesis in all animal systems is RPP control over the activity of ribosomal initiation and elongation factors. Critical loci for regulation include the ribosomal initiation factor 2 (eIF2), that brings the initiating methionine into the assembling ribosome, eIF4 that brings in the mRNA, and elongation factor 2 (eEF2). Studies with *O. lactea* showed that all of these are regulated during aestivation. When unphosphorylated, the alpha subunit of eIF2 forms a complex with GTP and methionine-tRNA and carries both into the assembling ribosome where the GTP is used to drive the attachment of

methionine as the first amino acid of the peptide chain. Subsequently, eIF2α-GDP dissociates and GDP must be recharged to GTP before the next methionine can be loaded. Phosphorylation of eIF2α blocks it from being recharged and thereby inhibits protein synthesis (Proud 2007). The relative amount of phosphorylated eIF2α is a sensitive indicator of protein synthesis activity in all eukaryotes. Analysis of the relative amount of P-eIF2α (Ser51) in *O. lactea* showed that levels soared when snails aestivated; amounts in hepatopancreas rose by 3.8-fold and 15-fold after 2 and 14 days of aestivation (Fig. 2.2) and foot muscle showed a 20-fold higher P-eIF2α (Ser51) content after 14 days (Ramnanan et al. 2009). Pakay et al. (2003) also found a approximately twofold increase in the relative amount of P-eIF2α in liver extracts from aestivating desert frogs (*Neobatrachus sutor*), but in *H. aspersa* the amount of P-eIF2α was below the detection limit in hepatopancreas of both aestivating and awake snails (Pakay et al. 2003).

The eIF4 initiation complex delivers the mRNA to the assembling ribosome and several subunits of the complex are subject to RPP regulation. Changes in the phosphorylation state of three of the eIF4 components were assessed in *O. lactea* (Ramnanan et al. 2009). Both eIF4GI and eIF4E can be phosphorylated and this is linked to enhanced translation (Proud 2007). Figure 2.2 shows that the amount of

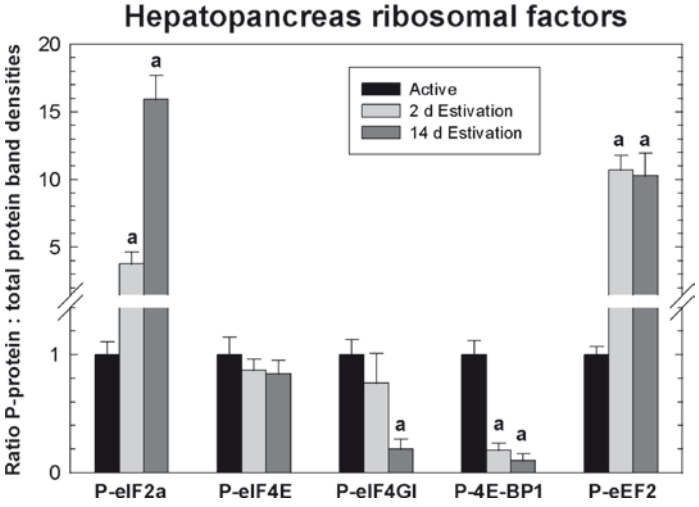

Fig. 2.2 Aestivation and the responses of ribosomal initiation and elongation factors in hepatopancreas of the *O. lactea*. Active snails are compared with those that aestivated 2 or 14 days. Immunoblotting was used to analyze both total protein and the amount of phosphorylated protein; phosphospecific peptide antibodies detected serine/threonine phosphorylation as follows: eIF2α (Ser51), eIF4E (Ser209), eIF4GI (Ser1108), 4E-BP1 (Ser65), and eEF2 (Thr56). Phosphoprotein band density was expressed as a ratio with total protein band density for the same sample; total protein did not change significantly during aestivation except for eIF4E where both total and P-eIF4E (Ser209) decreased by ~20% during aestivation. Data are shown relative to the active value; data are means ±S.E.M, $n = 4–5$ animals. (**a**) Significantly different from the value for active snails as determined by the Student's *t*-test, $P < 0.05$. Compiled from Ramnanan et al. (2009)

phospho-eIF4GI (Ser1108) decreased strongly in hepatopancreas during aestivation. Similarly, the content of phospho-eIF4E (Ser209) decreased strongly in foot muscle after 14 days of aestivation (Ramnanan et al. 2009). Reduced phosphorylation of both proteins is consistent with translational inhibition during aestivation. Furthermore, eIF4E is closely regulated by reversible association with a specific binding protein, 4E binding protein (4E-BP1). Binding to 4E-BP1 prevents eIF4E from interacting with EIF4GI to help deliver mRNA to the ribosome. Binding capacity is regulated by phosphorylation of 4E-BP1 which prevents it from binding and inhibiting eIF4E. Indeed, 4E-BP1 (like eIF2α) is one of the most important targets for RPP control over eukaryotic translation and is regulated by a variety of positive and negative effectors (Proud 2007). A strong reduction in amount of phospho-4E-BP1 (Ser65) content occurred in both hepatopancreas (Fig. 2.2) and foot muscle of aestivating *O. lactea* (Ramnanan et al. 2009). This means an increase in the amount of eIF4E bound to 4E-BP1 during estivation and is again indicative of translational inhibition in the hypometabolic state.

Translation is also regulated at the level of peptide elongation and several protein kinases focus their attention on the eukaryotic elongation factor 2 (eEF2) with phosphorylation inhibiting the factor (Browne and Proud 2002). This protein is also a target for phosphorylation in *O. lactea* with levels of P-eEF2 (Thr56) increasing ~10-fold in hepatopancreas (Fig. 2.2) and fivefold in foot muscle during aestivation (Ramnanan et al. 2009). Suppression of peptide elongation by enhanced phosphorylation of eEF2 is also well known in mammalian hibernation (Chen et al. 2001).

2.2.4 Protein Degradation

Given that rates of protein synthesis are strongly suppressed during aestivation, rates of protein degradation must also be suppressed, presumably by a similar amount, so that the amounts of most cellular enzymes/proteins are stabilized over what could be many weeks or months of hypometabolism. Reduced rates of protein degradation have been reported in several hypometabolic systems (summarized in Storey and Storey 2004) and this is also true of aestivation. In *O. lactea*, activity of the 20S proteasome in hepatopancreas decreased by 60–80% after 2 days of aestivation, although Western blotting showed that the content of selected subunits of the proteasome did not change (Ramnanan et al. 2009). Some subunits of the 20S proteasome are known to be phosphorylated (Pereira and Wilk 1990) and so in vitro incubations were used to test kinase and phosphatase effects on proteasome activity. Suppression of activity in vitro was mediated by protein kinase G whereas PP2A activated the proteasome (Ramnanan et al. 2009). Furthermore, reduced proteolytic activity in hepatopancreas during aestivation was associated with approximately twofold increases in K_m values (i.e., lower substrate affinity) for two out of three substrates tested.

However, although it is generally true that reductions in the rates of both protein synthesis and protein degradation would be needed to reduce ATP expenditure on protein turnover during aestivation, a situation where protein degradation is

specifically enhanced also occurs during long-term aestivation in anurans. Anurans enter aestivation with huge reserves of water in their bladders but after weeks or months they begin to experience water stress as the water potential of the soil declines. At this point, protein oxidation begins to supplement lipids as the fuel for metabolism (Jones 1980) and the nitrogen released is used to synthesize urea. Urea can build up as high as 300 mM and acts colligatively to defend against water loss from the body. It has been estimated that as much as 30% of total body protein may be catabolized for this purpose in *S. couchii* (McClanahan 1967; Jones 1980). The molecular regulatory mechanisms controlling this form of protein degradation have not yet been investigated for aestivating animals.

2.3 Signaling Cascades and Metabolic Control in Aestivation

The above discussion of reversible phosphorylation control suggests an important role for PKG in mediating various responses by metabolic enzymes during aestivation in *O. lactea*. This is also supported by a measured rise in cGMP levels in foot muscle and hepatopancreas over the first 24 h during entry into aestivation in these snails whereas, by contrast, tissue cAMP levels dropped (Brooks and Storey 1996). A study on spadefoot toads also indicated reduced reliance on cAMP signaling during aestivation with reductions (by one-third to one-half) in the percentage of PKA present as the active catalytic subunit in multiple tissues of aestivating animals (Cowan et al. 2000). Three organs also showed much lower percentages of active membrane-bound PKC during aestivation. Total tyrosine kinase activities were also reduced significantly during aestivation in most toad organs, including both membrane-bound receptor and cytoplasmic activities (Cowan and Storey 2001). In general, however, activities of protein phosphatases that oppose protein kinases were less affected during aestivation, including both serine/threonine and tyrosine phosphatases (Cowan et al. 2000; Cowan and Storey 2001). This subject is also reviewed in greater depth by Storey (2002). Recent studies have implicated additional signaling pathways in metabolic control during aestivation.

2.3.1 AMP-Activated Protein Kinase

A need to conserve energy (ATP) to permit long-term life extension during aestivation could implicate the AMP-activated protein kinase (AMPK) as another signalling kinase to consider in the control of aestivation. AMPK plays a central role in sensing cellular energy status and appropriately regulating the relative rates of catabolic ATP-generating pathways versus anabolic ATP-consuming processes; indeed, it is often called the “fuel gauge” of the cell (Hardie 2007; Hue and Rider 2007). AMPK is activated under various situations; it responds to hypoxia, glucose

deprivation, and muscle exercise (all situations that deplete ATP and elevate AMP), elevated intracellular Ca^{2+}, various cytokines (e.g., adiponectin, leptin) and tumor suppressors (e.g., LKB1) (Horman et al. 2002; Alessi et al. 2006; Hardie 2007). A recent study suggests AMPK involvement in aestivation in *O. lactea.* Tissue AMP levels do not rise during aestivation (Churchill and Storey 1989) but AMPK activity increased at least twofold, resulting from a strong increase in the amount of active AMPK as assessed by immunoblotting with antibody recognizing the phospho-Thr172 site on AMPK (Ramnanan and Storey, unpublished data). Because AMPK activation was due to phosphorylation, this implicated upstream kinases in AMPK regulation during aestivation. Three known upstream kinases that phosphorylate AMPK were then tested and one of them, LKB1, showed a parallel, approximately twofold, increase in activity (as assessed by the amount of active phospho-Ser428 LKB1) in snail tissues during aestivation. LKB1 is a tumor suppressor and, as the name suggests, is known to inhibit cell proliferation in other systems (Alessi et al. 2006). The LKB1-AMPK pathway functions as a cellular energy-sensing checkpoint, enabling growth and proliferation of cells to be coupled to the availability of fuel supplies; indeed, medical interventions that manipulate this signaling pathway have promise in cancer treatment (Alessi et al. 2006). The data for *O. lactea* suggest that this signaling system also has a natural action in suppressing biosynthesis and proliferation when organisms enter hypometabolic states. Downstream functions under AMPK control include inhibitory regulation of energy-expensive activities such as lipid biosynthesis via phosphorylation of acetyl-CoA carboxylase (ACC) and protein synthesis by regulating protein factors that control mTOR (Horman et al. 2002; Alessi et al. 2006; Hardie 2007). Not surprisingly, ACC activity was strongly inhibited in aestivating snails, as a result of a threefold increase in the amount of phospho-Ser79 ACC (Ramnanan and Storey, unpublished).

2.3.2 Akt Mediated Signaling

The cell signaling cascade consisting of phosphoinositide-3-kinase, 3-phosphoinositide-dependent kinase, and Akt (also called protein kinase B) is a major pathway involved in mediating survival and proliferation responses, most importantly transducing the action of insulin in vertebrates (Brazil et al. 2004). The pathway remains intact in invertebrates, triggered by different growth factors, and has been well characterized in *Caenorhabditis elegans* (Baumeister et al. 2006). Akt generally mediates a variety of actions that go forward when fuel/energy supplies to cells are plentiful while also inhibiting cell death signals. For example, Akt mediates the anabolic and growth-promoting effects of insulin and insulin-like growth factor by actions that include stimulating glucose uptake, promoting glycogen synthesis by inactivating glycogen synthase kinase 3 (GSK-3), and stimulating lipogenesis and protein synthesis (via regulation of mTOR) (Whiteman et al. 2002; Brazil et al. 2004). Oppositely, Akt inhibits apoptosis by control of the pro-apoptotic protein BAD and suppresses expression of forkhead box, class O transcription factors

(FOXOs) that are involved in cell-cycle arrest, quiescence, stress resistance, life extension, and apoptosis in different systems (Greer and Brunet 2005). Given the importance of these latter functions for long-term aestivation, the patterns of expression of Akt, downstream proteins and kinases under Akt control, FOXO transcription factors, and the genes that they control are clearly important in aestivating species. Indeed, numerous studies with *C. elegans* have documented the key role of these signaling pathways in regulating the dauer state, a developmental arrest (diapause) that occurs in the third larval stage when environmental conditions are not conducive to adult reproduction of the nematode (Burnell et al. 2005; Mukhopadhyay et al. 2006).

A recent study has explored the responses of Akt and various downstream targets under its control during aestivation in *O. lactea* (Ramnanan et al. 2007). Multiple criteria were consistent in showing that Akt was activated in foot and hepatopancreas of aestivating snails: measured Akt activity was approximately twofold higher, substrate affinities were lower, immunoblotting showed higher levels of the active phosphorylated form (Ser473), and incubation studies showed that only control Akt was susceptible to phosphorylation in vitro whereas aestivated Akt could be modified by protein phosphatases. Proteins under Akt control were also analyzed. A key regulator of protein translation, mTOR, showed no change in phosphorylation state during aestivation, which suggests both that it is not under Akt control in aestivation and that mTOR is not the regulator of the changes in 4E-BP1 phosphorylation, a known target of mTOR in other systems. Furthermore, the content of phospho-GSK-3β (Ser9) decreased by about half in both tissues during aestivation. Thus, it appears that growth/proliferation actions of Akt that are normally mediated via phosphorylation of mTOR and GSK-3 were uncoupled from Akt influence during aestivation. However, two other known targets of Akt action showed enhanced phosphorylation during aestivation: the amount of phospho-BAD (Ser136) was approximately twofold higher and phospho-FOXO3a (Ser253) was twofold and fourfold higher in hepatopancreas and foot muscle, respectively (Ramnanan et al. 2007). Phosphorylation of both of these proteins causes them to be sequestered in the cytoplasm. This prevents FOXO from moving to the nucleus to activate gene transcription and BAD from interacting with the apoptosis machinery in the mitochondria (Brazil et al. 2004). This suggests that proapoptotic signaling from either of these sources would be suppressed during aestivation. However, FOXO signaling also has key actions in cell-cycle arrest, enhancement of antioxidant defenses, stress survival, and life extension, and is key to dauer formation in *C. elegans* (Lam et al. 2006). These FOXO-mediated actions would predictably be beneficial during aestivation, so it remains to be determined if and how survival and life extension actions are accomplished in aestivating snails. It is possible that another protein kinase is involved such as the c-Jun activated kinase that has been linked to regulating FOXO with respect to its role in life extension in *C. elegans* and *Drosophila* (Lam et al. 2006). Thus, Akt and Akt-mediated downstream events are involved in snail aestivation, but there are clearly regulatory differences between aestivation and dauer formation that remain to be resolved.

2.4 Gene Regulation

2.4.1 Global Suppression of Gene Expression

Strong global suppression of gene expression is an integral part of metabolic rate depression in all systems of hypometabolism that have been studied to date (reviewed in Storey and Storey 2004, 2007). A variety of epigenetic controls are known that can mediate global gene silencing and have important roles to play in many aspects of life including development, differentiation, aging, and disease (Fraga et al. 2007). The best known epigenetic modifications are DNA methylation and posttranscriptional modification of histones by many methods including methylation, acetylation, phosphorylation, and SUMOylation, to name a few. Little is known to date about epigenetic controls in states of natural hypometabolism but the subject has begun to be explored in hibernating mammals (reviewed by Morin and Storey 2009) and comparable controls can be envisioned for aestivation. One method of global transcriptional repression is to reduce chromatin accessibility to the transcription apparatus. This is gated by histones which are subject to multiple forms of posttranslational modification that, in turn, affect chromatin packing. Both acetylation and phosphorylation of histones reduce chromatin packing to increase the accessibility. During mammalian hibernation, the level of histone acetylation and phosphorylation decreases consistent with translational silencing (Morin and Storey 2006). Furthermore, activities of histone deacetylases rose during torpor, providing the mechanism for reduced histone acetylation. Another factor is control over RNA polymerase II activity which is also reduced during hibernation, possibly via a RPP mechanism (Morin and Storey 2006). Global suppression of the action of transcription factors is another mechanism. Lee et al. (2007) showed that the levels of small ubiquitin-related modifier (SUMO) conjugated proteins rose dramatically in ground squirrel organs during hibernation. Transcription factors are primary targets of SUMOylation and this reversible posttranslational modification mainly has negative effects on gene expression (Girdwood et al. 2004). Hence, high levels of SUMOylation indicate strong global suppression of transcription factor action during torpor, and thus reduced transcription of the genes under their control. All of these methods of global transcriptional repression need to be explored in aestivation. A recent study took a specific look at epigenetic regulation in aestivation of green-striped burrowing frogs, *Cyclorana alboguttata* (Hudson et al. 2008). The authors quantified mRNA transcript abundance in cruralis muscle of control versus 6-month aestivated frogs to assess the expression of seven genes whose proteins have established roles in gene silencing: methyl CpG binding protein 2, chromodomain helicase DNA-binding protein 4, histone-binding protein rbbp4, histone deacetylase 1, nuclear receptor corepressor 2, transcriptional corepressor SIN3A, and DNA cytosine-5-methyltransferase 1. Transcript levels of the latter two were significantly elevated (by 1.7- and 3.5-fold, respectively) in aestivation, consistent with the idea that chromatin remodeling plays a significant role in long-term gene silencing in aestivators.

Gene expression is also regulated by mechanisms that are posttranscriptional but pretranslational – i.e., that affect mRNA processing and availability. One method is to sequester mRNA transcripts into protein–mRNA complexes (stress granules) or to store them in association with translationally silent monosomes (Bond 2006; Kedersha and Anderson 2007). This preserves existing mRNA transcripts so that they can be rapidly transcribed when organisms exit from stress conditions or a hypometabolic state. Polysome dissociation into monosomes is a documented feature of hypometabolism in hibernation and anaerobiosis (reviewed in Storey and Storey 2004). Anoxia-induced polysome dissociation occurs in marine snails (*Littorina littorea*) (Larade and Storey 2002) but only one study to date has examined aestivation. That study found that ribosomes were present almost exclusively as monosomes in *O. lactea* hepatopancreas from both active and aestivating snails (Hobbs et al. 1994).

Another principle of gene regulation that will probably prove to have a major influence in hypometabolic systems is the hot new topic in mRNA control. This is microRNA (miRNA). These small noncoding transcripts (19–25 nt long) regulate gene expression by binding to mRNA transcripts to block translation or target them for degradation (Bartel 2004). Dozens of studies are now linking miRNA to translational control in health and disease (O'Driscoll 2006) and new work on hibernators showed significant increases in the levels of selected miRNA types during torpor in ground squirrels, providing the first link between miRNA and hypometabolism (Morin et al. 2008). Comparable studies on aestivating species will be highly instructive.

2.4.2 Gene Hunting and Stress Response

Despite overall global suppression of transcription and translation in hypometabolic states, enhanced expression of selected genes and the proteins that they encode occurs in all hypometabolic systems that have been evaluated. Indeed, advances in molecular biology technologies in recent years have allowed huge leaps forward in our understanding not just of hypometabolism but of animal adaptation to environmental stress in general. A variety of approaches have been used for gene hunting in hypometabolism including screening of cDNA libraries, differential display PCR, and the now dominant technique, DNA array screening. Species-specific DNA arrays are now available for a variety of mammalian species as well as common "comparative" models including zebrafish (*Danio rerio*), African clawed frog (*Xenopus laevis*), fruit flies (*Drosophila melanogaster*), and the nematode (*C. elegans*). Furthermore, excellent results can be achieved by the gene hunter using heterologous screening (i.e., using an array developed for another species) (Eddy and Storey 2008). Although cross-reactivity is never 100% and additional validation is always needed to confirm gene/protein upregulation (via PCR or immunoblotting), heterologous probing of DNA arrays has been critical for identifying many genes whose protein products have never previously been linked

with hypometabolism and for finding conserved patterns of gene responses in multiple systems. For example, application of heterologous screening to several models of hypometabolism including hibernating ground squirrels, freeze-tolerant frogs, and anoxia-tolerant turtles has shown conserved gene expression responses that include enhanced expression of chaperones (heat shock proteins, glucose regulated proteins), antioxidant proteins, iron-binding protein, and serpins (serine protease inhibitors), among others (Storey 2004a,b; Storey 2006).

Several of the above gene expression responses are part of a conserved cellular stress response that is seen in all cells across phylogeny (Kültz 2003, 2005). The stress response includes (a) cell-cycle control including growth arrest through checkpoint control and translational controls, (b) proliferation of molecular chaperones to fold/refold and stabilize proteins, (c) DNA and chromatin stabilization and repair, and (d) degradation of damaged macromolecules. Although regulated entry into hypometabolic states would not be expected to generate cell stress, the coordinated implementation of the stress response could be of vital importance for two reasons. Firstly, the stress response could provide anticipatory preparation to deal with stresses that almost always accompany the hypometabolic state. For example, during aestivation this might include preparations to deal with anticipated stresses such as dehydration, wide variation in temperature, and oxidative stress. One of the earliest illustrations of this kind of anticipatory response was the upregulation of antioxidant enzymes in late gestation in mammalian lung preceding the oxidative stress that occurs when lung breathing begins at birth (Frank and Sosenko 1987). Secondly, activation of the stress response could aid life extension during dormancy by implementing or enhancing mechanisms that provide long-term stability of cellular macromolecules to deal with the realities of the hypometabolic state where energy/nutrient availability for macromolecular synthesis, repair, or degradation is limiting.

2.4.3 Aestivation-Responsive Gene Expression

Very little has been done to specifically explore aestivation-specific gene expression using molecular biology techniques. A recent study by Hudson et al. (2006) used PCR to evaluate mRNA transcript levels of selected genes in cruralis skeletal muscle of control versus 6-month aestivated *C. alboguttata*. Transcript levels of NADH ubiquinone oxidoreductase subunit 1, ATP synthase, and mitochondrial superoxide dismutase were suppressed by ~70%, consistent with mitochondrial quiescence during aestivation and reduced oxidant production, but transcripts of uncoupling protein type 2, catalase, and glutathione peroxidase were unchanged. PCR analysis of message levels for two extracellular matrix proteins in cruralis muscle also found no significant change during aestivation which supports the limited atrophy seen in this muscle (Hudson et al. 2007).

Enhanced expression of some specific genes during aestivation can be inferred in a number of cases. For instance, injection of ^{35}S-methionine into *O. lactea* followed by analysis of radiolabeled proteins by polyacrylamide gel electrophoresis

or isoelectrofocusing showed strong differential radiolabeling of a few selected proteins in aestivating snails as compared with active animals. Proteins of 91, 70, 50, and 30 kDa were prominently elevated and these molecular masses suggest that they may be chaperone proteins (Brooks and Storey 1995b). Some of the prosurvival defenses associated with the stress response (discussed above) are likely upregulated during aestivation. Indeed, activities of a number of antioxidant enzymes were higher in foot muscle and hepatopancreas of aestivating *O. lactea* compared with active snails whereas selenium-dependent glutathione peroxidase rose substantially in tissues of aestivating *H. aspera* (Hermes-Lima and Storey 1995; Ramos-Vasconcelos and Hermes-Lima 2003). By contrast, antioxidant enzyme activities were generally lower in tissues of aestivating versus active spadefoot toads (*S. couchii*) (Grundy and Storey 1998). Chaperone proteins also have a role to play. Levels of several heat-shock proteins (HSP10, HSP60, HSP90, HSP110) were significantly elevated in hepatopancreas of 14-day aestivated *O. lactea* (Ramnanan et al. 2009) but HSP70 protein and mRNA levels were not altered in another Mediterranean snail, *Cantareus apertus*, during aestivation (other chaperones were not assessed) (Reuner et al. 2008). Gene expression that deals with specific stresses associated with aestivation can also occur. The prime example known is urea cycle enzymes; activities of these enzymes are typically elevated in liver when osmotic or desiccation stress becomes an issue in both aestivating and nonaestivating anurans (Jones 1980; Lee et al. 1982). Since the regulation of urea cycle enzymes is at the transcriptional level in virtually all cell types (Morris 2002), it is logical to assume that upregulation of the genes encoding these enzymes occurs when needed during aestivation.

To our knowledge, the only direct use of gene screening techniques to search for aestivation-responsive gene expression involved the construction and screening of a cDNA library from liver of 2-month aestivated female *S. couchii*. Differential hybridization using cDNA probes made from active versus aestivated toads revealed strong upregulation of the gene for riboflavin-binding protein (RfBP) in aestivating animals (Storey et al. 1999). Indeed, this study also provided the first identification of RfBP in an amphibian species. The protein was previously known to be synthesized only by liver of female birds, reptiles, and mammals; it is then secreted, binds plasma riboflavin and loads the vitamin into eggs or fetus (White and Merrill 1988). The amino acid sequence of toad RfBP was 50% identical to chicken or turtle RfBP including key functional residues but lacked some of the phosphoserine residues that are involved in RfBP binding to the oocyte membrane in other species. Possible reasons for RfBP upregulation of RfBP in aestivating toads can be suggested. RfBP gene expression might be linked with the maturation of eggs in female toads prior to the explosive breeding that occurs immediately upon emergence from aestivation. Alternatively, enhanced levels of RfBP in adult toads could serve to cache endogenous riboflavin over the 9–10 months that the animals are in aestivation each year. All hypometabolic states have in common a need to achieve long-term life extension without an input of nutrients. As such, conservation and recycling programs for important nutrients would be valuable and could potentially be part of a

conserved stress response. It would be interesting for future research to determine whether there are universal mechanisms for conserving valuable nutrients such as vitamins during hypometabolism, particularly, in species that have extended seasonal dormancies.

2.5 Conclusion

Much is already known about the molecular mechanisms that regulate metabolic suppression and support long-term viability in aestivation. Other mechanisms are implicated from common principles that have emerged in other systems of aerobic hypometabolism including hibernation, diapause, and dauer state. However, a number of discrepancies have been pointed out in this chapter between the metabolic responses/controls seen in snail versus anuran aestivation or in aestivation versus dauer state. Hence, it is important to continue studies of aestivation in order to gain a full understanding of both the common principles and the diversity of mechanisms that achieve hypometabolism in different systems. There are still many new avenues to explore in the biochemistry and molecular biology of aestivation, and, indeed, in hypometabolism in general. Of particular interest, currently, are global mechanisms of gene silencing, cell cycle suppression, and mechanisms that aid life extension by minimizing degradative programs of atrophy or apoptosis. The clear participation of multiple intracellular signaling pathways in coordinating subcellular responses during aestivation also leads us to question what the extracellular signal(s) are that trigger metabolic suppression and/or coordinate responses among cells, tissues, and organs. Extracellular (blood or haemolymph borne) regulators of hypometabolism have been highly elusive in most models except, perhaps, for diapause hormone in insects. Evidence from both hibernating mammals and anoxia-tolerant turtles suggests a role for delta-opioids, at least in the suppression of brain activity (Borlongan et al. 2004; Pamenter and Buck 2008), and it would clearly be a major advance to discover an extracellular trigger factor for aestivation. Finally, aestivation is, perhaps, the "purest" of the hypometabolic states in nature – an aerobic dormancy that can be induced and sustained without complicating factors such as the large decrease in body temperature during mammalian hibernation, the oxygen deprivation of facultative anaerobiosis, or the genetic/seasonal programming that can make diapause difficult to manipulate experimentally. So, aestivation may, in fact, provide the best model for identifying the basic principles, common regulatory mechanisms, and the core proteome that define hypometabolism in animal systems. The future is exciting!

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Chapter 3
The Connection Between Oxidative Stress and Estivation in Gastropods and Anurans

Marlize Ferreira-Cravo, Alexis F. Welker, and Marcelo Hermes-Lima

Contents

Abstract In situations of food and water deprivation associated with unfavorable environmental conditions, a number of animal species undergo estivation. This state of locomotor inactivity involves a drastic reduction in the metabolic rate, allowing the estivator to survive long periods of adverse situations. However, the arousal from dormancy causes a rapid increase in oxygen consumption, which may elevate the production of oxygen radicals. Thus, it is expected that animals that arouse from estivation suffer a physiological oxidative stress. The reported mechanisms that protect estivators (anurans and gastropods) from the potential dangers of increased oxyradical formation are discussed. This includes the modulation of endogenous antioxidant defenses (enzymes and glutathione) of gastropods during dormancy, preparing them for arousal. A different strategy used for estivating anurans is also discussed.

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3.1 Biochemical and Physiological Adaptations for Estivation

The distribution and profusion of life on the planet are restricted in some degree by extreme temperatures, water, and food accessibility (Chown and Storey 2006). The fine-tunings for animal adaptations are in multiple grades: behavioral, physiological, and biochemical.

Estivation (also named aestivation) occurs in situations of food and water deprivation associated with other adverse factors, normally arid conditions and high temperatures. Many vertebrate (air breathing fishes, amphibians, reptiles, and small mammals) and invertebrate species experience this state of aerobic inactivity (Abe 1995; Storey 2002). The critical elements for long-term survival during estivation are water retention and plenty fuel reserves (Storey 2002). Moreover, other elements are related to the phenomenon of estivation, and these include metabolic suppression, tolerance of pH disturbances, hypoxia tolerance (at least in the case of gastropods, Hermes-Lima et al. 1998), and mechanisms to cope with reactive oxygen species (ROS) and oxidative damage (Bickler and Buck 2007).

Aerobic oxidation of lipids is the prime source of energy during estivation in vertebrate animals, jointly with protein catabolism and a moderately small input of carbohydrates (Storey 2002). In land snails, the main fuel is carbohydrate in the form of glycogen (Livingstone and de Zwaan 1983; Rees and Hand 1993); however, during extended periods of dormancy, proteins emerge as a significant fuel for catabolism (Rees and Hand 1993), much alike starvation in "regular mammals." In the case of metabolic arrest in estivation, a number of enzymes are regulated via reversible phosphorylation and others by alterations in the lipid environment. In addition, several processes are regulated by altering the concentration of enzymes, which is controlled by enzyme synthesis or degradation – contributing to the depression of different metabolic pathways (Stuart et al. 1998; Storey 2002).

Within just 2 days of the onset of estivation, land snails *Otala lactea* present marked changes in key biochemical parameters. Foot muscle and hepatopancreas extracts showed a reduction in the rate of in vitro protein translation by ~80%, which was associated with strong alterations in the phosphorylation state of ribosomal factors. Despite this, the expression of heat-shock proteins increased under estivation, which could be responsible for the lack of changes in the levels of oxidatively damaged protein, measured as carbonyl proteins (Ramnanan et al. 2009; Storey and Storey 2009). In addition, dormant land snails presented increased antiapoptotic pathways (Ramnanan et al. 2007), which could be operative during arousal – when physiological oxidative stress happens in land snails (Hermes-Lima and Zenteno-Savín 2002).

As an example of the changes in physiological parameters under metabolic depression, the VO_2 of land snails *Helix lucorum* becomes reduced at the onset of estivation and metabolic rates drop to just 10–30% of awaken individuals (Michaelidis 2002). In addition, isolated cells from estivating land snails show lower rates of oxygen consumption than cells from active ones, under the same experimental conditions (Bishop and Brand 2000; Guppy et al. 2000). These processes contribute to sparing fuel reserves under estivation (Herreid 1977; Bemis et al. 1987; Pinder et al. 1992; Pedler et al. 1996).

Some of these adaptations to estivation are regulated by alterations in the respiratory system. Estivating land snails show apnoic breathing, which diminishes water loss due to evaporation. This also causes fluctuations in CO_2 discharge and VO_2 (Barnhart and McMahon 1987). Oxygen availability under estivation increases after each breath and then drops again until a threshold value of pCO_2 is achieved – prompting the next breath (Herreid 1977; Barnhart 1986; Storey 2002). These alterations result in pH disturbances and hypoxia to internal organs. The reduced oxygen availability to estivating snails can be observed by the progressive fall in the pO_2 of the hemolymph throughout the period of estivation (Michaelidis 2002).

In a study by Pedler et al. (1996), it was reported that the extrinsic effects of pO_2 and pH are responsible for 70% of the total in vitro metabolic depression of mantle tissue of the land snail *Helix aspersa* whereas intrinsic effects contribute a further 30%. These extrinsic and intrinsic effects in land snails were also studied in hepatopancreas cells by Guppy et al. (2000). They observed that when the extrinsic effects of pH and pO_2 are excluded, the intrinsic effects are responsible for a 50% decrease in the metabolic rate of the cells from estivating snails in comparison to control animals.

All these adaptations seen in gastropods that experience estivation appear to bring advantages. Metabolic depression enables the preservation of endogenous fuel reserves, allowing estivators to survive periods of adverse situations. However, the adaptations can also be potentially dangerous, since the drastic changes in oxygen consumption are related to higher production of ROS – known to be involved in a number of human diseases and natural aging (Halliwell and Gutteridge 1999; Hermes-Lima 2004; Hulbert et al. 2007).

3.2 Oxidative Stress during Estivation and Arousal

ROS have the potential to harm macromolecules such as DNA, lipids, and proteins (Sies 1986; Halliwell and Gutteridge 1999). Superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) are some examples of ROS. Oxidative stress happens when the rate of ROS generation is higher than the rate of their decomposition, causing increased oxidative damage to biomolecules – such as proteins, DNA, and membrane lipids (Hermes-Lima 2004). Other reactive species of biological relevance are based on nitrogen – the reactive nitrogen species (RNS). Examples include nitric oxide, nitrogen dioxide, and peroxinitrite. ROS and RNS are also key players in cellular signaling processes of physiological (nonpathological) nature (Hermes-Lima 2004; Augusto 2006).

All organisms maintain antioxidant defenses to cope with ROS that are formed as a result of many physiological or pathological processes. Enzymatic defenses include superoxide dismutases, glutathione peroxidases, glutathione-*S*-transferases, catalase, glutathione reductase, thioredoxins, and several others. Endogenous nonenzymatic antioxidants include ascorbate, uric acid, and glutathione (GSH) (Hermes-Lima 2004).

At this point, we could ask how free radical metabolism is related to estivation. Within a few minutes after an increase in air humidity, estivating land snails (e.g., *O. lactea*, *H. pomatia*, *H. aspersa*, and *Pila ovata*) arouse from dormancy. The breathing pattern, which is apnoic under estivation, returns to normal and the oxygen consumption increases rapidly, returning to the values encountered in active animals (Herreid 1977). It is well known that a rapid elevation of oxygen metabolism results in an elevated production of ROS, partly because the rate of superoxide production at the mitochondrial level in many biological systems is proportional to oxygen tension (Turrens et al. 1982; González-Flecha and Demple 1995). Thus, it would be expected that physiological oxidative stress occurs in animals that arouse from estivation. Other animals that experience transient metabolic depression and then arousal and restoration of normal oxygen consumption also experience oxidative stress during arousal; for example, hibernating mammals, freeze-tolerant frogs, anoxia-tolerant turtles, and mollusks (Storey 1996; Hermes-Lima and Zenteno-Savín 2002; Bickler and Buck 2007). How do animals protect themselves against the high ROS production associated with arousal from estivation?

3.3 Dealing with ROS Associated to Estivation and Arousal

Several studies have investigated free radical metabolism of animals during estivation. These studies were performed with land snails, aquatic snails, and two anuran species (Hermes-Lima and Storey 1995a, 1995b Grundy and Storey 1998; Hermes-Lima et al. 1998; Ferreira et al. 2003; Ramos-Vasconcelos and Hermes-Lima 2003; Ramos-Vasconcelos et al. 2005; Hudson et al. 2006).

3.3.1 Free Radical Metabolism and Dormancy in Land Snails

By investigating the activities of antioxidant enzymes and the levels of GSH in foot muscle and hepatopancreas of the land snails *O. lactea*, Hermes-Lima and Storey (1995a) showed relevant changes in antioxidant enzymes in response to estivation. The activities of catalase, total-SOD, and GST were 62–94% higher in the foot muscle of estivating snails compared with active ones. In hepatopancreas of estivating snails, total-SOD was also 1.7-fold higher as compared with active snails whereas selenium-GPx was 2.2-fold higher. GR activity was not affected by estivation/arousal in either tissue. Within 40 min after arousal began, selenium-GPx activity in the hepatopancreas had fallen again to control values, but total-SOD showed a further 70% rise in activity before returning to control levels by 80 min (Fig. 3.1). Estivation had no effect on GSH-equivalent (GSH-eq) concentration in tissues, but GSSG content was about twofold higher in both organs of 30-day dormant snails (Hermes-Lima and Storey 1995a; Hermes-Lima et al. 1998). The observed increase in the GSSG/GSH-eq ratio during estivation was attributed to a reduction in the rate

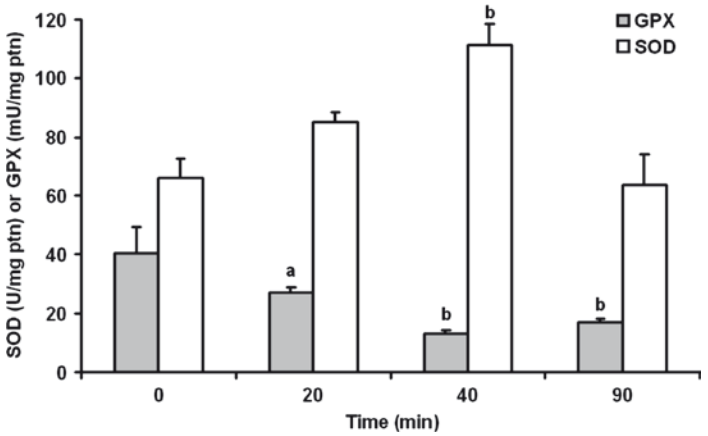

Fig. 3.1 Total SOD and selenium-GPX activities in hepatopancreas of land snails *Otala lactea* during arousal from 1 month of estivation. Modified from Hermes-Lima and Storey (1995a). (**a**) Significantly different from time zero, $P<0.01$; (**b**) $P<0.05$

of GSH recycling due to a possible decrease in NADPH supply in the hypometabolic state. On the other hand, recent work showed increased activity of G6PDH in the hepatopancreas of estivating *O. lactea* snails due to reversible phosphorylation of the enzyme (Ramnanan and Storey 2006). The observed higher G6PDH activity under estivation could be an indicative of increased NADPH production via the pentose phosphate pathway.

Lipid peroxidation, measured as TBARS concentration, was significantly increased by 25% in the hepatopancreas of *O. lactea* after 20 min of arousal from dormancy. With 40 min the levels of TBARS returned to those of estivating animals (Hermes-Lima and Storey 1995a). This suggests that oxidative damage in that organ occurs within minutes after arousal. Soon after that, aldehyde dehydrogenases and detoxification mechanisms should convert aldehydic products of lipid peroxidation into excretable metabolites. It is possible that lipid peroxidation could have been higher if antioxidant enzyme activities (total-SOD and selenium-GPx) were not increased in hepatopancreas during estivation.

In addition, xanthine oxidase, a potential in vivo ROS generator, showed very low activity in hepatopancreas of *O. lactea*, either after 1-month estivation or 24-h awakened (0.03 and 0.01 mUnits per milli gram protein, respectively). Only xanthine dehydrogenase presented high activity, which is important for nitrogen metabolism. The minimal ability of xanthine oxidase to induce oxidative stress may constitute an adaptive advantage for *O. lactea* during arousal periods (Hermes-Lima and Storey 1995b).

The response of free radical metabolism during estivation was also studied in *H. aspersa*. After 20 days of dormancy, the activities of hepatopancreas and foot muscle selenium-GPx were increased by 4.9-fold and 3.9-fold, respectively, compared with 24-h aroused animals (Ramos-Vasconcelos and Hermes-Lima 2003). In addition, levels of hepatopancreas GSH-eq were increased during estivation.

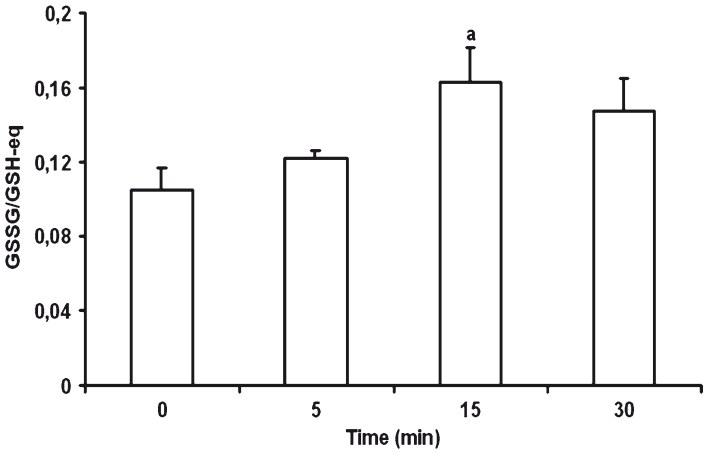

Fig. 3.2 Ratio of oxidized glutathione and glutathione equivalents (GSSG/GSH-eq) in hepatopancreas of land snails *Helix aspersa* during arousal from 20 days of winter-estivation. Modified from Ramos-Vasconcelos and Hermes-Lima (2003). (**a**) Significantly different from time zero ($P<0.05$)

During awakening, the ratio GSSG/GSH-eq increased significantly (by 1.6-fold) after 15 min and then returned to lower levels within 24 h (Fig. 3.2). This indicated that ROS overgeneration takes place in arousing snails. Moreover, levels of TBARS in the hepatopancreas increased by 1.3-fold from 5 to 30 min of arousal. There was, however, a 37% drop in TBARS levels from time-zero arousal to 5 min. Levels of lipid hydroperoxides decayed after 5 min of arousal in relation to 20-day estivanting snails and were maintained low afterwards (Ramos-Vasconcelos and Hermes-Lima 2003).

The determinations by Ramos-Vasconcelos and Hermes-Lima (2003) were conducted in the winter season of the South Hemisphere (in laboratory conditions). During summer (January to March in Brazil), estivating *H. aspersa* also showed an increase in selenium-GPx activity (by 3.6-fold) in the hepatopancreas, but not in foot muscle. No changes occurred in the activities of GR, GST, and G6PDH (as observed for winter-snails) in the two organs, but total-SOD and catalase activities diminished during summer estivation. Total GSH-eq concentration was increased (by 1.5-fold) only in foot muscle during estivation. Levels of TBARS and carbonyl proteins in foot and hepatopancreas were unchanged during estivation/arousal (Ramos-Vasconcelos et al. 2005). On the other hand, TBARS concentration in foot muscle was approximately two times lower in summer than in winter.

Seasonal variation of free radical metabolism has been seen in different animals, including rats (Belló-Klein et al. 2000), ground squirrels (Buzadzic et al. 1990; Carey et al. 2003), fish (Wilhelm Filho et al. 2001a), crocodilians (Furtado-Filho et al. 2007), polychaetes (Keller et al. 2004; Ferreira-Cravo et al. 2007), crustacea (Niyogi et al. 2001), and bivalve mollusks (Wilhelm Filho et al. 2001b). In the case of land snails, the few data available demonstrated marked seasonal changes in free radical metatolism in two species of *Helix* genera (Ramos-Vasconcelos et al. 2005; Nowakowska et al. 2009).

In *H. pomatia*, the onset and maintenance of winter torpor was considered a "passive" phenomena imposed by external conditions, while its spring interruption can be regarded as a precisely controlled endogenous mechanism, without influence of temperature (Wieser and Wright 1979; Bailey 1981; Vorhaben et al. 1984; Caputa et al. 2005).

The results from Ramos-Vasconcelos et al. (2005) clearly indicate important seasonal differences in the regulation of free radical metabolism of estivating snails. Since conditions of illumination, temperature, and humidity were very similar in the experiments with *H. aspersa* in winter and summer, it is possible that an endogenous mechanism (an "internal clock") is regulating the metabolism of free radicals in each season. One should remember that *H. aspersa*, originally from Mediterranean regions, undergo months of estivation and/or hibernation in the natural environment, depending on local conditions (Bailey 1981; Iglesias et al. 1996).

The elevation of selenium-GPx activity of *H. aspersa* during estivation in both seasons suggests an important role of this enzyme in protecting animals from oxidative stress following arousal. In the case of summer-snails, the absence of signs of oxidative stress in hepatopancreas during arousal was attributed to an exceptionally high activity of selenium-GPx, reaching up to 0.2 U mg^{-1} protein (hepatic GPx activity in rats are 0.6–1.2 U mg^{-1} protein) – as opposed to 0.02–0.03 mU mg^{-1} protein in dormant winter snails (Ramos-Vasconcelos et al. 2005).

Changes in the patterns of free radical metabolism during torpor at three different seasons were also observed in a recent study with land snails *H. pomatia.* Nowakowska et al. (2009) reported that during autumn (in the Northern hemisphere), the hepatopancreas from dormant snails showed a significant drop in GR activity and an augmentation (by threefold) of GST activity in estivating compared with active snails. No alterations were reported in catalase and GPx activities in the three organs analyzed (hepatopancreas, foot muscle, and nephridium). Moreover, TBARS concentration in the nephridium of torpid autumn snails was significant higher than in the active ones; no changes occurred in muscle or hepatopancreas.

At the end of winter, hepatopancreas catalase, GR, and GST activities in torpid snails were significantly higher than in the early phase of that season. Foot muscle and nephridium GPx activities were also increased (by 2–3 fold) in the final period of winter torpor. Nephridium catalase activity in torpid snails also rose about threefold by the end of the season. Moreover, levels of TBARS were increased in the foot muscle and nephridium by the final part of dormancy (Nowakowska et al. 2009).

In early spring, the activities of GST (from hepatopancreas) and catalase (from hepatopancreas and nephridium) of torpid *H. pomatia* continued to exhibit the same pattern of augmented activity observed at the end of winter torpor. These activities were also significantly higher (fourfold in the case of nephridium catalase) than that observed in active spring snails. On the other hand, nephridium GPx activity increased upon arousal in spring. Moreover, no changes were observed for GR activity and TBARS levels in the three organs. The concentration of GSH was unchanged in all seasons when comparing active with torpid animals (in spring and fall) and during the stages of winter torpor (Nowakowska et al. 2009).

These results show relevant seasonal alterations in the control of free radical metabolism in active and dormant snails. An important difference in these observations

for *H. pomatia* as compared with those of Ramos-Vasconcelos et al. (2005) is that land snails were kept under natural environmental conditions. However, the observations in *H. pomatia* generally show evidence for increased antioxidant potential in dormant animals in comparison with active ones. This adds to similar observations on antioxidant regulation in *O. lactea* and *H. arsersa* (Hermes-Lima and Storey 1995a; Hermes-Lima et al. 1998; Ramos-Vasconcelos et al. 2005).

The increase in activity of antioxidant enzymes or GSH levels in animals under very low metabolic rates (or under anoxia/hypoxia exposure) was considered to be a form of "preparation for oxidative stress." Oxidative stress of a physiological nature has been shown to occur during arousal in dormant snails and hibernating ground squirrels (Orr et al. 2009), or during reoxygenation in hypoxic tolerant species (Hermes-Lima and Zenteno-Savín 2002).

3.3.2 Free Radical Metabolism and Dormancy in a Freshwater Snail

Other work on estivation was conducted with *Biomphalaria tenagophila,* a freshwater mollusc species known as an intermediate host of *Schistosoma mansoni* in Brazil, which also undergoes estivation (Paraense 1986; Teles and Marques 1989). The activities of antioxidant enzymes and one marker of oxidative stress were evaluated in the hepatopancreas of this freshwater snail (Ferreira et al. 2003).

After 15 days of estivation, total-SOD activity was significantly lowered whereas selenium-GPx activity was significantly elevated (by 14%) in comparison to controls. The activities of GST, GR, and G6PDH were unchanged by estivation. After 24 h of arousal, SOD activity was recovered whereas catalase, Se-GPX, and GST activities were significantly lower than those of the respective controls. This part of the study was conducted during summer (Ferreira et al. 2003).

The evaluation of a marker of oxidative stress took place in the fall season (in Brazil) after 15 days of estivation and 26 h of recovery from estivation. TBARS levels in hepatopancreas did not change during estivation/recovery. It was not possible to establish a relationship between the modulation of antioxidant enzymes during estivation/recovery with the lack of lipid peroxidation because TBARS and antioxidant enzymes were determined in different seasons.

3.3.3 Reduction of ROS Production

Another possible defense against the dangers of wide fluctuations in oxygen availability (and consumption) is to use mechanisms that reduce ROS production. It was demonstrated that slices of turtle brain produce less ROS when the animals are exposed to progressive hypoxia and anoxia, a situation that also causes severe metabolic depression (Pamenter et al. 2007). Under aerobic conditions, mitochondria produce

high quantities of ATP through the continuous phosphorylation of ADP by ATP synthase, which allows the passage of protons through the inner membrane of the organelle coupled with ATP synthesis. A portion of the protons crosses the inner membrane through uncoupling proteins (UCPs), promoting the phenomenon known as proton leak.

Lower rates of ROS production are achieved through higher rates of proton leak in mitochondria of rats and mice (Brand 2000; Speakman et al. 2004; Harper et al. 2008). Recently, the induction of proton leak by an uncoupling agent reduced the rates of ROS production in mice (Caldeira da Silva et al. 2008). Therefore, it can be hypothesized that the induction of proton leak under hypometabolic states – despite being energetically costly – could be a protective process not only during dormancy but also during arousal. Interestingly, higher gene expression of UCPs was found in the muscles of lizards and in the brain of fish subjected to low temperatures (Jastroch et al. 2007; Rey et al. 2008). However, the available data obtained from estivators do not confirm the hypothesis that higher rates of proton leak really happen under natural conditions (Bishop and Brand 2000).

In fact, the kinetics of proton leak in *H. aspersa* remains unchanged during estivation in relation to active snails (Bishop et al. 2002). In *Cepaea nemoralis* snails, estivation for 6 weeks altered the lipid composition of mitochondria, which could reduce proton conductance (Stuart et al. 1998). Considering that some authors argue that lower rates of ROS production are actually associated with diminished rates of proton leak (Rolfe and Brown 1997; Ramsey et al. 2004; Philipp et al. 2005; Ramos-Vasconcelos 2005), the exact relationship between proton leak and oxidative stress in dormant estivators remains to be elucidated.

3.3.4 Desert Toads, Estivation, and Oxidative Stress

Spadefoot toads, *Scaphiopus couchii*, spend 9–10 months of every year estivating under the soil of the American southwest desert. During estivation, oxygen consumption is reduced to about 20–30% of the normal metabolic rate. Principal fuel utilization under these conditions consists of lipid reserves and structural proteins (Seymour 1973; Hermes-Lima et al. 2001). Urea rises in the toad during the dormant months and provides colligative resistance to the loss of body water. This loss can reach 60% of total body water after several months (McClanahan 1967).

When the animal leaves the state of dormancy, oxygen uptake and consumption increase rapidly, which might be accompanied by a higher rate of ROS generation. Examination of the antioxidant defenses and oxidative stress markers in tissues of toads showed how they respond to the broad variations in tissue oxygen levels that come with arousal from months of estivation. Antioxidant enzymes, GSH, GSSG, conjugated dienes, and lipid hydroperoxides were measured in 2-month estivating toads (burrowed at 21°C) versus 10-day awakened toads (Grundy and Storey 1998).

The authors observed that catalase activity increased in heart and liver (by 1.4 and 1.7 fold) after awakening but decreased by 40% in kidney. SOD activity rose

in kidney and heart (by 1.5 and 2.1 fold) after arousal but decreased in liver and muscle by 50 and 30%, respectively (Grundy and Storey 1998).

Moreover, the activity of total-GPx (selenium plus nonselenium dependent GPx activity) increased after arousal in liver, heart, and lung (by 1.7–2.3 fold) and selenium-GPx increased more than double times in liver and gut but decreased by 50% in kidney. GR activity rose significantly after awakening in heart, kidney, and liver. GST activity doubled in liver, heart, and gut and increased 1.5-fold in lung and kidney after awakening. In addition, GSH-eq rose in skeletal muscle, liver, and lung after arousal, by 1.2–1.7 fold (Grundy and Storey 1998).

Overall, toad organs showed evidence that antioxidant capacity was increased after arousal (and therefore was diminished during estivation) ranging from only an upward adjustment of GSH-eq in muscle to an extensive restructuring in liver (increases in five enzymes and GSH-eq after arousal).

The lower antioxidant capacity in dormant toads might also be the cause of the increased GSSG/GSH ratio (1.2–3.5 fold higher) in all organs, except muscle, of estivating spadefoot toads. An increased GSSG/GSH ratio suggests a higher use of GSH for peroxide (organic or inorganic) detoxification during dormancy, which is an indication of physiological oxidative stress (Grundy and Storey 1998; Hermes-Lima et al. 2001). Such a rise in GSSG/GSH was also observed in arousing *H. aspersa* – see Fig. 3.2.

Conjugated dienes and lipid hydroperoxides levels were higher in most organs of estivating toads than the control ones, suggesting accumulated oxidative damage to lipids during the dormant state (Grundy and Storey 1998). Levels of lipid peroxidation byproducts normally correlate positively with the rates of oxidative metabolism in various species (Adelman et al. 1988). Therefore, at first glance, it would be expected that a drop in lipid peroxidation levels would occur in torpid spadefoot toads. However, the results clearly indicate oxidative stress in most toad organs during dormancy. This may be caused by the decreased levels of antioxidant defenses – enzymes and GSH. Toads may have to cope with oxidative damage during estivation. It is possible that repair systems are upregulated when normal metabolic rates resume during arousal.

In the green-striped burrowing frog (*Cyclorana alboguttata*), estivation for 6 months prompted a 67% drop in the expression of mitochondrial SOD (SOD-2 mRNA transcript) in the skeletal muscle (Hudson et al. 2006). On the other hand, gene expression of muscle catalase and GPx was kept constant during estivation. In addition, water-soluble and membrane-bound antioxidants from muscle were also unchanged in dormant and active frogs. The expression of UCP type-2, which is suggested to be a possible way to diminish mitochondrial ROS production, was also maintained in the frog muscle (Hudson et al. 2006). The authors also normalized their antioxidant determinations to the animal´s metabolic rate, and interpreted this to suggest that estivating *C. alboguttata* could actually experience a relative increase in the antioxidant potential in muscle. One question not addressed by the authors is how frog muscle would cope with a potential rise in ROS formation upon arousal from dormancy (tissues other than muscle were not analyzed).

A reduction in ROS production in estivating amphibians is another possible mechanism that may minimize oxidative stress. During hibernation and hypoxia

exposure, the proton conductance in the muscle of *Rana temporaria* frogs is maintained, but the lower mitochondrial activity reduces the rates of proton leak (St-Pierre et al. 2000). Similar results were found in mitochondria of *Xenopus laevis* and *Bufos marinus* (Brookes et al. 1998).

Moreover, the increase in UCP-2 expression in white adipose tissue and UCP-3 in muscle of hibernating arctic ground squirrels (Boyer et al. 1998) may mitigate any rise in mitochondrial ROS generation during thermogenic arousal. The investigation of UCPs in nonmammalian hibernators/estivators is still in its infancy and deserves a lot more attention.

3.4 Conclusions

Estivation induced the response of *preparation for oxidative stress* in four different species of snails (Hermes-Lima and Storey 1995a; Ferreira et al. 2003; Ramos-Vasconcelos et al. 2005; Nowakowska et al. 2009). Among the enzymes involved in this process, selenium-GPx was observed to be a key player. The activation of selenium GPx (activity or gene expression) has also been reported in tissues of other animals under different situations of metabolic depression and/or low oxygen availability. The list includes garter snakes and wood frogs during freezing-exposure, leopard frogs during severe dehydration or anoxia exposure, goldfish during anoxia (Hermes-Lima and Zenteno Savín 2002), marine giant oysters, common carp, tilapia, and hatchling painted turtles during hypoxia exposure (Cardoso 2005; David et al. 2005; Lushchak et al. 2005; Storey 2006), as well as supercooling in a lizard (Voituron et al. 2006), and hibernation in bats *Myotis lucifugus* (Storey 2003) and 13-lined ground squirrels (Page et al. 2009). Increased activities of selenium-GPx were also observed in snails *B. tenagophila* and *H. aspersa* under anoxia exposure (Ferreira et al. 2003; Welker 2009). Most of the works quoted above reported either organ-specific increases in GPx activity or a rise in gene expression of specific GPx isoforms.

These observations make a strong body of evidence for a particular protective role of selenium-GPx – protection against peroxide-induced stress – in situations of wide changes in metabolic rate and oxygen availability in vertebrates and invertebrates. Moreover, it is intriguing that the same enzyme (selenium-GPx) is useful for adaptation under different environmental extremes in animals that do not share recent evolutionary ancestors. It is possible that such an adaptive role of GPx appeared in evolution before the origin of vertebrates.

On the other hand, estivating spadefoot toads presented diminished levels of antioxidant parameters in different organs and, at the same time, increased signs of oxidative stress (Grundy and Storey 1998). In green-striped burrowing frogs, estivation had no effect on most of the antioxidant parameters that were measured in skeletal muscle, except for a drop in SOD-2 expression (Hudson et al. 2006). In both anurans, it seems that the antioxidant status is in accordance with the suppression of most metabolic processes, contributing for energy sparing. In any case, accumulated oxidative damage in tissues of estivating anurans (analyzed only in spadefoot toads) must be dealt with upon arousal. Indeed, in spadefoot

toads, there was a decrease in the levels of oxidative stress markers in 10-day active animals as compared with the previously estivating animals (Grundy and Storey 1998). The study of repair mechanisms – and how they are regulated – in estivating vertebrates is the logical next step for investigation.

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Chapter 4
Nitrogen Metabolism and Excretion During Aestivation

Y.K. Ip and S.F. Chew

Contents

Abstract In this chapter, up-to-date information on nitrogen metabolism and excretion in various aestivators is presented. Although aestivation involves long-term fasting and corporal torpor, adaptive responses with regard to excretory nitrogen

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metabolism exhibited by aestivators during aestivation differ from those exhibited by nonaestivators undergoing fasting or immobilization. Special efforts were made to address current issues pertaining to excretory nitrogen metabolism and related phenomena in aestivators. Adaptations exhibited by aestivators were discussed in relation to the induction, maintenance, and arousal phases of aestivation. For the induction phase, we included topics like urea as an internal induction signal for aestivation, alteration in the permeability of the skin to ammonia, and changes in rate of ammonia production and urea synthesis. For the maintenance phase, the emphasis was on protein synthesis and degradation, ammonia production, and urea synthesis and accumulation. For the arousal phase, the focus was on rehydration, urea excretion, and phenomena related to feeding. Adaptations exhibited by aestivators specifically to each of these three phases of aestivation are essential to the understanding of the overall aestivation process, but, at present, only limited information is available on excretory nitrogen metabolism in animals during the induction or arousal phases of aestivation. Therefore, future efforts should be made to identify adaptive responses particular to each of the three phases of aestivation in various aestivators.

4.1 Introduction

Aestivation involves corporal torpor at high environmental temperature with absolutely no intake of food and water for an extended period. Such conditions have profound effects on nitrogen metabolism and excretion in animals capable of aestivation. As a result, aestivators exhibit adaptations that differ from fed or fasted nonaestivators. These adaptations include control of protein synthesis, modification and preservation of proteinaceous structures, suppression of ammonia production, and regulation of rate of nitrogenous waste excretion. They are essential to the understanding on how suspended animation can be achieved through aestivation at high environmental temperatures, but many of them have not been studied in detail. In this chapter, we will make an attempt to present up-to-date information on nitrogen metabolism and excretion, and certain closely related phenomena, in various aestivators during the induction, maintenance, and arousal phases of aestivation. Special efforts will be made to address some controversial and enigmatic issues currently confronting researchers in the area of excretory nitrogen metabolism in aestivators.

4.2 Nonaestivating Animals and Feeding

4.2.1 Excess Dietary Protein and Gluconeogenesis

Amino acids have numerous functions; they are the building blocks of proteins that are needed for survival, growth, and development. Dietary protein is a major source of amino acids in animals. Under normal circumstances, most animals take in

amino acids in excess of what is needed to sustain growth and protein turnover. Unlike carbohydrates and lipids, which can be stored as glycogen and triglycerides, respectively, amino acids are not stored to any great extent and animals are not known to possess protein stores solely for the purpose of energy metabolism (Campbell 1991). Therefore, excess amino acids from diets are preferentially degraded, and their carbon skeletons can be channeled directly into the tricarboxylic acid cycle or converted to glucose through gluconeogenesis (Campbell 1991). Besides diet, amino acids can also be released by muscles and other tissues, especially, during physiological states of increased protein catabolism. In vertebrates, the liver acts as the "glucostat" where amino acid catabolism and gluconeogenesis take place (Campbell 1991). Amino acids reaching the liver via the hepatic portal system from the intestine or via the systemic circulation from the extra-hepatic tissues serve as major gluconeogenic substrates. Glucose can then be supplied to other tissues or stored as glycogen.

The first step in amino acid catabolism involves the removal of the α-amino nitrogen as ammonia. For some amino acids, deamination involves specific deaminases, but many amino acids are deaminated through transdeamination (Campbell 1973, 1991). Transdeamination of amino acids usually occurs in the liver and requires an initial transamination of the amino acid with α-ketoglutarate in the cytosol to form glutamate, which then enters the mitochondria and is oxidatively deaminated by glutamate dehydrogenase (GDH). GDH is, therefore, crucial to the regulation of amino acid catabolism, and hence ammonia production. It also plays an important role in integrating nitrogen and carbohydrate metabolism. Amino acid catabolism releases ammonia which, because of its toxicity, must be disposed of or detoxified.

4.2.2 Ammonia is Toxic

Ammonia is toxic for many reasons (see Cooper and Plum 1987 for a review). At the molecular level, NH_4^+ can substitute for K^+ in Na^+/K^+-ATPase and in $Na^+/K^+/2Cl^-$ cotransport, and for H^+ in Na^+/H^+ exchanger. In neurons, NH_4^+ can substitute for K^+ and permeate through K^+ background channels, affecting the membrane potential. Ammonia can interfere with energy metabolism through inhibiting certain glycolytic enzmes and impairment of the tricarboxylic acid cycle. In vertebrates, ammonia toxicity normally manifests as encephalopathy at the organismal level; the animal enters into a coma and succumbs to the deleterious effects of ammonia. Two distinct mechanisms underlying ammonia toxicity in the central nervous system of mammals have been identified. The primary and rapid event involves the over-activation of NMDA receptor in neurons (Hermenegildo et al. 2000), and probably also in astrocytes (Schliess et al. 2004). The second mechanism of ammonia neurotoxicity is attributable to the ammonia-induced increases in glutamine synthesis and accumulation, resulting in astrocytic swelling and cerebral edema (Albrecht and Norenberg 2006). Unlike mammals, some tropical air-breathing fishes can tolerate high levels of ammonia and/or accumulate high levels of glutamine in their brains (see Ip et al. 2001, 2004a, 2004b, and Chew et al. 2006 for reviews).

Thus, the mechanisms of ammonia toxicity in the brains of fish species with high ammonia tolerance are likely to be different from those in mammalian brains.

4.2.3 Excretory Nitrogenous End-Products

Since ammonia is toxic, it must be excreted or converted into less toxic compounds for transient storage before excretion. Ammonotelic species prevent the buildup of ammonia in their bodies by efficiently excreting ammonia, usually in an aquatic medium. Some animals can facilitate NH_3 excretion by increasing H^+ excretion (Chew et al. 2003a; Ip et al. 2004c; Tay et al. 2006; Wood et al. 2005a), while a few animals are known to be capable of actively excreting ammonia against an unfavorable NH_4^+ gradient (Randall et al. 1999; Ip et al. 2004c, 2004d; Tay et al. 2006; Chew et al. 2007). In terrestrial species that are ureotelic and/or uricotelic, cooperativity between enzymes in the mitochondrial and cytosolic compartments leads to the formation of urea and uric acid, respectively. For ureotelic and uricotelic species, the transient accumulation of end-products in their body fluids posts a much lesser problem than ammonia since urea and uric acid are relatively less toxic. Ammonia can be detoxified to urea through the ornithine–urea cycle in certain land snails, African and South American lungfishes, coelacanths, amphibians, chelonid and rhynchocephalid reptiles, and mammals. Urea transporters that facilitate the permeation of urea across membranes have been identified in the mammalian kidney, amphibian bladder, elasmobranch kidney, and teleost gills and kidney (see Sands 2003a, 2003b; McDonald et al. 2006 for reviews) and there are indications that active urea transport systems may exist in animals (Sands 2003a; McDonald et al. 2006). Excretion of urea requires at least a limited supply of water. Perhaps, because of that, the ornithine–urea cycle became dysfunctional in the reptilian line (Squamata and Crocodilia) leading to the birds, and these animals detoxify ammonia to uric acid instead of urea (Campbell 1973, 1995). Uric acid is highly insoluble in water and can, therefore, be excreted in a semi-solid state.

4.3 Aestivation Involves Fasting, Desiccation, High Temperature and Corporal Torpor

Suspended animation has long fascinated scientists because of its great application potentials in fields ranging from medicine to space travel. Animals enter into a state of inactivity during suspended animation. They have absolutely no intake of food and water, and hence produce minimal or no urine and fecal materials for an extended period. In nature, suspended animation is expressed in animals undergoing hibernation or aestivation. Aestivation occurs widely in both vertebrates and invertebrates to survive arid conditions (except for aquatic aestivators like certain

sponges and sea cucumbers) at high temperature, in many cases during summer. In comparison with hibernation, which occurs in response to cold temperature, aestivation is more intriguing and fascinating because a state of corporal torpor is achieved at high environmental temperature.

From the behavioral point of view, aestivation could be defined as inactivity at high environmental temperature, particularly during dry seasons for terrestrial animals (Gregory 1982; Peterson and Stone 2000). Ultsch (1989) advanced the all-behavior position, calling aestivation "a nonmobile fossorialism." From the physiological point of view, aestivation has often been associated with metabolic depression (Storey 2002), because conservation of metabolic fuels has been regarded as an important adaptation during long periods of aestivation without food intake. While this association is clearly present in endothermic mammals during aestivation, it is debatable whether it can be universally applied to aestivating ectothermic animals. For instance, it has been proposed that metabolic depression (Storey and Storey 1990; Guppy and Withers 1999) would decrease both urea production and respiratory water loss, in addition to conserving metabolic fuels, in aestivating turtle (Kennett and Christian 1994; Hailey and Loveridge 1997). However, whether metabolic depression in turtles is an adaptation to aestivation per se or simply a response to fasting (Belkin 1965; Sievert et al. 1988) remains an open question. In fact, the decrease in oxygen consumption in laboratory-aestivating yellow mud turtle *Kinosternon flavescens* is identical to that of fully hydrated turtles that are fasted for an equivalent period (Seidel 1978; Hailey and Loveridge 1997).

For African lungfishes, it has long been accepted that a profound decrease in metabolic rate occurs in association with aestivation in a mud cocoon or an artificial substratum (Smith 1935; Janssens and Cohen 1968a, 1968b), but without any knowledge on whether aestivation takes place in hypoxia or normoxia. Recently, it was demonstrated that the slender lungfish, *Protopterus dolloi*, aestivating in a completely dried mucus cocoon in air (normoxia) had a respiratory rate comparable to that of control fish immersed in water (Perry et al. 2008; the application of the term "terrestrialization" to these fish was inappropriate; see comments by Loong et al. 2008a and Sect. 5.1.2). By contrast, the respiratory rate of fish immersed in water was greatly reduced by aerial hypoxia (Perry et al. 2005). Loong et al. (2008b) therefore reasoned that there could be a greater reduction in metabolic rate in fish aestivating in hypoxia than in normoxia, resulting in a greater suppression in nitrogen metabolism in the former than in the latter. They (Loong et al. 2008b) determined ATP and creatine phosphate in three different regions of *Protopterus annectens* based on ^{31}P NMR spectroscopy. Indeed, their results support the proposition that metabolic depression in aestivating African lungfish is triggered by hypoxia and may not be an integral part of aestivation. Therefore, aestivation can be regarded as a state of summer corporal torpor with or without metabolic rate reduction, depending on the environmental conditions and the animal species involved. This is an important point for excretory nitrogen metabolism because metabolic rate reduction encompasses processes like ammonia production (see Sect. 5.2.3) and urea synthesis which are energy dependent (see Sect. 5.2.4), and it has been conceptually linked in part with suppression of protein synthesis (see Sect. 5.2.1).

4.4 Current Issues on Excretory Nitrogen Metabolism and Related Phenomena in Aestivators

4.4.1 Aestivation in Normoxia or Hypoxia?

It is difficult to interpret information available in the literature on nitrogen metabolism in aestivating animals because, over many instances, it is uncertain whether the aestivating animal was being exposed to hypoxia and, if so, the degree of hypoxia involved. As a result, it is difficult to analyze phenomena incidental to aestivation independent of hypoxia. However, it is important to do so because aestivation in mud or an artificial substratum may prescribe exposure to hypoxia and Loong et al. (2008b) have demonstrated recently that aestivation in normoxia or in hypoxia exerts different effects on nitrogen metabolism in the African lungfish *P. annectens*. Furthermore, there are differences in excretory nitrogen metabolism between the swamp eel, *Monopterus albus*, aestivating in mud and exposed to air (Chew et al. 2004), and between the African lungfish, *P. annectens*, aestivating in mud and aestivating in air (Loong et al. 2008a).

4.4.2 Induction, Maintenance and/or Arousal?

There was a lack of effort in the past to identify and examine phenomena associated specifically with a certain phase of aestivation, and hence it becomes difficult to evaluate the physiological implications of the observed phenomena. Aestivation comprises three phases: induction, maintenance, and arousal. During the induction phase, animals detect environmental cues and turn them into some sort of internal signals that would instill the necessary changes at the behavioral, structural, physiological, and biochemical levels in preparation of aestivation. After entering the maintenance phase, they have to preserve the biological structures and sustain a slow rate of waste production to avoid pollution of the internal environment. Upon the return of favorable environmental conditions, they must arouse from aestivation, excrete the accumulated waste products, and feed for repair and growth. Completion of aestivation occurs only if arousal is successful; if not, the animal have had apparently succumbed to certain factors during the maintenance phase. It can, therefore, be deduced that adaptive changes in nitrogen metabolism, especially protein synthesis and degradation, would vary in different phases of aestivation, although studies in the past focused largely on the maintenance phase.

4.4.3 Preservation of Biological Structures or Conservation of Metabolic Fuels?

During long-term fasting, animals incapable of aestivation or hibernation enter into a protein catabolic state, mobilizing amino acids as metabolic fuels and releasing

ammonia of endogenous origins. However, unlike carbohydrates and lipids, there is no known protein store in animals (see Sect. 2.1), and proteins have to be mobilized from biological structures that have specific functions. Skeletal, smooth, and cardiac muscles are protein structures with contractile properties but cardiac muscles must be spared from the catabolic process until very critical moments. Although skeletal muscle is the most prominent protein source, aestivating animals have to preserve muscle structure and strength in preparation of arousal. This has to be achieved in spite of the aestivating animal being in a state of corporal torpor which is associated with skeletal muscle disuse. Muscle disuse can lead to a decrease in protein synthesis and an increase in protein degradation, resulting in muscle atrophy (Childs 2003). However, a drastic increase in proteolysis, as in the case of fasting alone, does not occur in aestivating animals, and they can effectively preserve muscle structure and strength through suppression of protein degradation and amino acid catabolism. Therefore, suppression of protein degradation during the maintenance phase of aestivation should be regarded primarily as an adaptation to preserve proteinaeous structures and functions (Hudson et al. 2005; Symonds et al. 2007), and conservation of metabolic fuel stores can at best be regarded as a secondary phenomenon.

4.4.4 Modifications of Structures/Functions or Static Preservation of Structures?

In the past, the occurrence of organic structural modifications in aestivating animals has been largely neglected; but to date, aestivation in African lungfishes is known to be associated with structural and functional modifications in at least the heart and the kidney (Icardo et al. 2008; Ojeda et al. 2008; Amelio et al. 2008). Recently, Icardo et al. (2008) reported that the myocytes in the trabeculae associated with the free ventricular wall of *P. dolloi* showed structural signs of low transcriptional and metabolic activity (heterochromatin, mitochondria of the dense type) while in water (Icardo et al. 2008). These signs are partially reversed in aestivating fish (euchromatin, mitochondria with a light matrix), and paradoxically, aestivation appears to trigger an increase in transcriptional and synthetic myocardial activities, especially, at the level of the ventricular septum (Icardo et al. 2008). In addition, Ojeda et al. (2008) demonstrated structural modifications in all the components of the renal corpuscle of aestivating *P. dolloi*. These changes can be reversed after arousal, indicating that the renal corpuscle is a highly dynamic structure capable of modifying its architecture in response to different phases of aestivation. Studies in progress indicate that morphological downregulation and quick restoration of morphology during the maintenance phase and arousal phase, respectively, also occur in the intestine of *P. annectens* (J. Icardo, personal communication). Thus, aestivation cannot be regarded as the result of a general depression of metabolism, but it involves the complex interplay between upregulation and downregulation of diverse cellular activities (Icardo et al. 2008). Unlike fasting in nonaestivators, aestivation could involve variations in rates of protein degradation

and protein synthesis, reconstructing and regenerating cells and tissues during the induction and arousal phases, respectively, through a rapid protein turnover with little production of nitrogenous wastes.

4.4.5 *Increased Detoxification of Ammonia or Decreased Ammonia Production?*

Due to the lack of water to facilitate nitrogenous waste excretion, ammonia must be turned into less toxic products for retention. In the past, ammonia detoxification took center stage in nitrogen metabolism in aestivating animals (Wither 1998; Wright 2007), but the conversion of ammonia to less toxic products, e.g. glutamine, urea, and uric acid, is energy intensive. More importantly, since aestivating animals undergo long-term fasting, problems associated with toxic ammonia being released from excess amino acids as in fed animals (see Sect. 2.1) no longer prevail, and there would be a low demand for ammonia detoxification. Furthermore, modification and preservation of biological structures during the induction and maintenance phases of aestivation, respectively, prescribe a low rate of ammonia production which would further ameliorate the demand for ammonia detoxification through energy-intensive processes. Thus, the focus should be on decreased ammonia production instead of increased detoxification of ammonia.

4.4.6 *Nitrogenous Wastes for Excretion or Nitrogenous Products with Specific Functions?*

To date, the intrinsic mechanisms by which cells, tissues, and organs are able to adapt and match their function to the environmental cues during aestivation are still enigmatic. Röszer et al. (2004, 2005) reported that nitric oxide (NO) was involved in the neural transmission to intestinal muscles of the snail *Helix lucorum*. During dormancy, enteric release of NO was blocked and the L-arginine/NO conversion ability of nitric oxide synthase (NOS) was apparently inhibited. Results obtained recently from African lungfish indicate that NO and urea can act as signaling molecules in various phases of aestivation. Amelio et al. (2008) demonstrated that cardiac eNOS expression increased in *P. dolloi* after 6 days of aestivation but decreased in those aestivated for 40 days. Furthermore, both renal localization and expression of eNOS increased with aestivation. They (Amelio et al. 2008) concluded that NO contributed, probably in an autocrine–paracrine fashion, to cardiac and renal readjustments during aestivation. On the other hand, Ip et al. (2005b) reported that increased tissue urea contents could be one of the essential factors in initiating and maintaining aestivation in *P. dolloi* (see Sect. 5.1.1), and there are indications that accumulated urea could facilitate rehydration during the arousal phase of aestivation (see Sects. 5.2.6 and 5.3.1). In addition, Muir et al. (2008)

reported that urea depressed the metabolism of living organs in vitro, although its effect varied with temperature and seasonal acclimatization. Thus, the conception that urea is accumulated simply as an end-product of ammonia detoxification, pending excretion during subsequent arousal, needs to be reevaluated.

At present, why aestivators generally prefer to accumulate urea instead of other nitrogenous products during aestivation is debatable. So far, only some phyllomedusid tree frogs are known to coat their body surface with skin secretion and excrete uric acid to minimize water loss during aestivation (Shoemaker et al. 1972; Abe 1995). Urea accumulation in aestivating animals has been proposed to serve the purpose of reducing evaporative water loss (Campbell 1973; Storey 2002), but reports on this phenomenon are controversial (see Sect. 5.2.6). Storey (2002) proposed that a gradual increase in protein catabolism would occur in aestivating animals as the demand for urea synthesis increases, presumably to facilitate retention of tissue water (Storey 2002). However, urea synthesis is an energy-intensive process, utilizing 4 and 5 moles of ATP per mole of urea synthesized in animals possessing carbamoyl phosphate synthetase I (CPS I; e.g., mammals) and CPS III (e.g., snails and African lungfishes), respectively. An upregulation of urea synthesis during aestivation would, therefore, increase energy expenditure and contribute negatively to metabolic depression. More importantly, the mobilization of nitrogen for increased urea synthesis to reduce water loss would contradict the fundamental principles of preservation of biological structures and metabolic fuels during suspended animation. The importance of preservation of nitrogen during suspended animation is evidenced from hibernating bears, in which urea recycling occurs between animal tissues and the intestinal microbial fauna (Barboza et al. 1997). Urea recycling effectively prevents the build up of urea in the body during hibernation. It minimizes body protein loss and conserves mobility, providing greater flexibility during winter and facilitating rapid resumption of foraging and growth in spring (Barboza et al. 1997). By contrast, urea recycling has not been demonstrated definitively in aestivating animals, indicating that urea accumulated during aestivation may have important functions.

4.5 Excretory Nitrogen Metabolism in Aestivators

In the following sections, attempts will be made to address excretory nitrogen metabolism in animals during the three phases of aestivation, based mainly on results reported in the literature on snails, African lungfishes, and amphibians. Occasionally, works on hibernating animals will be cited because of the vast amount of information available on hibernation. Special emphases will be given to African lungfishes, which hold an important position in the evolutionary tree with respect to water–land transition. African lungfishes are obligatory air-breathers. They are ureogenic and possess a full complement of hepatic ornithine–urea cycle enzymes (Janssens and Cohen 1966, 1968a; Mommsen and Walsh 1989) that comprises CPS III instead of CPS I (Chew et al. 2003b; Loong et al. 2005). However, they are ammontelic in water, and would turn transiently ureotelic after

feeding (Lim et al. 2004; Iftikar et al. 2007). African lungfishes (*Protopterus* spp.) can undergo aestivation in mud cocoons during desiccation (Smith 1930; Janssens 1964; DeLaney et al. 1974; Fishman et al. 1987), and they can aestivate for as long as 3–5 years (Smith 1930), which happens to be the longest aestivation period known among vertebrates. Recently, we have succeeded in inducing African lungfishes to aestivate in completely dried mucus cocoon in plastic boxes in the laboratory (Chew et al. 2004; Ip et al. 2005c; Loong et al. 2005, 2007, 2008a, 2008b). During the induction phase, the fish hyperventilates and secretes a lot of mucus which turns into a dry mucus cocoon within 6–8 days. Aestivation begins when the fish is completely encased in a cocoon, and there is a complete cessation of feeding and locomotor activities. The fish can perpetuate to aestivate under such conditions for more than a year in the laboratory. The aestivating lungfish can be aroused by the addition of water. Upon arousal, the fish struggles out of the cocoon and swims, albeit sluggishly, to the water surface to gulp air. Feeding begins approximately 7–14 days after arousal, and the fish grows and develops as normal thereafter.

4.5.1 Nitrogen Metabolism and Excretion during the Induction Phase

4.5.1.1 Urea as an Internal Signal in the Induction Process

Although aestivation normally occurs in association with summer heat, it is not part of a chronobiological rhythm but an episodic event that requires an inducing stimulus. Several inducing factors of aestivation have been proposed for African lungfishes (Fishman et al. 1987), which include (1) dehydration, leading to oliguria/anuria and metabolic acidosis, (2) air-breathing on land, leading to CO_2 retention and respiratory acidosis, (3) starvation, affecting the metabolic, circulatory and respiratory changes and (4) stress, leading to release of neurohormonal mediators and/or affecting thyroid function. Recent works reveal that increases in environmental ammonia concentration (Chew et al. 2005b; Ip et al. 2005b) and ambient salinity (Ip et al. 2005a) could be important environmental cues for the induction of aestivation in African lungfish.

Naturally, aestivation occurs when an African lungfish is stranded in a puddle of water or in semi-solid mud during the dry season. The continual excretion of ammonia into a small volume of external medium would lead to high concentrations of environmental ammonia. The situation would be aggravated by the constant evaporation of the external medium under high temperature, further concentrating ammonia and other ions and resulting in high ambient salinity. Indeed, Ip et al. (2005a) demonstrated that *P. dolloi* exposed to water of salinity 3 for 6 days exhibited consistently lower daily urea excretion rate as compared with the freshwater control. Simultaneously, there were decreases in urea contents in various tissues and organs. Ip et al. (2005a), therefore, concluded that *P. dolloi* could respond to salinity changes in the external medium as it dried up, suppressing ammonia production in preparation of aestivation. In a separate study, Chew et al. (2005b)

discovered that *P. dolloi* was capable of maintaining low concentrations of ammonia in its body by upregulating the rate of urea synthesis to detoxify ammonia when exposed to environmental ammonia. Simultaneously, *P. dolloi* was able to increase its rate of urea excretion, but urea accumulated in the muscle, liver, and plasma of specimens exposed to environmental ammonia despite the significant increase in urea excretion rate (Chew et al. 2005b). Similar observations were made on *P. dolloi* fasted for 40 days, and urea contents in various tissues increased significantly in fasted *P. dolloi* (Chew et al. 2004), despite being immersed in water and having the capacity to upregulate urea excretion under certain conditions (Lim et al. 2004; Wood et al. 2005b). Since fasting is known to be one of the inducing factors of aestivation, urea accumulation could be an important part of the induction mechanism.

Ip et al. (2005b) undertook a series of experiments that aimed to determine whether ammonia (as NH_4Cl) injected intraperitoneally into *P. dolloi*, would be excreted directly instead of being detoxified to urea, and to examine whether injected urea would be retained in this lungfish, leading to decreases in liver arginine and brain tryptophan levels as observed during aestivation on land. Despite being ureogenic, *P. dolloi* rapidly excreted the excess ammonia within the subsequent 12 h after NH_4Cl was injected into its peritoneal cavity. By contrast, when urea was injected intraperitoneally into *P. dolloi*, only a small percentage (34%) of it was excreted during the subsequent 24 h. At hour 24, significant quantities of urea were retained in various tissues of *P. dolloi*. Injection with urea led to an apparent reduction in endogenous ammonia production, a significant decrease in the hepatic arginine content, and a significantly lower level of brain tryptophan in this lungfish. All these three phenomena had been observed in aestivating *P. dolloi* (Chew et al. 2004). Therefore, Ip et al. (2005b) concluded that urea synthesis and accumulation could be one of the essential factors in initiating and maintaining aestivation.

Similarly, Hiong et al. (2005) reported that the giant African snail, *Achatina fulica*, accumulated urea progressively not only during 23 days of aestivation, but also during 23 days of fasting (Hiong et al. 2005). Fasting did not impede ammonia or urea excretion in *A. fulica* and fasting snails moved around in the containers actively with part of their bodies fully extended out of the shell. Yet, urea accumulation occurred in the hepatopancreas and foot muscle, with ammonia levels remained relatively unchanged. Hence, similar to African lungfishes (Chew et al. 2005b; Ip et al. 2005c), urea might be involved as part of the induction mechanism of aestivation, which is unrelated to nitrogenous excretion and water retention, in *A. fulica* (Hiong et al. 2005).

4.5.1.2 Changes in the Permeability of the Skin to Ammonia and its Implications

Since African lungfishes would have to defend against environmental ammonia toxicity during the induction phase of aestivation, Loong et al. (2007) undertook a study to determine how the African lungfish, *Protopterus aethiopicus*, defended

against ammonia toxicity when confronted with high concentrations (30 or 100 mmol l^{-1}) of environmental ammonia. Using an Ussing-like apparatus, they (Loong et al. 2007) reported that the skin of *P. aethiopicus* had low permeability (1.26×10^{-4} μmol $min^{-1} cm^{-1}$) to NH_3 in vitro. Indeed, the influx of exogenous ammonia into fish exposed to 30 mmol l^{-1} NH_4Cl was low (0.117 μmol min^{-1} 100 g^{-1} fish). As a result, *P. aethiopicus* could afford to maintain relatively low ammonia contents in plasma, muscle, liver, and brain even after 6 days of exposure to 100 mmol l^{-1} NH_4Cl. In addition, Loong et al. (2007) obtained results which suggest that *P. aethiopicus* was capable of decreasing the NH_3 permeability of its body surface in response to ammonia exposure. After 6 days of exposure to 100 mmol l^{-1} NH_4Cl, the NH_3 permeability constant of the skin (0.55×10^{-4} μmol min^{-1} cm^{-1}) decreased to half of the control value. A decrease in the already low cutaneous NH_3 permeability and an increased urea synthesis, working in combination, allowed *P. aethiopicus* to effectively defend against environmental ammonia toxicity without elevating the plasma ammonia level. Hence, unlike other fishes (see Ip et al. 2001; Ip et al. 2004a, 2004b; Chew et al. 2006 for reviews), glutamine and alanine contents did not increase in the muscle and liver, and there was no accumulation of glutamine in the brain, even when the fish was immersed in water containing 100 mmol l^{-1} NH_4Cl (Loong et al. 2007).

The mechanisms involved in the alteration of NH_3 permeability in the skin of *P. aethiopicus* are unclear at present, but results obtained by Loong et al. (2007) imply that the permeability of the skin to water and ions can be altered during the induction and, perhaps, also during the arousal phases of aestivation. Similar to the low NH_3 permeability in the skin of *P. aethiopicus*, the body surface of *P. dolloi* has low permeability to water and ions (Wilkie et al. 2007). Recently, Wilkie et al. (2007) and Staples et al. (2008) studied *P. dolloi* in water (control) or exposed to air for an extended period (5 months) without the formation of a complete cocoon. In essence, the fish was sustained in a prolonged induction phase of aestivation which they described as "terrestrialization" (Wood et al. 2005b; Wilkie et al. 2007). "Terrestrialization" was achieved by spraying water onto the bottom of the container in which *P. dolloi* was induced to aestivate. Since the bottom of the container was wet, an incomplete cocoon was formed only along the dorsal–lateral cutaneous surface, with the ventral surface in direct and constant contact with water throughout the 5-month period. Unlike aestivating fish, those undergoing terrestrialization exhibited occasional movement and were not confronted with desiccation which should theoretically lead to tissue dehydration. Thus, it is unsurprising that Wilkie et al. (2007) and Staples et al. (2008) reported a substantial increase instead of a decrease in muscle water content in fish exposed to air for 5 months, and their results suggest that water was absorbed through the ventral cutaneous surface. During the initial phase of air exposure, water and ion fluxes in the experimental fish were comparable to those in fish kept in water. However, the water flux declined over time, indicating once again that adaptive changes could have occurred in the skin under such experimental conditions (Wilkie et al. 2007).

It is important to mention that observations made by Wilkie et al. (2007) and Staples et al. (2008) would not be manifested by fish during the maintenance phase

of aestivation, whereby the ventral surface is encased completely in a dry mucus cocoon. In addition, if not because of the artificial extension of the induction phase to 5 month, tissue urea content would not have built up to high concentrations (13-fold). Without high levels of tissue urea, the magnitude of water retention in the muscle would have been dismal during an induction period of 6–8 days. On the contrary, a rapid rehydration is expected to occur in fish aroused from long-term aestivation when water becomes available to the ventral body surface because of high concentrations of urea being accumulated in the body (see Sect. 5.3.1). At present, no information is available on changes in permeabilities of the skin to NH_3, water, or ions in African lungfishes during the transition from the induction phase to the maintenance phase (when the body is encased completely in a dried mucus cocoon), or from the maintenance phase to the arousal phase, of aestivation. Perhaps, efforts should be made in the future to examine such changes and to elucidate the mechanisms involved.

4.5.1.3 A Decrease in Ammonia Production and an Increase in Urea Synthesis

The traditional focus of nitrogen metabolism in aestivating lungfishes was on increased urea synthesis (Smith 1930, 1935; Janssens 1964; Janssens and Cohen 1968a, 1968b). Although decreased ammonia production was suspected to occur during aestivation (Janssens and Cohen 1968a; Carlisky and Barrio 1972), its importance during both the maintenance and the induction phases has not been confirmed until recently (Chew et al. 2003b, 2004; Loong et al. 2005; Ip et al. 2005d). Chew et al. (2003b) demonstrated that there were significant increases in urea levels in the muscle (8-fold), liver (10.5-fold), and plasma (12.6-fold) of *P. dolloi* exposed to air for 6 days without entering to aestivation. There was also a significant increase in the urea excretion rate in fish exposed to air for 3 days or more. Taken together, these results indicate that *P. dolloi* increased the rate of urea synthesis to detoxify ammonia during this period. Aerial exposure also led to an increase in the hepatic ornithine–urea cycle capacity, with significant increases in the activities of CPS III (3.8-fold), argininosuccinate synthetase + lyase (1.8-fold) and glutamine synthetase (2.2-fold). In addition, the ammonia excretion rate in the experimental fish decreased significantly but there were no significant increases in ammonia contents in the muscle, liver, or plasma, indicating that endogenous ammonia production was drastically reduced. In a separate study, Loong et al. (2005) reported that the rates of urea synthesis in *P. aethiopicus* and *P. annectens* exposed to air for 6 days increased only 1.2- and 1.5-fold, respectively, which were smaller than that in *P. dolloi*. However, unlike *P. dolloi*, aerial exposure had no significant effects on the hepatic CPS III activities of *P. aethiopicus* and *P. annectens*. Rather, aerial exposure induced relatively greater degrees of reductions in ammonia production in *P. aethiopicus* (34%) and *P. annectens* (37%) compared with *P. dolloi* (28%). Thus, Loong et al. (2005) concluded that there were subtle differences in responses by various species of African lungfishes to aerial exposure, and it would

appear that *P. aethiopicus* and *P. annectens* depended more on a reduction in ammonia production than an increase in urea synthesis to ameliorate ammonia toxicity during the induction phase of aestivation.

In the case of *P. dolloi* exposed to air, the apparent decrease in ammonia production was associated with significant decreases in contents of glutamate, glutamine, lysine, and total free amino acid in the liver (Chew et al. 2003b). Therefore, Chew et al. (2003b) interpreted that a decrease in proteolysis and amino acid catabolism could have occurred. However, in retrospect, the reduction in ammonia production during the induction phase of aestivation should not be viewed as an adaptation responding solely to ammonia toxicity and conservation of metabolic fuels (Chew et al. 2003b). There could actually be an increase in protein synthesis, which would also result in decreases in ammonia production and in the total free amino acid content. Some animals (African lungfishes, Chew et al. 2004; Loong et al. 2005, 2008b; green-striped burrowing frog, Booth 2006) are known to secrete large quantities of mucus before aestivation. The mucus subsequently dries up to form a mucus cocoon which presumably functions to reduce evaporative water loss. The composition of the mucus cocoon is unclear at present, but there are indications that it comprises nitrogenous compounds (Bayomy et al. 2002). Thus, it is logical to deduce that there could be an increase in the synthesis of certain proteins during the induction phase. Furthermore, structural changes have been identified recently as important facets of aestivation in African lungfishes (Icardo et al. 2008; Ojeda et al. 2008), and structural changes cannot occur without increased protein synthesis. Hence, results obtained by Chew et al. (2004) could be interpreted as the occurrence of increased protein synthesis and turnover instead of decreased protein degradation during the induction phase of aestivation. Since African lungfishes hyperventilate during the initial period of the induction phase of aestivation, the reduction in ammonia production may not occur in association with metabolic depression, and there could be an increase in metabolic rate instead.

4.5.2 Nitrogen Metabolism During the Maintenance Phase

4.5.2.1 A Decrease in Protein Synthesis in General and Increases in Syntheses of Certain Proteins in Specific Organs

Protein synthesis is an energy-intensive process, requiring ~5 ATP equivalents per peptide bond formed (Storey and Storey 2004), and is therefore sensitive to the availability of energy and amino acids. The rate of protein synthesis is suppressed during fasting and during severe hypoxia in oxygen-sensitive systems. Although metabolic depression may not be universally exhibited by all aestivators, it has been unequivocally demonstrated in some aestivating animals (Guppy et al. 2000), and suppression of protein synthesis contributes substantially to metabolic depression. For instance, aestivation in the Australian desert frog, *Neobatrachus centralis*,

is accompanied by an in vivo metabolic depression of 77% (Withers 1993; Fuery et al. 1998). Using an in vitro liver slice preparation, Fuery et al. (1998) reported a 55% depression in metabolic rate with a concomitant 67% decrease in the rate of protein synthesis. The decrease in protein synthesis accounted for 52% of the metabolic depression of the tissue, but only 4.9% of the metabolic depression of the whole animal. Similarly, Pakay et al. (2002) concluded that metabolism depression in aestivating land snail, *Helix apersa*, was accompanied by a downregulation of protein synthesis in hepatopancreas (23%) and foot muscle (53%). However, it should not be generalized that syntheses of all proteins are suppressed in every organ during the maintenance phase of aestivation because there are results that indicate otherwise.

At the onset of aestivation, Australian desert frogs (*Neobatrachus* sp. and *Cyclorana* sp.) assume a water-conserving posture and become inactive (Withers 1995). A thin, transparent cocoon is observed to form within a week of onset of inactivity, and becomes progressively thicker and opaque. The entire body surface is covered with cocoon, which consists of an accumulation of multiple layers of single-cell-thick sheets of outer epidermal cells, formed at regular periods of about every 2 (*Cyclorana* sp.) to 4 days (*Neobatrachus* sp.) that correspond to the normal shedding frequency. As a result, the thickness and the number of layers of the cocoon increase progressively with the duration of aestivation. Hence, normal or increased rate of protein synthesis is likely to be maintained in the cutaneous epithelium in desert frogs during aestivation. Additionally, Bayomy et al. (2002) demonstrated that there were changes in acid mucopolysaccharide components in the liver, kidney, skin, and cocoon of aestivating desert frogs, which presumably helps to retain water and to protect the frogs from desiccation. Acid mucopolysaccharide in the cocoon probably functions as cement between the cocoons' layers and its physical presence presumably contributes to prevent evaporative water loss.

On the other hand, Shalan et al. (2004) monitored changes in testis size, histological status, and plasma testosterone level in three species of desert frogs during aestivation. They reported that relative testis mass declined gradually in all species during the first 7 months of aestivation and then increased significantly after 6–9 months of aestivation in two species (*Cyclorana maini* and *Neobatrachus sutor*). Early spermatocytogenesis were evident in all three species after 7 months of aestivation. Shalan et al. (2004), therefore, concluded that testicular recrudescence occurred after approximately 1 year of aestivation in desert frogs, which prepared them to breed again, once rain falls. These results indicate that increased protein synthesis must have occurred in the testes of desert frogs during the maintenance phase of aestivation in preparation for arousal.

The definitive evidence of increased synthesis of certain proteins during aestivation has been demonstrated in aestivating snails. Brooks and Storey (1995) examined patterns of ^{35}S-methionine-labeled protein in land snail *Otala lactea* exposed to four different conditions. Increases in the radioactivity of four proteins were observed in the hepatopancreas and foot muscle of snails undergoing short- or long-term aestivation, indicating that there were increases in synthesis of these proteins. The functions of these proteins were uncertain

but Brooks and Storey (1995) proposed that they were involved in metabolic depression in relation to aestivation.

Taken together, it can be concluded that besides acting as metabolic fuels, endogenous amino acids continue to act as building blocks for protein synthesis in certain organs during aestivation in spite of fasting. Since there is no known protein stores in animals, amino acids are likely to be mobilized from proteolysis of muscles which happen to be the most prominent source of protein in the body.

4.5.2.2 Protein/Amino Acids as Metabolic Fuels Versus Preservation of Muscle Structure and Strength

During aestivation, energy can be produced from the catabolism of lipids, ketone bodies, carbohydrates, and/or proteins/amino acids (Frick et al. 2008b), and the primary fuel preference varies between species. Large amounts of metabolic reserves, in the form of lipids and glycogen, accumulate in the prepupae of the arctiid moth, *Cymbalophora pudica*, prior to aestivation, and glycogen serves as the main metabolic fuel for aestivating prepupae (Kostal et al. 1998). Aestivating land snails (*Oreohelix strigosa* and *O. subrudis*) utilize predominately carbohydrates during the initial 2–4 months of aestivation, and protein is the primary metabolic substrate after polysaccharide reserves are depleted, with lipid being catabolized at a low rate throughout aestivation (Rees and Hand 1993). For aestivating sea cucumber, *Apostichopus japonicus*, lipid, and protein act as energy sources for large-size specimens while protein is the major fuel for small-size individuals (Yang et al. 2005, 2006). Many vertebrate species accumulate large glycogen stores prior to aestivation, but unlike hypoxic exposure, there is usually no instantaneous decrease in tissue glycogen contents during the maintenance phase of aestivation. Hence, either glycogen degradation rate is extremely low, or glycogenolysis is compensated constantly by gluconeogenesis and glycogen synthesis. The continuous build up of urea in the body of aestivating African lungfishes suggests protein/amino acids as the major metabolic fuel during long-term aestivation (Janssens and Cohen 1968a, 1968b; Chew et al. 2004; Frick et al. 2008a), and the relatively unchanged levels of glycogen (Janssens and Cohen 1968a, 1968b; Frick et al. 2008a) indicates that a portion of the carbon skeletons derived from amino acid catabolism is channeled to gluconeogenesis. The yellow mud turtle, *K. flavescens*, has very high lipid content prior to aestivation (Long 1985). Although catabolism of proteins and amino acids occur during aestivation, the rate of nitrogenous waste production is dependent on initial lipid stores (Peterson and Stone 2000). Overall, it can be generalized that proteins/amino acids act as the major metabolic fuel in animals undergoing long-term aestivation. However, despite the metabolic demand for protein and amino acids, prominent increases in rates of proteolysis and amino acid catabolism do not occur in muscle during the maintenance phase of aestivation.

Skeletal muscle atrophy refers to a loss of muscle volume and strength due to decreases in the size (hypotrophic) and/or the number (hypoplastic) of muscle

fibers, resulting in a compromised capacity for physical work. Disuse muscle atrophy occurs as a result of immobility (Childs 2003). To date, disuse muscle atrophy has only been examined in detail in one aestivator, i.e., the green-striped burrowing frog, *Cyclorana alboguttata* (Hudson and Franklin 2002a, 2002b, 2003; Hudson et al. 2006; Symonds et al. 2007). It has been reported that *C. alboguttata* can preserve muscle structure and function after 6–9 months of aestivation. At present, the actual mechanisms behind the preservation of muscle structure and function in aestivators and hibernators are uncertain. For hibernating mammals, some interesting hypotheses have been raised, which include the positioning of the immobilized limb muscle in the over-wintering bears (Tinker et al. 1998) and the use of "shivering thermogenesis" as an exercise cue in ground squirrels (Wickler et al. 1991). In addition, it has been hypothesized that hibernating bears can retain their skeletal muscle protein and strength by lowering the energy demand for proteolysis through metabolic rate reduction, drawing on labile protein reserves such as visceral smooth muscle and extracellular matrix, and/or synthesizing new amino acids and protein from urea nitrogen (Harlow et al. 2001). For the aestivating frog, *C. alboguttata*, Hudson et al. (2006) reported that transcriptional silencing of bioenergetic genes, such as NADH ubiquinone oxidoreductase 1, ATP synthase, and superoxide dismutase 2 occurred in the skeletal muscle. They (Hudson et al. 2006) suggested that defenses against oxidative stress could be involved in the suppression of disuse muscle atrophy. Recently, Hudson et al. (2008) demonstrated that increases in expression of seven genes, which code for proteins with established roles in epigenetically mediated gene silencing, occurred in the muscle of *C. alboguttata* after 6 months of aestivation, and therefore concluded that transcriptional silencing of skeletal muscle bioenergetic genes could involve chromatin remodeling.

4.5.2.3 Suppression of Ammonia Production and Changes in Hepatic GDH Activity

The swamp eel, *M. albus,* inhabits muddy ponds, swamps, canals, and rice fields, where it can burrow into the moist earth and survives for long periods during the dry summer season. However, Tay et al. (2003) reported that mortality increased when *M. albus* was exposed to air for 8 days or more. Thus, Chew et al. (2005a) undertook a study with the objective of elucidating the strategies adopted by *M. albus* to defend against ammonia toxicity during 6 or 40 days of aestivation in mud and to evaluate whether these strategies were different from those adopted by fish to survive 6 days of aerial exposure. Ammonia and glutamine accumulations occurred in the muscle and liver of fish exposed to air (normoxia) for 6 days, indicating that ammonia was detoxified to glutamine under such conditions. In contrast, ammonia accumulation occurred only in the muscle, with no increases in glutamine or glutamate contents in all tissues, of fish aestivated in mud for 6 or 40 days. While aestivation in mud prevented excessive water loss through evaporation, *M. albus* was exposed to hypoxia, as indicated by significant decreases in blood PO_2, muscle energy charge, and ATP content. Glutamine synthesis is energy

intensive, and that could be the reason why *M. albus* did not depend on increased glutamine synthesis to defend against ammonia toxicity when a decrease in ATP supply occurred. Instead, suppression of endogenous ammonia production was adopted as the major strategy to ameliorate ammonia toxicity when *M. albus* aestivated in mud. Results obtained by Chew et al. (2005a) suggest that suppression of ammonia production in *M. albus* could be induced more effectively by environmental hypoxia than increased tissue ammonia concentrations. This may explain why *M. albus* is able to aestivate in mud for long periods (40 days) but can survive in air for only 8–10 days.

For the African lungfish *P. dolloi*, Chew et al. (2004) demonstrated that the rate of ammonia production reduced to 26 and 28% during the first 6 and the subsequent 34 days of aestivation, respectively, as compared with the day 0 control value of 6.83 μmol day^{-1} g^{-1}. For *P. aethiopicus* that underwent 46 days of aestivation, there was only a 20% decrease in the rate of ammonia production during the initial 12 days, but a profound decrease (96%) in ammonia production occurred during the final 12 days of aestivation (day 34 to day 46) (Ip et al. 2005d). Hence, different African lungfish species exhibit different capacity of reduction in ammonia production. In another study, Loong et al. (2008a) examined the importance of increased urea synthesis and decreased ammonia production in *P. annectens* aestivating in air versus those aestivating in mud. Twelve days of aestivation in air led to significant increases in contents of urea, but not ammonia, in tissues of *P. annectens*. The estimated rate of urea synthesis increased 2.7-fold despite the lack of changes in the activities of hepatic ornithine–urea cycle enzymes, but there was only a minor change in the estimated rate of ammonia production. After 46 days of aestivation in air, the ammonia content in the liver decreased significantly and contents of urea in all tissues studied increased significantly, indicating that the fish shifted to a combination of increased urea synthesis (1.4-fold of the day 0 value) and decreased ammonia production (56% of the day 0 value) to defend against ammonia toxicity. By contrast, 12 days of aestivation in mud produced only minor increases in tissue urea contents, with ammonia contents remained unchanged. This was apparently achieved through decreases in urea synthesis and ammonia production (40 and 15%, respectively, of the corresponding day 0 value). Surprisingly, 46 days of aestivation in mud resulted in no changes in tissue urea contents, indicating that profound suppressions of urea synthesis and ammonia production (2.6 and 1.2%, respectively, of the corresponding day 0 value) had occurred. Since fish aestivated in mud had relatively low blood pO_2 and muscle ATP content, they could have been exposed to hypoxia, which induced reductions in metabolic rate and ammonia production. Consequently, fish aestivating in mud had a lower dependency on increased urea synthesis to detoxify ammonia, which is energy intensive, than fish aestivating in air.

Since transdeamination is an important pathway of amino acid catabolism, GDH is in a crucial position to regulate ammonia production (see Sect. 2.1). Janssens and Cohen (1968b) suspected, but without supportive data, that reduction in ammonia production in aestivating *P. aethiopicus* was achieved through the regulation of GDH activities. GDH is known to be activated by ADP (Campbell 1973), the concentration

of which may change during hypoxic exposure, and GDH can also be modified by ADP-ribosylation (Herrero-Yraola et al. 2001). Recently, Loong et al. (2008a) undertook a study to examine whether there would be changes in specific activity and kinetic properties of GDH from the liver of *P. annectens* during the induction and maintenance phases of aestivation, and whether these changes would be different between normoxic and hypoxic fishes, especially with regard to ADP activation in vitro. They discovered that the activities of hepatic GDH, in the amination and deamination directions, remained relatively constant in fish exposed to normoxia during the induction phase (3 or 6 days) of aestivation (Loong et al. 2008a). However, there was a significant increase in the GDH amination activity, with the deamination activity remained unchanged, in fish aestivating in normoxia on day 12. Hence, GDH would act less favorably in the deamination direction during the maintenance phase of aestivation to reduce the production of ammonia through transdeamination. Simultaneously, the hepatic GDH amination activity, but not the deamination activity, from fish aestivating in normoxia on day 12 became highly dependent on the presence of ADP. These results indicate that transdeamination of amino acids through the hepatic GDH became responsive mainly to the cellular energy status of the fish during the maintenance phase of aestivation (day 12) in normoxia. Since ammonia concentrations in various tissues of *P. annectens* exposed to normoxia (or hypoxia) remained relatively unchanged, Loong et al. (2008a) concluded that changes in the activity of hepatic GDH occurred primarily to reduce ammonia production, and not to detoxify ammonia during aestivation. In comparison, for fish exposed to hypoxia, significant increases in the hepatic GDH amination activity, the amination/deamination ratio and the dependency of the amination activity on ADP activation occurred much earlier on day 6; i.e., at the onset of aestivation, instead of day 12 (Loong et al. 2008a). These results indicate that decreased ammonia production through changes in the activity of hepatic GDH in *P. annectens* could be more effectively induced and exacerbated by a combination of aestivation and hypoxia than aestivation alone (in normoxia). In addition, they suggest that GDH was critically regulated in fish during the transition between the induction and the maintenance phases of aestivation in hypoxia, suppressing ammonia production in order to reduce the dependency on increased urea synthesis to detoxify ammonia.

4.5.2.4 Changes in the Rate of Urea Synthesis and Activities of Ornithine–Urea Cycle Enzymes

In spite of suppressing ammonia production during the maintenance phase of aestivation, endogenous ammonia must be detoxified because its excretion would have been completely impeded during long periods of desiccation. In many cases, ammonia is detoxified to urea through the hepatic ornithine–urea cycle. By synthesizing and accumulating the moderately less toxic urea, animals can carry out protein catabolism for a longer period without being intoxicated by ammonia. Chew et al. (2004) reported that urea synthesis rates of *P. dolloi* increased 2.4-fold and

3.8-fold during the first 6 and the subsequent 34 days of aestivation, respectively, compared with the day 0 control value; and urea accumulated in various tissues of fish aestivated for 6 or 40 days. Although activities of ornithine–urea cycle enzymes in fish aestivated for 6 days remained unchanged, the activities of several ornithine–urea cycle enzymes increased significantly in fish aestivated for 40 days.

Previous works by Janssens and Cohen (1968a) also showed that urea accumulation occurred in *P. aethiopicus* aestivated for 78–129 days in an artificial mud cocoon. However, by injecting ^{14}C-bicarbonate into fish aestivated for 78–129 days and quantifying radiolabeled urea during a subsequent 60-h period, Janssens and Cohen (1968a) concluded that urea accumulation in *P. aethiopicus* did not involve an increase in the rate of urea synthesis, even though the fish appeared to be in continuous gluconeogenesis throughout aestivation. Subsequently, Ip et al. (2005c) undertook a study to test the hypothesis that the urea synthesis rate in *P. aethiopicus* was upregulated to detoxify ammonia during the initial period of aestivation (day 0 to day 12), and that a profound suppression of ammonia production occurred at a later period of aestivation (day 34 to day 46) which eliminated the need to sustain the increased rate of urea synthesis. Contrary to the report of Janssens and Cohen (1968a), Ip et al. (2005c) demonstrated a drastic increase in urea synthesis (3-fold) in *P. aethiopicus* during the initial 12 days of aestivation, although the magnitude of the increase in urea synthesis decreased over the next 34 days. Between day 34 and day 46 (12 days), the rate of urea synthesis decreased to 42% of the day 0 control value, instead. There were significant increases in tissue urea contents and activities of some ornithine–urea cycle enzymes from the liver (Ip et al. 2005c). Since there was a meager 20% decrease in the rate of ammonia production in *P. aethiopicus* during the initial 12 days, as compared to a 96% decrease during the final 12 days of aestivation (day 34 to day 46), Ip et al. (2005c) concluded that *P. aethiopicus* depended mainly on increased urea synthesis to ameliorate ammonia toxicity during the initial period of aestivation, but during prolonged aestivation, it suppressed ammonia production profoundly, eliminating the need to increase urea synthesis which is energy intensive.

Recently, Loong et al. (2008a) reported that *P. annectens* exhibited different adaptive responses during aestivation in normoxia and in hypoxia. Ammonia toxicity was avoided by increased urea synthesis and/or decreased endogenous ammonia production, but the dependency on these two mechanisms differed between the normoxic and the hypoxic fish. The rate of urea synthesis increased 2.4-fold, with only a 12% decrease in the rate of ammonia production in the normoxic fish. By contrast, the rate of ammonia production in the hypoxic fish decreased by 58%, with no increase in the rate of urea synthesis. Using in vivo ^{31}P NMR spectroscopy, it was demonstrated that hypoxia led to significantly lower ATP concentration on day 12 and significantly lower creatine phosphate concentration on days 1, 6, 9, and 12 in the anterior region of the fish as compared with normoxia. Additionally, the hypoxic fish had lower creatine phosphate concentration in the middle region than the normoxic fish on day 9. Hence, lowering the dependency on increased urea synthesis to detoxify ammonia by reducing ammonia production would conserve cellular energy during aestivation in hypoxia.

4.5.2.5 Levels of Accumulated Urea and Mortality

Rees and Hand (1993) studied biochemical changes occurring over 7 months of aestivation in two species of land snails, *O. strigosa* and *O. subrudis*, to determine whether differential mortality during aestivation was related to different energetic strategies. Laboratory-maintained snails, which were fed ad libitum prior to aestivation, were compared with snails collected from the field and induced to aestivate without augmenting their energy reserves. If the duration of aestivation was limited by the depletion of energy storage compounds during aestivation, then snails with larger stores prior to aestivation would be predicted to survive aestivation proportionately longer. Indeed, snails with elevated level of polysaccharide had lower mortality during aestivation as compared to snails collected from the field, and the negative correlation between preaestivation polysaccharide stores and mortality was statistically significant. However, the observation that polysaccharide stores were exhausted several months prior to the onset of mortality suggests that mortality was not due to the depletion of this substrate. On the other hand, the tissue urea concentrations (150–300 mM) were positively correlated with mortality in these snails. Since methylamine compounds that can offset disruptive effects of elevated urea, were present in low concentrations, Rees and Hand (1993) suggested that in the absence of elevated levels of counteracting compounds, urea might reach toxic levels and might be one factor limiting the duration of aestivation without mortality. If urea does reach toxic levels, then it raises the question: why do aestivating snails synthesize and accumulate urea?

4.5.2.6 Accumulation of Urea – Why?

Some snails and turtles that accumulate urea during aestivation appear to have the capacity to synthesize purines and uric acid, which are considered completely innocuous as nitrogenous wastes. Therefore, it is paradoxical that ammonia detoxification occurs mainly through increased urea synthesis, rather than increased purine synthesis, in these animals during aestivation. Perhaps, urea synthesis is a compromise between the toxicity of the terminal end-product during the maintenance phase of aestivation and the loss of organic carbon and energy equivalents through excretion upon arousal from aestivation, both of which are greater in purine synthesis.

Many aestivators accumulate urea in their body fluids, and urea concentrations can often reach several hundred millimolar (Jones 1980; Grundy and Storey 1998). Urea is well known for its ability to denature proteins and exerts disruptive effects on enzymes, (Hochachka and Somero 1984), and there is little evidence of the presence of methylamines or other potential counteracting solutes in aestivating animals that accumulate urea (McClanahan 1967; Withers and Guppy 1996). Fuery et al. (1997) proposed that an adaptation for tolerance of high concentrations of urea existed in the lactate dehydrogenase of the aestivating Australian desert frog as compared with those of nonaestivating species. On the other hand, it has been suggested that accumulated urea contributes to metabolic depression in dormant animals (Griffith 1991) by reversibly inhibiting key metabolic enzymes (Hand and

Somero 1982; Yancey et al. 1982). Indeed, recent studies (Costanzo and Lee 2005; Muir et al. 2007) on hibernating wood frogs (*Rana sylvatica*) suggest a link between urea accumulation and metabolic depression. Muir et al. (2008) measured aerobic metabolism of isolated organs from the wood frog in the presence or absence of elevated urea at various temperatures using frogs acclimatized to different seasons. When organs from winter frogs were tested at 10°C, metabolism was significantly decreased in urea-treated liver and stomach by ~15% and in urea-treated skeletal muscle by ~50%. Therefore, Muir et al. (2008) concluded that the presence of urea depressed the metabolism of living organs, and thereby reduced energy expenditure.

Grundy and Storey (1994, 1998) analyzed the effects of urea on selected enzymes involved in intermediary metabolism and antioxidant defense in spadefoot toad organs and compared these with the effects of KCl. Urea (200 mM) had no effect on pyruvate kinase, phosphofructose kinase 1, and isocitrate dehydrogenase but lowered GDH activity to 65% of control values. By contrast, 200 mM KCl inhibited all four enzymes with a particularly strong effect on GDH activity, indicating that spadefoot toad enzymes are much more sensitive to high KCl concentrations than to high urea concentrations. Therefore, it would appear that urea accumulation minimizes the elevation of cellular ionic strength that would otherwise occur and affect enzyme activities (Grundy and Storey 1998; Cowen and Storey 2002).

Riddle (1983) proposed that urea accumulated during the maintenance phase of aestivation could facilitate water uptake from the environment upon rehydration during arousal. In addition, there is also evidence which suggests that urea accumulation is essential to hydration status of tissues during aestivation. Arad (2001) studied the physiological responses to combinations of desiccation and rehydration in a Mediterranean land snail, *Theba pisana*. Their results indicated that urea was transported from the pallial fluid to the soft body tissue, facilitating water movement to and maintaining the hydration status of the latter. On the other hand, the burrowing frog, *Cyclorana australis*, digs shallow burrows while the soils are still quite moist at the beginning of the dry season. It spends 2–3 months underground without cocoons, and cocoon formation occurs only when the soils dry to an extent that would dehydrate the frogs. During the first part of aestivation before the formation of cocoon, it absorbs water from the environment (Booth 2006; Tracy et al. 2007), probably as a result of accumulation of urea in tissues and body fluids. Increased urea levels in tissues also facilitate the mobilization of water stored in the urinary bladder during the later phase of aestivation (Cartledge et al. 2008).

It has been previously suggested that aestivation in air entails desiccation, and increased tissue urea contents might serve the secondary function of facilitating water retention in tissues through vapour pressure depression (Horne 1971; Campbell 1973; Land and Bernier 1995; Withers and Guppy 1996; Withers 1998; Storey 2002). However, this proposition may be invalid for several reasons. First, it has been established that urea concentration of 300 mM has only minor contribution to the gradient for water movement between tissues and dry air (Machin 1975). Secondly, contrary to the suggestions that urea may have an osmotic role in aestivating snails (Campbell 1973; Horne 1971; Bishop et al. 1983); Horne (1973a) demonstrated that *B. dealbatus* which underwent aestivation in a 85%

relative humidity remained active for a longer period before aestivation and accumulated urea at a faster rate than those aestivated in 14% relative humidity. Thus, Horne (1973a, b) concluded that increases in urea synthesis and accumulation in aestivating *B. dealbatus* were unrelated to water conservation, but occurred as a result of ammonia detoxification in conjunction with an increase in protein degradation during fasting. Furthermore, Hiong et al. (2005) reported that the giant African snail, *A. fulica*, accumulated urea progressively not only during 23 days of aestivation, but also during 23 days of fasting (Hiong et al. 2005). Since *A. fulica* was fasted in an ample supply of water and since fasting had no significant effects on the tissue water contents, urea accumulation did not occur because of the need to retain water through a decrease in the partial pressure of water vapor. Thirdly, Loong et al. (2008a) experimented on two groups of *P. annectens* that underwent aestivation in closed boxes with similar flow rates of air or 2% O_2 in N_2, and hence experienced similar magnitudes of desiccation. However, fish aestivating in hypoxia suppressed ammonia production and consequently accumulated much less urea. Therefore, increased urea synthesis in *P. annectens* is an adaptation responding primarily to rates of protein degradation/synthesis and amino acid catabolism (Loong et al. 2008a).

4.5.3 Nitrogen Metabolism and Excretion during Arousal from Aestivation

4.5.3.1 Rehydration

The environmental cues that signal animals to arouse from aestivation have not been explicitly defined but water availability can naturally be regarded as one of the most important factors involved. In our laboratories, we routinely arouse African lungfish from aestivation in air by reimmersion. Also, it has been suggested that the burrowing frog, *C. australis*, emerges from soils when the osmoticity of soil water becomes low enough to permit water absorption (Tracy et al. 2007).

Although Wilkie et al. (2007) studied *P. dolloi* during prolonged (5 months) exposure to air, their results actually offered insights into what would happen during the arousal phase when water becomes once again available to aestivating African lungfishes (see Sect. 5.1.2). Their results (Wilkie et al. 2007) indirectly support the proposition of Riddle (1983; see Sect. 5.2.6), because the 13-fold increase in muscle urea content was the likely explanation for the 56% increase in muscle water content observed after 5 months of air exposure. However, the phenomena reported by Wilkie et al. (2007) that muscle acted as a "water reservoir" during air exposure and that the body mass decreased by 20% during subsequent reimmersion in water could not be regarded as the real situation of arousal from aestivation. Firstly, the level of urea accumulated in the body during the normally short period of induction phase of aestivation would not lead to such a large increase in muscle water content (see Sect. 5.1.2). Secondly, after a long period of

aestivation, there should be a decrease, and not an increase, in the muscle water content. Thirdly, during arousal, it is essential for the fish to gain water from, instead of loosing it to, the environment. Although it is unlikely that fish can accumulate such high levels of urea during a 6–8 day induction period, similar magnitude of increase in urea content can be expected to have occurred in fish that underwent an equivalent period (i.e., 5 months) of aestivation. Therefore, it can be deduced from results reported by Wilkie et al. (2007) that water absorption occur through the ventral body surface of African lungfish as water becomes available during arousal. Upon arousal, water absorption must precede urea excretion because urea is crucial to this osmotic phenomenon (see Sect. 5.2.6).

4.5.3.2 Excretion of Accumulated Urea

Urea accumulated in the body of aestivating lungfishes can be excreted effectively during arousal in water (Smith 1930; Janssens 1964). Chew et al. (2003b), working on *P. dolloi* exposed to terrestrial conditions for 6 days, demonstrated that the urea excretion rate increased 22-fold during reimmersion as compared to the control specimen. This is the greatest increase in urea excretion amongst fishes during emersion–immersion transition, and suggests that *P. dolloi* possesses transporters which facilitate the excretion of urea in water. Subsequently, Wood et al. (2005b) reported that after 21–30 days of aestivation in air or exposed to air without aestivation, the urea excretion rate was greatly elevated in *P. dolloi* during reimmersion, reaching 2,000–6,000 μmol N h^{-1} kg^{-1} at 10–24 h after return to water. A divided chamber experiment demonstrated that 72% of the urea-N efflux occurred through the posterior 85% of the body, with minimal involvement of the kidney, thereby pointing to the skin as an important site of urea-N excretion. Wood et al. (2005b) discovered that urea was excreted by *P. dolloi* in pulses during reimmersion but the reason behind such a phenomenon was unclear. Perhaps, it is an adaptation to assure complete rehydration, which is dependent on tissue urea content, and to minimize instantaneous osmotic shock to cells, upon arousal. Through the injection of NH_4Cl + urea, Ip et al. (2005c) concluded that excretion of accumulated urea in *P. dolloi* was regulated by the level of internal ammonia. There could be an increase in ammonia production through increased amino acid catabolism upon arousal, and the increased production of endogenous ammonia could act as a signal to enhance urea excretion.

Recently, the full-length cDNA sequence of a putative urea transporter (lfUT) of the UT-A type has been cloned from *P. annectens* (Hung et al. 2009). The lfUT cDNA is 1990 bp in length and its open reading frame encodes a 409 amino acid long protein, with a calculated molecular weight of 44,723 Da. The sequence is closest to those of amphibians (~65% amino acid homology), followed by mammals and elasmobranchs (~60%), and then teleosts (~50%). lfUT was clearly expressed in gill, kidney, liver, skeletal muscle, and skin. Upon reimmersion in water after 33 days of air exposure, *P. annectens* exhibited a massive rise in urea-N excretion which peaked at 12–30 h with rates of 2,000–5,000 μmol N kg^{-1} h^{-1}

and persisted until 70 h. Quantitative RT-PCR revealed significant elevation of lfUT expression in the skin between 14 and 48 h of reimmersion. Thus, it can be deduced that transcriptional activation of lfUT would occur in the skin of African lungfishes to facilitate urea excretion during the arousal phase of aestivation.

4.5.3.3 Feeding, Tissue Regeneration and Protein Synthesis

The increased excretion of urea during rehydration and changes in osmolality of tissue fluids may be involved indirectly in tissue regeneration upon arousal from aestivation. Working on *P. dolloi* during the maintenance and arousal phases of aestivation, Icardo et al. (2008) reported that the heart had high capacity for functional recovery. They proposed that the drastic reduction in the amounts of urea accumulated in the body tissues that occurs upon arousal (Wood et al. 2005a) may produce an osmotic imbalance that eventually results in rupture of the membranes and the massive accumulation of the vacuolized cytoplasm components in the septal myocytes of the heart. Subsequently, these areas attract the macrophages involved in debris clearance, and such a process may facilitate tissue regeneration.

During aestivation, the intestine of the green-stripped burrowing frog, *C. alboguttata*, undergoes significant morphological downregulation, but there is rapid restoration of intestinal morphology upon arousal from aestivation and during the initial stages of refeeding (Cramp and Franklin 2005; Cramp et al. 2005). Arousal alone has a marked impact on many morphological parameters, including small and large intestine masses, small intestinal length, enterocyte cross-sectional area, and microvilli height and density (Cramp and Franklin 2005). Such structural changes would require increased syntheses of certain proteins, and since they occur before refeeding, it would imply the mobilization of amino acids of endogenous origin. Upon feeding, *C. alboguttata* employs reduced digesta passage rates so as to maximize nutrient assimilation efficiency following prolonged food deprivation during aestivation (Cramp and Franklin 2003). However, at present, there is a dearth of knowledge on protein degradation and synthesis during the arousal of aestivating animals. Furthermore, no information is available on postprandial nitrogen metabolism and excretion in animals upon arousal from aestivation.

4.6 Conclusion

In summary, adaptive responses exhibited by aestivators with regard to excretory nitrogen metabolism during the induction, maintenance, and arousal phases of aestivation differ from those exhibited by nonaestivators undergoing fasting or immobilization, although aestivation involves long-term fasting and corporal torpor.

At present, only limited information is available on excretory nitrogen metabolism in animals during the induction or arousal phase of aestivation. Therefore, future efforts should be made to identify adaptive responses particular to each of the three phases of aestivation. Since structural changes could occur during the induction and arousal phases, it would be essential to study the intricate relationship between protein synthesis and protein degradation and the resulting rapid turnover of nitrogen in certain organs of animals going through these two phases of aestivation. In addition, efforts should be made in the future to study mechanisms involved in the suppression of protein degradation to preserve proteinaceous structures and the regulation of GDH to reduce ammonia production. As for nitrogenous products, it would be important to further elucidate the functional roles of urea, and also other nitrogen containing compounds, during the induction and maintenance phases, and to examine how urea excretion is regulated to facilitate complete rehydration upon arousal.

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Chapter 5
Aestivation in Mammals and Birds

Fritz Geiser

Contents

Abstract Aestivation, which in the context of this paper refers to avian and mammalian torpor in summer/at high ambient temperatures (T_a), does not appear to differ functionally from other forms of torpor, and to a large extent reflects the higher body temperatures (T_b) caused by high T_a. However, from an ecological point of view, aestivation results in different challenges and requirements than does torpor use in winter, because heat can cause reduced food and water availability in many regions, but without the access to low T_a for a substantial reduction of T_b. Aestivation is used by a diversity of adult mammals and birds both in the field and laboratory, as well as by growing young to reduce thermoregulatory energy expenditure. Torpor occurs at high T_a including the thermo-neutral zone and even under these conditions the reduction in energy expenditure and water requirements or water loss is substantial. Although data from the laboratory and, especially, from the field are limited, they show that torpor at high T_a is an effective survival strategy and suggest that it is employed by many mammals and birds in a diversity of habitats.

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5.1 Introduction

Endothermic mammals and birds have the ability to maintain a constant high body temperature (T_b) over a wide range of ambient temperatures (T_a) primarily by producing heat internally by combustion of fuels. Because the surface area/volume ratio of animals increases with decreasing size, the amount of heat loss, and therefore the amount of endogenous heat required per gram tissue for maintenance of a stable T_b, increases with decreasing size. While heat loss is most pronounced at low T_a, energy expenditure in small endotherms even at mild T_a is still much higher than in ectotherms (Hulbert and Else 1989; Withers 1992). These high metabolic rates (MR) in small endotherms demand intake of large amounts of food, and, when food supply is low or fluctuating, energy requirements may exceed energy availability.

To cope with these challenges, not all mammals and birds are permanently homeothermic (i.e., maintain a constant high T_b), but during certain times of the day or the year enter a state of torpor (Lyman et al. 1982; Geiser and Ruf 1995; Boyer and Barnes 1999; Carey et al. 2003; Geiser 2004; Withers and Cooper 2009). Torpor in these "heterothermic endotherms" is characterized by a controlled reduction of T_b, MR, and other physiological functions, which importantly also include water loss. During torpor, T_b falls from high normothermic values of ~32–42°C to values between −3 and ~30°C, but during steady-state torpor, T_b always remains above the T_a, even when the animals are thermo-conforming (Barnes 1989; Geiser and Ruf 1995). The minimum MR during torpor (torpor metabolic rate (TMR)) is, on average, reduced to 5 to 30% of the basal metabolic rate (BMR) and in some species TMR can be less than 1% of the resting MR (RMR) in normothermic animals at low T_a, emphasizing the effectiveness of torpor in reducing MR (Geiser and Ruf 1995; Boyer and Barnes 1999). Although torpid mammals and birds usually thermoconform over a wide range of temperatures, thermoregulation is not abandoned (Florant and Heller 1977; Withers and Cooper 2009). The T_b during torpor is regulated by a proportional increase in heat production at a minimum that differs widely among species, apparently to avoid organ or tissue damage caused by low temperatures, or to prevent cooling to T_bs from which they cannot arouse.

Cold exposure in high latitudes/altitudes is widely seen as the main challenge to small mammals and birds because increased heat loss often is associated with low food availability. However, low food and water availability are not restricted to high latitudes or altitudes, but also are pronounced in desert regions to some extent due to extreme heat during summer, and even may be experienced in tropical and subtropical regions during the dry winter season. Recent evidence suggests that low food and water availability, not cold exposure, are the primary reasons for employment of torpor in mammals and birds. Torpor can be induced by withdrawal of food and water at relatively high T_a and even within the thermo-neutral zone (TNZ) at maintenance BMR, without thermoregulatory heat production, although cold exposure obviously will enhance torpor use because of increased energetic demands for thermoregulation (Geiser 2004).

The purpose of this review is to provide a brief summary of torpor in mammals and birds in general, and then to synthesize what is known about "aestivation" specifically. Aestivation (from Latin aestas, summer) is often defined as "torpor during heat and drought during summer in some animals" (Lawrence 2008) i.e., with a seasonal connotation, or a "form of torpor usually in response to high temperatures or scarcity of water" (Pough et al. 2009), i.e., simply with reference to thermal and hygric conditions. Therefore, in this review, aestivation in endotherms will be viewed as shallow torpor at relative mild T_a at all times of the year; nevertheless, torpor in summer specifically will also be discussed. Suitable examples to illustrate both have been selected from the literature, but this paper does not aim to be exhaustive.

5.2 Prolonged, Multiday Torpor or Hibernation

Most heterothermic mammals and birds appear to use one of two common patterns of torpor: (a) hibernation or prolonged, multiday torpor is employed by the "hibernators," and (b) daily torpor is employed by the "daily heterotherms." Hibernation, often, is seasonal and usually lasts from late summer/autumn to late winter/spring. However, hibernators do not remain torpid throughout the hibernation season. Bouts of torpor, during which T_b are low and bodily functions are reduced to a minimum, last for several days or weeks, but are interrupted by periodic rewarming and brief (usually less than 1 day) normothermic resting periods with high T_b and high energy turnover (French 1985; Geiser and Kenagy 1988; Körtner and Geiser 2000; Carey et al. 2003; Geiser 2004). Torpor bouts of hibernators at low T_b usually last for several days to several weeks, but importantly bout duration is strongly temperature-dependent. Torpor bout duration decreases with increasing T_a over the T_a range that torpid hibernators are thermo-conforming and, of special interest to the present review, a high T_a around 20 to 30°C torpor bouts even in hibernators often last for less than a day (French 1985; Geiser and Kenagy 1988; Song et al. 1997; Geiser and Brigham 2000). Hibernation is currently known to occur in many mammalian orders (Lyman et al. 1982; Geiser 1998), but only in a single bird species, the poorwill, *Phalaenoptilus nuttallii* (Withers 1977; Brigham 1992; Geiser and Ruf 1995; Geiser 1998; McKechnie and Lovegrove 2002; Woods and Brigham 2004). Hibernators that reduce their T_b by >10°C during torpor are generally small (median mass 85 g), most weigh between 10 and 1,000 g, and all weigh <10 kg (Geiser and Ruf 1995). Many hibernators fatten extensively before the hibernation season, refuse to hibernate when lean, and rely to a large extent on stored fat (or in some species stored food) for an energy source in winter.

Hibernating species usually reduce their T_b to below 10°C, with a minimum of −3°C in arctic ground squirrels, *Spermophilus parryii* (Barnes 1989), and most, with the exception of large carnivores (which express shallow torpor with <10°C T_b reduction) and perhaps tropical hibernators, have T_b minima of around 5°C (Geiser and Ruf 1995; Fietz et al. 2003; Grigg et al. 2004; Dausmann et al. 2005). The

TMR in hibernators is on average reduced to about 5% of the BMR, but can be as little as 1–2% of the BMR in small hibernating species that employ extensive metabolic inhibition to reduce MR in addition to temperature effects due to the low T_b (Storey and Storey 1990; Geiser and Ruf 1995; Guppy and Withers 1999; Geiser 2004; Willis et al. 2005). Even when the high cost of periodic arousals is considered, energy expenditure during the mammalian hibernation season is still reduced to 4–15% of that of an animal that would have remained normothermic throughout winter (Wang 1978; Geiser 2007). This enormous reduction in energy expenditure is, perhaps, best illustrated by the fact that many hibernating mammals can survive for 6–8 months or more entirely on body fat that has been stored prior to the hibernation season (French 1985; Geiser 2007; Bieber and Ruf 2009).

5.3 Daily Torpor

Daily torpor in the daily heterotherms is the other widely used pattern of torpor in mammals and, in contrast to hibernation, also is commonly found in birds (Geiser and Ruf 1995; McKechnie and Lovegrove 2002). Daily heterotherms, in contrast to the hibernators, are incapable of multiday torpor bouts. Daily torpor lasts only for hours rather than days and is usually, but not always (Körtner and Geiser 2009), interrupted by daily foraging and feeding. Nevertheless, daily torpor may occur continuously over several days and up to months (Körtner and Geiser 2009). On average, daily heterotherms are smaller (median mass 19 g) than hibernators, most weigh between 5 and 100 g and their overall mass range is ~2–9,000 g (Geiser and Ruf 1995). In diurnal species, such as many birds, daily torpor is usually restricted to the night, whereas in nocturnal mammals and birds it is common in the second part of the night and/or the early morning. Generally, daily torpor is less seasonal than hibernation and can occur throughout the year, although its use often increases in winter. Although daily torpor may be expressed as a response to acute energy shortage, in some species, it is employed regularly to balance energy budgets, even when food availability appears favorable. For example, several small arid zone marsupials regularly enter daily torpor in the laboratory when food is freely available (spontaneous torpor), which appears to reflect the generally low energy availability in their natural desert environment where they may enter torpor on every night in winter (Geiser and Baudinette 1987; Geiser 2003; Warnecke et al. 2008; Körtner and Geiser 2009). Moreover, both in the laboratory and field, some of these desert marsupials, as for example the mulgara (*Dasycercus cristicauda*), appear to use daily torpor during pregnancy to facilitate fat storage for the following energetically demanding period of lactation (Geiser and Masters 1994; Körtner et al. 2008).

Many daily heterotherms, unlike most hibernators, do not show extensive fattening before the season when torpor is most commonly employed, and mainly enter torpor at times when body mass is low (Heldmaier and Steinlechner 1981; Geiser 1988; Holloway and Geiser 1996). Administration of the hormone leptin, which simulates

the presence of high fat stores, inhibits daily torpor, further supporting the interpretation that daily torpor is employed predominantly when animals are lean (Geiser et al. 1998). When food is withheld from small daily heterotherms for several days in captivity they will perish (Kennedy and MacFarlane 1971), whereas small hibernators can survive for months and up to an entire year (Geiser 2007; Bieber and Ruf 2009). The main energy supply of daily heterotherms, even in the main torpor season, remains food rather than stored body fat, and they appear to attempt to balance energy expenditure and uptake on a daily or at least short-term basis (Körtner and Geiser 2000). In most of the daily heterotherms, such as small insectivorous/carnivorous marsupials (e.g., *Sminthopsis* spp.) and mice (e.g., *Peromyscus* spp.), T_b usually falls to between 10 and 20°C (mean 17°C), whereas in some hummingbirds values below 10°C have been reported (Carpenter 1974; Geiser and Ruf 1995; McKechnie and Lovegrove 2002). In others, mainly large species, such as tawny frogmouths (*Podargus strigoides*) or American badgers (*Taxidea taxus*), T_b is maintained just below 30°C (Harlow 1981; Geiser and Ruf 1995; Körtner et al. 2000). The TMRs of daily heterotherms are, on average, reduced to about 30% of BMR although this percentage is strongly affected by body mass and other factors. When RMR at low T_a is used as a point of reference, reductions of MR during daily torpor to about 10 to 20% of that in normothermic individuals at the same T_a are common. Overall, daily energy expenditure is usually reduced to 10–80% on days when daily torpor is employed in comparison to days when no torpor is used, primarily depending on the duration and intensity of activity, the duration of the torpor bout and torpor depth, and whether or not they employ basking during rewarming from torpor and during normothermia (Ruf et al. 1991; Holloway and Geiser 1995; Geiser et al. 2002; Warnecke et al. 2008; Körtner and Geiser 2009).

5.4 Aestivation

While hibernation and daily torpor differ functionally with regard to fat storage, metabolic rate reduction, and, perhaps most importantly, the ability to express or not express multiday torpor bouts, aestivation or torpor at high T_a or in summer does not appear to differ functionally from other forms of torpor and to a large extent reflects the higher T_b caused by high T_a (Song et al. 1997; Geiser and Brigham 2000; Wilz and Heldmaier 2000). Moreover, hibernation and aestivation are often difficult to distinguish temporally because some hibernators begin to aestivate in late summer, often around the hottest part of the year, but then continue to hibernate throughout the autumn and the winter and some species even into the spring. Clearly, it is of no significance to the hibernator whether they "aestivate" before 22 September (first day of northern hemisphere autumn) or "hibernate" thereafter; rather, what is of significance to the hibernator is that soil temperatures in late summer/autumn will be substantially higher than in winter (e.g., Nicol and Andersen 2007).

Thus, from an ecological point of view, aestivation in summer has different challenges and requirements than torpor in winter. Summer, often, is hot, which can

reduce food and water availability in many regions, but without access to low T_a for a substantial reduction of T_b. However, in comparison to studies on torpor at low T_a, quantitative information on torpor at high T_a or during summer is limited and in the past was to a large extent, based on laboratory investigations as reviewed by Hudson and Bartholomew (1964). New data from the laboratory and also from the field are now available and these suggest that use of torpor during mild thermal conditions is far more common than is widely believed at present (Song et al. 1995, 1997; Turbill et al. 2003a, 2003b; Stawski et al. 2008).

5.4.1 Torpor in Summer

Animals often enter torpor in secluded places, including burrows and caves where T_a is buffered from thermal extremes and therefore well below that of the maximum daily T_a of the surrounding air. Thus, torpor does not appear to be induced by heat stress *per se*, but rather a reduction of food and water caused by heat, and the animals often make use of cool underground conditions to lower T_b. If animals do not shelter in thermally stable and relatively cool refugia in summer, as for example, bats roosting under leaves or in tree hollows, then they enter torpor during the coolest, not hottest, part of the day (Turbill et al. 2003a; Stawski et al. 2008).

Torpor in summer occurs in diverse mammals including monotremes (echidna), marsupials (small dasyurids and possums), placentals (bats, rodents), and birds (nightjars and hummingbirds), as is described below.

Monotremes: Free-ranging echidnas (*Tachyglossus aculeatus*) in Tasmania commence hibernation during the warmest part of the year (in late summer) with initial minimum T_b of around 20°C and short torpor bouts and continue to hibernate for up to 7 months (Nicol et al. 2008). Even in warm areas such as southwestern Queensland, echidnas occasionally enter torpor for 1 day in summer (Brice et al. 2002).

Marsupials: Use of summer torpor in marsupial mammals is well documented. Captive arid zone insectivorous/carnivorous marsupials (*Sminthopsis* spp. and *Dasyuroides byrnei*) display torpor throughout the year, although use of spontaneous torpor (food ad libitum) is reduced in summer and torpor is generally deeper in winter than in summer (Geiser and Baudinette 1987; Geiser 2003). Pygmy-possums (*Cercartetus* spp.) also display torpor, including prolonged torpor, in summer (Geiser and Körtner 2004); however, a detailed seasonal investigation has not been conducted in this group to see whether there are functional changes during the year. In the wild, torpor in the eastern pygmy-possum *Cercartetus nanus* occurs in all seasons including in early summer, but not in late summer to midwinter, when banksia trees (*Banksia integrifolia*), a major source of nectar, are flowering and animals reproduce (Bladon et al. 2002). Torpor in spring and autumn also occurs in honey possums (*Tarsipes rostratus*) captured in pit traps (Collins et al. 1987).

Placentals: Summer torpor in placental mammals is best known for bats. It was already recognized over 170 years ago that small insectivorous bats (Microchiroptera or microbats) display prolonged torpor in winter and brief bouts of torpor in summer

(Hall 1832). Nevertheless, detailed information on torpor in free-ranging bats in summer has only recently become available with the development of small temperature-sensitive radio transmitters. Long-eared bats (*Nyctophilus* spp.), which hibernate and display bouts of torpor lasting up to 2 weeks in winter in a cool-temperate area of Australia (Turbill and Geiser 2008), enter torpor essentially every day in summer, even when daily maximum T_a reaches 30°C (Turbill et al. 2003b). On cool summer days, they may extend torpor bouts for up to 2 days (Turbill et al. 2003b). Similarly, a Canadian bat (*Myotis evotis*) uses torpor every day between May and August and even when reproductively active (Chruszcz and Barclay 2002). Female *Myotis daubentoni* in central Germany enter torpor mainly during postlactation in late summer, whereas males use torpor frequently even during their reproductive period in early summer (Dietz and Kalko 2006). Pregnant female hoary bats (*Lasiurus cinereus*) enter prolonged torpor in southern Canada during inclement weather in late spring/early summer; this is likely not just to conserve energy, but also to delay parturition until conditions are more favorable for neonatal survival (Willis et al. 2006). Even in subtropical areas of Australia and South Africa, small insectivorous bats display short bouts of torpor in summer in the wild (Turbill et al. 2003a; Vivier and van der Merwe 2007; Stawski et al. 2008). Although torpor is widely used by insectivorous microbats in summer, their torpor bouts are generally shorter and are not as deep as in winter, apparently reflecting ambient thermal conditions, rather a seasonal change in physiology (Geiser and Brigham 2000).

Interestingly, seasonal changes in the use of torpor by the nectarivorous blossom-bat *Syconycteris australis* (Megachiroptera) from the subtropical east coast of New South Wales were the opposite of those observed for insectivorous microbats and many other heterothermic mammals. For *S. australis* captured in winter, average torpor bout duration was short (5.5 h) and shallow, whereas in summer torpor was deep and bouts lasted for 7.3 h on average (Coburn and Geiser 1998). While these findings may seem counterproductive at first glance, different day length and food availability in summer and winter appear to explain the unusual seasonal response. In winter, T_a on the New South Wales north coast is relatively mild and bats can forage for prolonged periods during long nights and have access to an abundance of flowering plants (Coburn and Geiser 1998). In summer, nights, and thus foraging times, are brief and the availability of nectar is substantially reduced (Coburn and Geiser 1998). Thus, the unusual seasonal pattern of torpor use by *S. australis* appears to be an appropriate physiological adaptation to ecological constraints of their subtropical habitat.

Rodents are another group of placental mammals known to aestivate. During a severe drought in Pennsylvania, free-ranging woodchucks (*Marmota monax*) entered short bouts of torpor in August with T_b fluctuating between ~25 and 38°C when T_a ranged from 20 to 30°C; after rainfall some individuals remained normothermic, whereas others continued to exhibit torpor (Zervanos and Salsbury 2003). For five ground squirrel species (*Spermophilus* spp.) from a wide range of North-American habitats, the period of heterothermy begins in late summer/early autumn (Barnes 1996) and often this coincides with the reduction of green vegetation.

For the small South-American cricetid rodent *Calomys musculinus*, torpor was employed in summer at T_a 25°C when MR fell by 75% in comparison to resting values at the same T_a (Bozinovic and Rosenmann 1988). European dormice (*Glis glis*) hibernate for 7–8 months from autumn to spring, but after their final arousal in spring, nonreproductive individuals may reenter a period of dormancy from early or midsummer (Bieber and Ruf 2009). Summer-acclimated African dormice (*Graphiurus murinus*) exhibit torpor at T_a 25°C (Webb and Skinner 1996), and other dormice (*Muscardinus avellanarius*, *Glirulus japonicus*, *Dryomys nitedula,* and *Eliomys quercinus*), as well as birchmice, *Sicista betulina,* also are known to display torpor in summer (French 2008).

Birds: Although it is widely believed that birds migrate to avoid adverse conditions and employ torpor less frequently than mammals, several birds are known to display torpor use even in summer. Free-ranging American poorwills (*P. nuttallii*) occasionally enter torpor in June (Woods and Brigham 2004). They also used torpor when incubating, but only rarely and during inclement weather (Kissner and Brigham 1993). Summer torpor also has been observed for a captive spotted nightjar (*Eurostopodus argus*) in Australia in response to a 13% loss of body mass (Dawson and Fisher 1969). Andean humming birds (*Oreotrochilus estella*) roosting in caves at about 4,000 m elevation will enter torpor in both winter and summer, although summer torpor is less frequent and shorter than winter torpor (Carpenter 1974). Migrant North American hummingbirds (*Selasphorus rufus*) use nocturnal torpor extensively in summer, not in response to energy shortage, but rather to reduce thermoregulatory energy expenditure and enhance fat storage for migration (Carpenter and Hixon 1988; Hiebert 1993). Captive passerine sunbirds (*Nectarina famosa*) from South Africa also enter nocturnal torpor in summer at low T_a (Downs and Brown 2002).

5.4.2 Torpor during Development and Growth

A substantial part of the development of birds and mammals occurs during summer when the young ones are small and often not well insulated, and require substantial amounts of energy for thermoregulation. Although high energy costs are likely to provide a strong selection pressure for heterothermy in these young ones during this time, only limited data are available on torpor during development in summer.

Torpor during development and growth has been observed for small (adult body mass 25–110 g) insectivorous/carnivorous marsupials (family Dasyuridae) (Geiser et al. 2006; Geiser 2008). For the four species examined (*Sminthopsis macroura*, *Antechinus* spp., and *D. byrnei*), torpor was deeper and longer at some stage of juvenile development, usually in summer, compared with adults in other parts of the year (Geiser 2008), suggesting that it may be an important measure for energy conservation during growth.

In placental mammals, torpor has been described during development in the insectivores, bats, and rodents. Juveniles of the insectivorous shrew (*Crocidura russula*) entered daily torpor at a body mass of ~5 g (~40% adult mass) from day 7

postpartum (Nagel 1977). Free-ranging early volant juvenile bats (*Eptesicus fuscus*), which hibernate for prolonged periods in winter, entered shallow bouts of torpor in summer (Hollis and Barclay 2008). Captive juveniles of the herbivorous hamster *Phodopus sungorus* displayed daily torpor in response to food restriction by 2 weeks after they became endothermic (Bae et al. 2003).

Avian species known to be heterothermic during development include the orders Charadriiformes (shorebirds), Coliiformes (mousebirds), Apodiformes (swifts) and Passeriformes (songbirds). Juvenile storm-petrels (*Oceanodroma furcata*), in their natural burrows in Alaska, employed torpor soon after becoming endothermic over a period of ~2 weeks during the growth phase when body mass increased from ~10 to the adult mass of 60 g (Boersma 1986). In mousebirds (*Urocolius macrourus*) from the Afrotropics, torpor was first observed at 55% of adult mass, 10 days after hatching, essentially at the same time as they were capable of endothermic thermoregulation (Finke et al. 1995). Juvenile swifts (*Apus apus*) from Finland (Koskimies 1948) displayed torpor after 6 days of fasting. They were able to survive fasting for up to 12 days, likely because of their large storage of fat and use of nocturnal torpor. Juvenile martins (*Delichon urbica*) from cool-temperate Europe were able to enter and arouse from torpor by 11 days posthatching, 1 day after endothermic thermoregulation was established (Prinzinger and Siedle 1988). Torpor could be induced by starvation at a body mass of about 12 g (~60% of adult mass).

Thus, although not widely investigated, it appears that torpor is an important adaptation during the development of mammals and birds in summer to minimize energy loss and, perhaps, to spare valuable nutrients for the growth of young ones.

5.4.3 Torpor in or Near the TNZ

Within the T_a range in which torpor is usually expressed, use of torpor is generally less frequent at high T_a than at low T_a. For example, dunnarts (*S. macroura*), under a range of food and water regimes, have a higher torpor frequency at T_a 18°C than at T_a 28°C, which is near their lower critical T_a of the TNZ (Song and Geiser 1997). Nevertheless, the species still used torpor at T_a 28°C, especially when food and water were withheld, but to a lesser extent than when they were available (Song and Geiser 1997). *S. macroura* even enter daily torpor at and slightly above T_a 30°C within the TNZ, with a reduction of T_b by about 5°C and MR by about 33% in comparison to the BMR (Song et al. 1995). Pygmy-possums *C. nanus* also enter torpor in the TNZ at T_a 29°C; in this species, a reduction of T_b by only 2.9°C results in a reduction of MR in comparison to BMR by ~50% (Song et al. 1997), emphasizing the effective metabolic savings possible even at high T_b in hibernating species.

Bats also use torpor at T_a slightly below or within the TNZ. For example, small tropical megabats, the blossom bats (*Macroglossus minimus*), enter daily torpor at T_a of 25°C only slightly below the TNZ (Bartels et al. 1998). It has been known for some time that small micobats, such as *Myotis lucifugus*, which are capable of hibernation, may successfully employ small reductions of T_b for energy conservation

at high T_a (Hock 1951; Studier 1981). Torpid long-eared bats (*Nyctophilus geoffroyi*), like pygmy-possums, can reduce MR by >2/3 at high T_a near the lower critical T_a of the TNZ, with only a small reduction of T_b by about 6°C (Geiser and Brigham 2000; Turbill et al. 2008). If these micobats relied on temperature (Q_{10}) effects alone, then MR would be reduced by only one-third and this would limit the potential for energy and water conservation.

Other placental mammals that employ torpor at high T_a include spiny mice (*Acomys russatus*) held at T_a 30°C, that by employing torpor can maintain a more or less stable body mass with only 50% of ad libitum food (Gutman et al. 2006). Gerbils (*Gerbillus pusillus*) use daily torpor at about T_a 30°C to substantially reduce energy expenditure in comparison to normothermic resting individuals (Buffenstein 1985). Pouched mice (*Saccostomus campestris*) enter torpor near their TNZ at T_a 25°C when food is reduced to 70% of ad libitum rations (Lovegrove and Raman 1998). Moreover, gray mouse lemurs (*Microcebus murinus*) express short bouts of shallow torpor at T_a 30°C when acclimated to long summer photoperiod and food is reduced by 80% (Giroud et al. 2009). The most extreme cases in this regard are free-ranging fat-tailed lemurs (*Cheirogaleus medius*) from tropical Madagascar and long-eared bats (*Nyctophilus bifax*) from subtropical Australia (Dausmann et al. 2005; Stawski et al. 2009). Both species use multiday torpor bouts when hibernating in rather exposed conditions with T_b fluctuating daily by up to 15–20°C, but without obvious endothermic arousals even when T_a and T_b rise to about 30°C. Similarly, the Madagascan tenrec (*Echinops telfairi*) occasionally used multiday torpor bouts at high T_a with pronounced daily fluctuations of T_b, but mainly displayed short bouts of torpor with daily arousals (Lovegrove and Génin 2008).

In birds, limited information is available on torpor at high T_a. However, captive cloven-feathered doves (*Drepanoptila holosericea*) enter shallow torpor at T_a 27°C even when food is available (Schleucher 2001).

5.4.4 Torpor Induction via Water Restriction

As we have seen in the previous section, torpor can be induced even under thermoneutral or near thermo-neutral conditions mainly by restriction of food. If aestivation, as often stated, is a response to water shortage, then we would assume that withdrawal of water has a similar effect as withdrawal of food. Surprisingly, information on this topic is limited and the results are equivocal.

Cactus mice (*Peromyscus eremicus*), when water deprived in midwinter, did not enter torpor, lost weight and died after 6–11 days, although they had free access to mixed bird seeds. In contrast, food restriction resulted in torpor use in other individuals (MacMillen 1965). In summer, similar results were obtained after water deprivation for eight out of ten individuals. However, the two individuals that regularly displayed torpor did survive, suggesting that torpor use due to water deprivation allows some individuals to survive water shortages (MacMillen 1965).

For gerbils (*G. pusillus*), 20% of individuals with access to dry food but without water entered torpor, although torpor use increased to 88% when both food and water were withheld (Buffenstein 1985). Similarly, shallow torpor was observed for Inca doves (*Scardafella inca*) with access to food but experiencing water deprivation, although simultaneous withdrawal of food and water resulted in deeper torpor (MacMillen and Trost 1967). For dunnarts (*S. macroura*), daily torpor was expressed at T_a 18 (63%) and 28°C (35%) when food and water were available; when food and water were withheld, torpor use increased substantially, whereas withdrawal of water in the presence of moist food had no effect (Song and Geiser 1997). However, when dry food was offered, withdrawal of water resulted in an increase of torpor use in comparison to animals with access to food and water, although this may have reflected reduced consumption of dry food by the animals without access to water rather than the lack of water *per se* (Song and Geiser 1997).

5.4.5 *Energy Conservation at High T_b*

High T_a prevents T_b from falling to low levels and therefore should limit the energy-conserving potential of torpor. Nevertheless, even small reductions in T_b result in substantial reductions in energy expenditure (Fig. 5.1). In comparison to BMR, a reduction of TMR associated with an approximately 10°C fall of T_b (i.e., at T_a 25°C) amounts to a 79% saving in an 85-g (average mass) hibernator and 58% in a 19-g (average mass) daily heterotherm (calculated from Geiser and Ruf 1995; Geiser 2004).

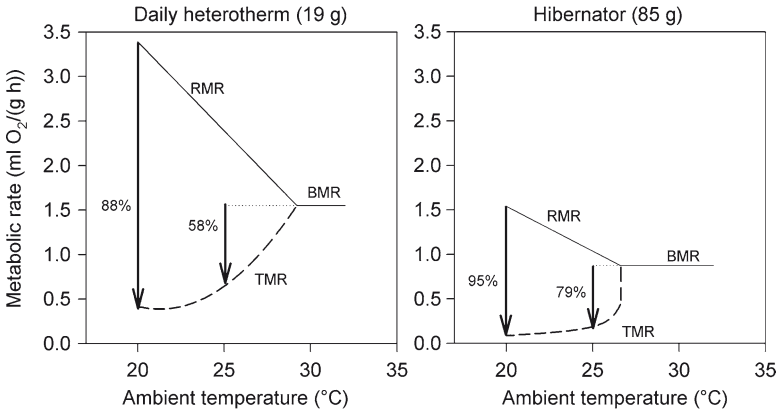

Fig. 5.1 Metabolic rates measured as the rate of oxygen consumption in an average daily heterotherm (body mass 19 g) and an average hibernator (85 g). Note the substantial reduction of metabolic rate from normothermic resting values at BMR (within TNZ) to torpid values (TMR) even at a high T_a of 25°C, which is associated by a fall of T_b by about 10°C. In comparison to RMR at T_a 20°C, which also entails a thermoregulatory component, and therefore is higher in the small daily heterotherm in comparison to the hibernator, the substantial fall of MR is associated by a fall of T_b by about 15°C

The greater reduction in the hibernator is due to the pronounced metabolic inhibition. In comparison to RMR of normothermic individuals at T_a 20°C, the reduction of TMR associated with a 15°C fall of T_b (i.e., at T_a 20°C) amounts to 95% in an average hibernator and 88% in an average daily heterotherm (calculated from Geiser and Ruf 1995; Geiser 2004; Geiser and Körtner 2004). The more pronounced MR reduction in the daily heterotherm in this case is largely due to its increased thermoregulatory cost (RMR) due to its small size (Withers 1992). These simple examples emphasize the enormous energy-conserving potential of both forms of torpor even at relatively mild T_a, even without considering the reduced costs of rewarming at high T_a and the possibility of passive rewarming (Lovegrove et al. 1999; Geiser et al. 2004).

5.4.6 Water Conservation

Although a reduction in energy expenditure is generally considered to be the major role of torpor, torpor also appears to be important for water conservation, especially, in species with limited access to free water. Free-ranging mouse-lemurs (*M. murinus*) using torpor have lower rates of water turnover than individuals remaining normothermic (Schmid and Speakman 2000). Evaporative water loss of torpid cactus mice (*P. eremicus*) is about 37% of that of normothermic individuals at the same T_a (MacMillen 1965). Similarly, for gerbils (*G. pusillus*) and marsupial dunnarts (*S. macroura*) daily torpor reduced evaporative water loss to about 20 to 40% of that in normothermic individuals (Buffenstein 1985; Cooper et al. 2005). More extreme reductions in evaporative water loss were observed for torpid bats (*Chalinolobus gouldii*) hibernating at T_a 10°C, which reduced evaporative water loss by almost 90% in comparison to normothermic individuals (Hosken and Withers 1997). Further, evaporative water loss of torpid honey possums (*T. rostratus*) during torpor was so low that is was not detectable with the available equipment (Withers et al. 1990).

Thus, torpor clearly reduces water loss and water turnover in mammals and is, therefore, of ecological importance. However, it would be worthwhile to unravel how much of the reduced water loss simply reflects a reduction in metabolism and T_b rather than being a selective reduction in water requirements.

5.5 Conclusions

Torpor (i.e., aestivation) is widely used by mammals and birds in summer as well as at high T_a. Torpor during development in summer may be an important survival mechanism that not only helps in reduction of thermoregulatory energy expenditure but also may spare valuable nutrients for growth and therefore warrants further investigation. In adults, torpor even at relatively high T_a results in a substantial reduction of energy and water use and therefore plays a vital role in the survival of mammals and birds in a variety of habitats.

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Chapter 6
Metabolic Rate Suppression as a Mechanism for Surviving Environmental Challenge in Fish

Jeffrey G. Richards

Contents

Abstract The ability to reduce metabolic rate during exposure to environmental stress, termed metabolic rate suppression, is thought to be an important component to enhance survival in many organisms. Metabolic rate suppression can be achieved through modifications to behavior, physiology, and cellular biochemistry, all of which act to reduce whole organisms energy expenditure. This chapter will critically evaluate the use of metabolic rate suppression as a response to environmental challenge in fish using three metabolic states: aestivation, hypoxia/anoxia exposure, and diapause.

6.1 Introduction

The earth's biosphere is rife with inhospitable environments from the perspective of an endothermic homeotherm, yet almost all these environments contain organisms that have adapted to thrive. Variation in the environment condition, even within these

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so-called extreme environments necessitates in some cases periods of dormancy to potentially limit the impacts of environmental variation on organism survival. In the simplest sense, exposure to environmental challenge imposes a stress on energy balance and the maintenance of integrated physiological and biochemical systems. Over the past several decades, it has emerged that modifications to metabolic rate, in particular, the ability to reduce overall energy demands during periods of environmental stress, termed metabolic rate suppression, is a unifying strategy for surviving environmental challenge (e.g., Boutilier 2001; Buck et al. 1993b; Hochachka and Somero 2002). As pointed out by numerous authors (e.g., Guppy 2004), the use of metabolic rate suppression to survive environmental challenge is a normal part of the life cycle of many animals including representatives from most of the major invertebrate phyla and all vertebrate classes.

Metabolic rate suppression has been documented in response to numerous environmental challenges including exposure to low or a complete lack of O_2 (termed hypoxia and anoxia, respectively; Boutilier and St-Pierre 2000; Buck et al. 1993b; Nilsson and Lutz 2004), cold temperatures (Campbell et al. 2008), desiccation (Smith 1930), lack of food availability, and almost any environmental change that results in a disturbance to cellular homeostasis. Environmental variation can occur either on predictable circa annual or circadian rhythms, therefore, allowing an animal to prepare in advance for these events or occurs on irregular or unpredictable rhythms. The precise cues for the initiation of metabolic rate suppression are unknown, but can be broadly categorized as exogenous; for example, changes in O_2 tensions, where an animal responds to the environmental insult, or endogenous, where an animal prepares for the environmental insult as seen in the developmentally cued diapause.

There is a considerable wealth of published literature outlining the use of metabolic rate suppression as a response to environment change across broad phylogenetic groups. This chapter, however, will focus primarily on fish. Fish present a particularly tractable model with which to explore the general utility of metabolic rate suppression as a response to environmental challenge because one could argue that fish are far more impacted by changes in their environment than other animals. Almost every phylogenetic order has its exceptions in terms of a species ability to survive environmental challenge, but amongst fish species, numerous examples exists of survival in some of the most extreme and inhospitable environments imaginable. In order to explore the general utility of metabolic rate suppression as a response to environmental change, the current definitions of metabolic rate suppression will be first reviewed and a working model for what constitutes metabolic rate suppression is then developed. Following the development of a model, this chapter will critique the available literature on the use of metabolic rate suppression during three metabolic states that are believed to involve metabolic rate suppression as the primary response enhancing survival: aestivation, hypoxia/anoxia exposure, and diapause.

6.2 Defining Metabolic Rate Suppression

Before we proceed with a discussion of the general utility of metabolic rate suppression as a response to environmental challenge, we must first develop a definition for the term. To accomplish this task, we must first establish a “bench-mark”

metabolic state from which to compare the environmentally imposed metabolic rate in order to elucidate the degree of metabolic rate suppression. Loosely, metabolic rate can be defined as the sum of all energy-consuming processes occurring over a given time. Under almost all metabolic states, cellular energy demand in terms of ATP utilization is closely matched to ATP production, and, therefore, metabolic rate can be defined as the sum of the cellular ATP turnover rates in various organs within an intact animal (Hochachka and Somero 2002). Clearly, the rate of ATP turnover is subject to variation in ATP demand, and, therefore, different levels of metabolic rate must be defined. For the purposes of this chapter, we will use and build upon the definitions of metabolic rate put forward by F.E.J. Fry (1971). Fry (1971) defined three levels of metabolism, mostly dependent upon the mode of measurement, including "standard," "routine," and "active" levels of metabolism.

Before we begin to define the levels of metabolic rate, it must be clearly noted that metabolic rate defined as ATP turnover cannot be measured directly. In order to measure metabolic rate indirect measures must be used. The two predominately used indirect measures of metabolic rate are heat dissipation and O_2 consumption. The best indirect measure of metabolic rate (often referred to as the "direct" measure of metabolic rate to demonstrate its superiority) is the measurement of heat dissipation. Metabolic heat is the product of inefficient energy transduction during ATP turnover and the rate of heat production is directly proportional the ATP turnover rate. Heat production is apparently not influenced by variation in the contributions of aerobic and anaerobic metabolism to overall ATP turnover; however, modifications to futile cycling, such as proton leak in the mitochondria, could have substantial effects on heat production that are possibly independent of ATP turnover. A reduction in heat dissipation and, therefore, heat production by an animal can be interpreted as a reduction in metabolic rate. Measurements of O_2 consumption rate are far more common in the scientific literature because of its ease of measure, but it must be noted that these measurements only estimate the contributions of aerobic metabolism to overall ATP turnover and do not take into account the contributions of anaerobic metabolism to ATP turnover or metabolic rate. As a result, measurements of O_2 consumption rate could underestimate metabolic rate during periods of environmental stress where animals may activate O_2-independent means of ATP generation through substrate-level phosphorylation (e.g., glycolysis alone or phosphocreatine hydrolysis). Measurements of O_2 consumption during periods of environmental stress, can be improved and yield a better estimate of metabolic rate if experimenters also measure the typical end products of substrate-level phosphorylation. In the case of fish, measure-ments of lactate combined with O_2 consumption rates can give an approximation of metabolic rate.

Standard metabolic rate (SMR), termed basal metabolic rate (a term more common in literature on mammals) is defined as the minimum rate of metabolism for an intact animal. SMR is determined on resting, unstressed, adult animals in their postabsorptive state under normothermic conditions. For ectothermic animals, normothermic would be considered a temperature well within the species tolerance limits and for which the animal has had ample time to acclimate. Of importance to note and as pointed out by Hochachka and Somero (2002), the contribution

of SMR is tissue specific. For example, in humans, the contributions to SMR of liver, gastrointestional tract, kidney, lung, central nervous system, heart, and muscle are 17, 10, 6, 4, 20, 11, and 25%, respectively. It is often assumed that SMR cannot be measured directly because of the difficulty of meeting the criteria setout above in fish.

Routine metabolic rate (RMR) is the mean rate of ATP turnover observed in fish whose metabolic rate is influenced by random activity under experimental conditions in which movements are presumably somewhat restricted and the fish is protected from outside stimuli. RMR, as pointed out by Fry (1971) gives a range of metabolic rates dependent on activity level within the above outlined confine, and an extrapolation through the resulting measured metabolic rates and possibly including metabolic rates measured during forced activity gives similar values to measured SMR when the fish is not disturbed.

Maximal of active metabolic rate (MMR) has been defined as the maximum sustained ATP turnover rate for a fish swimming steadily (Fry 1971). Measured as O_2 consumption, MMR is equivalent to $\dot{V}o_{2max}$ in mammalian studies and represents the maximum aerobic metabolic rate. Of course, during forced swimming, for example, a fish can exceed the maximum aerobic metabolic rate by utilizing "anaerobic" pathways for ATP generation, but this would only be evident if heat dissipation is measured or the accumulation of anaerobic end products were measured. The difference between the aerobic MMR and SMR is the metabolic scope for activity.

6.3 Metabolic Rate Suppression as a Response to Environmental Stress

In contrast to the metabolic scope of activity, metabolic rate suppression can be thought of as the metabolic scope for survival (Hochachka and Somero 2002) and, in general, this phenomenon is primarily thought of at a cellular and biochemical level representing a controlled reduction in the ATP-consuming processes contributing to SMR. The cellular capacity to reduce SMR during environmental challenge extends the time that an organism can survive on stored fuel during periods of environmental stress. Typical definitions of metabolic rate suppression note an 85–100% reduction in SMR (Guppy 2004) and this degree of suppression has been noted in many species of mammals, anurans, and fish in response to cold (e.g., hibernation in squirrels), hypoxia/anoxia (e.g., intertidal mollusks, goldfish), dehydration (e.g., snails, frogs, etc), and diapause (embryos of annual killifish). These states can last for periods of hours, days, a complete season, or years in some cases. At the heart of these extreme cases of metabolic rate suppression (e.g., decreases in metabolic rate to nearly undetectable levels of life), there is a cellular reorganization to reduce ATP demands to the lowest possible level. This phenomenon has fascinated comparative biochemists for decades. It has long been conjectured, and indeed there is accumulating evidence, that survival of some environmental challenges are dependent on the degree of cellular metabolic rate suppression or the metabolic scope for survival.

This is most certainly the case for hypoxia/anoxia survival, but as discussed in the sections below this may be an overly simplistic view.

Although the majority of the work on metabolic rate suppression has focused on the cellular and biochemical mechanisms underlying reductions in SMR, there are many possible mechanisms that facilitate a reduction in overall energy demands of an animal entering into or experiencing environmental challenge. In the next few paragraphs, some of the possible mechanisms, from the whole animal to the cell, that collectively achieve metabolic savings and contribute to metabolic rate suppression will be outlined. A proposed progression of responses to an environmental stress is first behavioral, facilitating a reduction in metabolic scope for activity followed by physiological responses leading to alterations in organ function and finally cellular and biochemical changes that reduce ATP consumption at the ATPase (Fig. 6.1). In order to survive periods of environmental stress, a coordinated

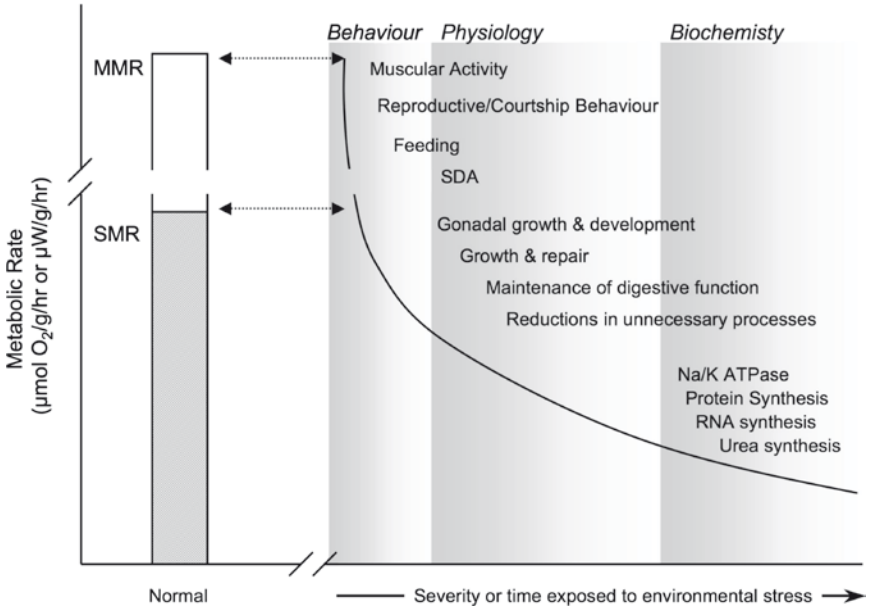

Fig. 6.1 Metabolic response during exposure to an environmental challenge. During exposure to environmental challenge of increasing severity or duration, metabolic rate is decreased by a variety of behavioral, physiological, and biochemical adjustments. At the onset of an environmental challenge, behavioral adjustments of decreasing muscular activity, reproductive/courtship, feeding, and other voluntary activities facilitates metabolic savings by reducing metabolism from a maximum metabolic rate (MMR) toward a standard metabolic rate (SMR). As the environmental challenge persists or grows more intense, physiological adjustments that include reductions in gonadal development, growth, and energy allocated for the maintenance of nonessential physiological functions (e.g., digestive function) allow SMR to decrease below levels that would otherwise be maintained under normal conditions. Biochemical adjustments at the cellular level including controlled reductions in Na^+/K^+ ATPase activity, protein synthesis, RNA synthesis, and urea synthesis, to name a few, further decrease overall SMR and enhance survival

response that maintains cellular energy balance is essential to extent the period over which a set amount of stored fuel can be used to support life. However, in a context where an individual's survival is at stake, responses at the cellular and biochemical level cannot be considered in isolation from the whole organism. For example, changes in behavior such as swimming activity, most certainly affects cellular ATP demands, which in the case of swimming manifest as reductions in the activity of actin–myosin ATPase and Ca^{2+} ATPase activities. Reductions in ATP consumption by the muscle during periods of inactivity make available circulating fuels for other more essential tissues. Prolonged inactivity of muscle could result in cellular reorganization that further reduces ATP demands (see biochemical aspects below).

6.3.1 *Behavior Metabolic Rate Suppression Responses can Reduce Metabolic Demands*

The first and most obvious response to an environmental challenge is a forced or voluntary reduction in aerobic scope for activity, courtship behavior, or feeding (Fig. 6.1). Voluntary reductions in swimming activity have been noted in response to many environmental perturbations and within fish dramatic reductions in activity have been noted during exposure to hypoxia (Chapman and McKenzie 2009). Low levels of activity are also an important part of reducing metabolic rate during aestivation where lungfish and other aestivating fishes are known to burrow into the mud and remain inactive until the return of favorable conditions. Quantifying the metabolic saving associated with reductions in activity has received little attention. During exposure to anoxia, the crucian carp (*Carassius carassius*) reduced spontaneous swimming activity by ~50% which yielded a ~35% decrease in energy use (Nilsson et al. 1993).

6.3.2 *Physiology Metabolic Rate Suppression Responses can Reduce Metabolic Demands*

Beyond the behavioral components that reduce metabolic demands, exposures to a variety of environmental stressors are known to elicit physiological and biochemical responses that yield a reduction in metabolic rate. Physiological adjustments, including the cessation of digestion and the associated reductions in specific dynamic action (Fitzgibbon et al. 2007) as well as changes in cardio-respiratory function, to name just a few, can also yield dramatic metabolic savings. There are many described cases where during periods of environmental stress, fish and other organisms will cease gonadal development (Wu 2009), growth (Wang et al. 2009), and shift metabolic resources from one organ to another depending on the relative importance of each organ system to survival. It is not hard to imagine, although it has not specifically been experimentally addressed, that the physiological shifts described above could yield substantial reductions in energy demand and facilitate metabolic rate suppression. One example that is relevant to this chapter is the

potential metabolic saving associated with the cost of ventilation in fish. It has been estimated that ventilation in water represents as substantial contribution to SMR (Steffensen and Lomholt 1983) and a reduction in the need to ventilate water would facilitate metabolic rate suppression. During aestivation in lungfish for example, gill ventilation is reduced to nearly undetectable levels, which would clearly impact SMR relative to active water ventilating animals.

6.3.3 *Biochemistry Metabolic Rate Suppression Responses can Reduce Metabolic Demands*

The biochemical underpinnings of metabolic rate suppression have received considerable attention over the past several decades and the literature has been reviewed several times. The reader is directed toward any one of many excellent reviews for a more indepth discussion of the biochemical aspects of metabolic rate suppression (Guppy 2004; Hand and Hardewig 1996; Hochachka and Somero 2002; Hochachka et al. 1996; Hochachka and Guppy 1987; Land and Bernier 1995). Here, a brief overview of processes that are most often involved in metabolic rate suppression at the cellular level is provided. Suppression of cellular metabolic rate is achieved through the regulated decreases in the activity of ATP demand processes. The most commonly quoted cellular and biochemical responses yielding decreases in cellular metabolism in response to environmental challenges are decreases in the rates of protein synthesis, membrane ion movement (regulated primarily by the activity of Na^+/K^+ ATPase), gluconeogensis, RNA synthesis, urea synthesis, and any anabolic process that consumes ATP could conceivably be downregulated to achieve reductions in cellular metabolic rate. For example, in response to hypoxia exposure, the anoxia-tolerant turtle (*Chrysemys picta)*, shows a 94% suppression in metabolic rate during anoxia exposure and this was achieved through the dramatic downregulation of Na pumping, protein turnover, urea synthesis, and gluconeogensis (Buck and Hochachka 1993; Buck et al. 1993a; Buck et al. 1993b; Hochachka et al. 1996; Hochachka and Lutz 2001; Land et al. 1993). It is now clear that cellular mechanisms underlying metabolic rate suppression are similar across broad taxonomic groups (Buck and Hochachka 1993; Hochachka et al. 1996; Lewis et al. 2007; Richards et al. 2007; Wieser and Krumschnabel 2001). The combination of which ATP-demand processes are affected by any specific environmental stressor will be dependent upon which processes are essential for survival and not all environmental perturbations will necessarily elicit the same cellular response.

With this general concept for the mechanisms involved in metabolic rate suppression (Fig. 6.1), the available data on utility of metabolic rate suppression as a response to environmental change during aestivation, hypoxia/anoxia exposure, and the developmentally cued diapause state will be reviewed. The metabolic responses observed during aestivation and hypoxia/anoxia exposure are responses to exogenous cues, whereas the responses observed during diapause are cued to

endogenous factors. Organisms typically enter diapause before experiencing an environmental challenge and will remain in the diapaused state even when exposed to environmental conditions that would normally promote an active lifestyle.

6.4 Aestivation

Aestivation is a metabolic response to drought. Any fish species living in an area with circa annual rhythms in rainfall could experience a loss of water habitat as a result of long periods without rainfall. Fish inhabiting these environments are presented with two options. First, they can relocate to new, more permanent bodies of water, or second, they can survive the period of drought until the rains return. In order to survive drought, fish must overcome a myriad of problems including loss of habitat, increased predation risk, reductions in food availability, a potential loss of effective gas exchange surface area, accumulation of toxic metabolic end products, and desiccation. Fish that are unable to avoid these environmental conditions utilize aestivation as a metabolic response to enhance survival. Aestivation necessitates behavioral, physiological, and morphological adjustments to limit water loss and prevent the accumulation of toxic metabolic waste products, and, perhaps, most importantly, adjustments that reduce metabolic rate to extend the period over which stored substrates can support metabolic function. It is this last point, the role of metabolic rate suppression in aestivation that this portion of the chapter will focus on.

There are only a handful of fish species that have been documented to undergo aestivation including several species of African lungfish (*Protopterus* sp.; Greenwood 1986), some South American lungfish (*Lepidosiren paradoxa*; Mesquita-Saad et al. 2002), swamp eels (*Synbranchus marmoratus*; Eduardo et al. 1979), Salamander fish (*Lepidogalaxias salamandroides*; Pusey 1990), and several other documented fish species that survive drought (see Land and Bernier 1995). Common to all these species is the capacity for air breathing, which is clearly a prerequisite for the maintenance of at least a modicum of O_2 uptake during drought. The metabolic responses and, more importantly, the capacity for metabolic rate suppression have only been examined extensively in the lungfishes, which will be the primary focus of this portion of the chapter.

There are six species of lungfishes (subclass: Sarcopterygii) in the world including the South American *L. paradoxa* (Family: Lepidosirenidae), the Australian *Neoceratodus forsteri* and four species of the African including *Protopterus aethiopicus*, *P. annectens*, *P. amphibious,* and *P. dolloi*. Among the extant species of lungfish, all have reduced gills; however, the dependence on gill versus lung respiration differs between species. The Australian *N. forsteri* is not known to aestivate and is highly dependent on gill respiration as evidenced by the fact that this fish can remain submerged in normoxic water for several hours (Kind et al. 2002). The South American lungfish *L. paradoxa* has only rudimentary gills and is thought to aestivate without cocoon formation. The

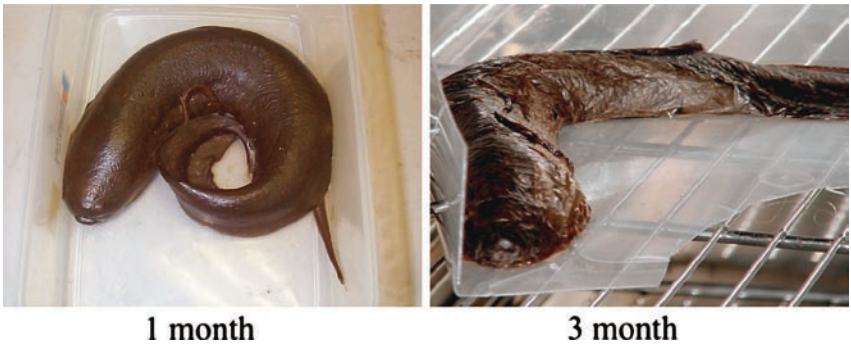

Fig. 6.2 *Propterus dolloi* after 1 and 3 month of "terrestrialization" in the laboratory. *Propterus dolloi* do not suppress O_2 consumption in the laboratory; therefore, the term "terrestrialization" is used rather than aestivation. Photo courtesy of Prof. Steve Perry, University of Ottawa

champions of aestivation are the African lungfishes from the genus *Protopterus*. These species possess reduced gills, but well-developed lungs, which has inspired considerable research into the evolution of air breathing (Graham 1997). All four species of *Protopterus* are thought to utilize aestivation to survive drought, but to varying degrees. As lakes dry out, lungfish, typically, expect *L. paradoxa*, dig a burrow in the sediment where they secrete copious amounts of mucous that gradually harden to form a cocoon (see Fig. 6.2 for an image of a cocooned lungfish in the laboratory). *Protopterus amphibius* has been reported to aestivate for up to 7 years (Lomholt 1993).

Since the original observation by Homer Smith (1930), it has been agreed upon that aestivation involves metabolic rate suppression; however, considerable controversy exits about the underlying cause of the reduction in metabolic rate. Table 1 outlines some of the available data on O_2 consumption rates in several species of African lungfish during periods of aestivation and starvation. Several species of lungfish have been shown to suppress O_2 consumption rates by up to 95% over prolonged periods of time (*P. amphibious* survived 6 years of aestivation and reduced O_2 consumption by 95% compared with active-fed animals; Lomholt 1993), whereas other species (*P. dolloi)* show a 27% increase in O_2 consumption rate during 30–60 days of aestivation (in this case, termed terrestrialization; Perry et al. 2008). Even within a single species, *P. aethiopicus*, there is considerable variation among studies in the degree of suppression of O_2 consumption during aestivation from roughly a 60% decrease following 18 months of aestivation to the 95% suppression reported earlier in this paragraph (Table 1). Clearly, there is inter and intraspecific variation in the use of metabolic rate suppression during aestivation and further research is needed to isolate the underlying causes of this variation.

During aestivation in lungfish, numerous behavioral, physiological, and potential biochemical changes occur that may facilitate reductions in O_2 consumption. Since the early works of Smith (1930, 1935), it has been proposed and supported by data that the decline in metabolic rate during aestivation was primarily due to

Table 6.1 The effects of aestivation and starvation of oxygen consumption rates in various species of lungfish

Scientific name	Temp. (°C)	Size (g)	Active aquatic Vo_2	Aestivation duration	Aestivation lowest VO_2	Starvation duration	Starvation lowest VO_2	Reference
P. aethiopicus	~25		13–31 ml $kg^{-1}h^{-1}$	60 days	5.05 ml $kg^{-1}h^{-1}$	–	–	(Delaney et al. 1974)
P. aethiopicus		2000–6000	74 ml $kg^{-1}h^{-1}$	130 days	~10 ml $kg^{-1}h^{-1}$	33 days	~10 ml $kg^{-1}h^{-1}$	(Fishman et al. 1986)
P. aethiopicus			37 ml $kg^{-1}h^{-1}$	235 days	~10 ml $kg^{-1}h^{-1}$			(Fishman et al. 1986)
P. aethiopicus	~25	~200	~20 ml $kg^{-1}h^{-1}$	390–540 days	~8 ml $kg^{-1}h^{-1}$			(Smith 1930)
P. aethiopicus	20	220	33.8 ml $kg^{-1}h^{-1}$[a]			58 days	20.4 ml $kg^{-1}h^{-1}$	(Smith 1930)
P. aethiopicus	N/A	N/A	20–40 ml $kg^{-1}h^{-1}$	~200 days	3.5–8 ml $kg^{-1}h^{-1}$		12–17 ml $kg^{-1}h^{-1}$	(Fishman et al. 1992)
P. aethiopicus			20.7 ml $kg^{-1}h^{-1}$	30 days	4.6 ml $kg^{-1}h^{-1}$			(Swan et al. 1969)
P. amphibious			50 ml $kg^{-1}h^{-1}$	6 years	2.3 ml $kg^{-1}h^{-1}$			(Lomholt 1993)
P. annectens	~27	151	2.5 mmol $kg^{-1}h^{-1}$[b]			0.5–4 days	0.5–1.0 mmol $kg^{-1}h^{-1}$	(Iftikar et al. 2008)
P. dolloi	25	146	0.95 mmol $kg^{-1}h^{-1}$	30–60 days	1.21 mmol $kg^{-1}h^{-1}$			(Perry et al. 2008)

All measurements were made using respirometry

[a]These estimates are for a fish that had been fasted for 2 days after 90 days of regular feeding

[b]Oxygen consumption rate 5–8 h after feeding

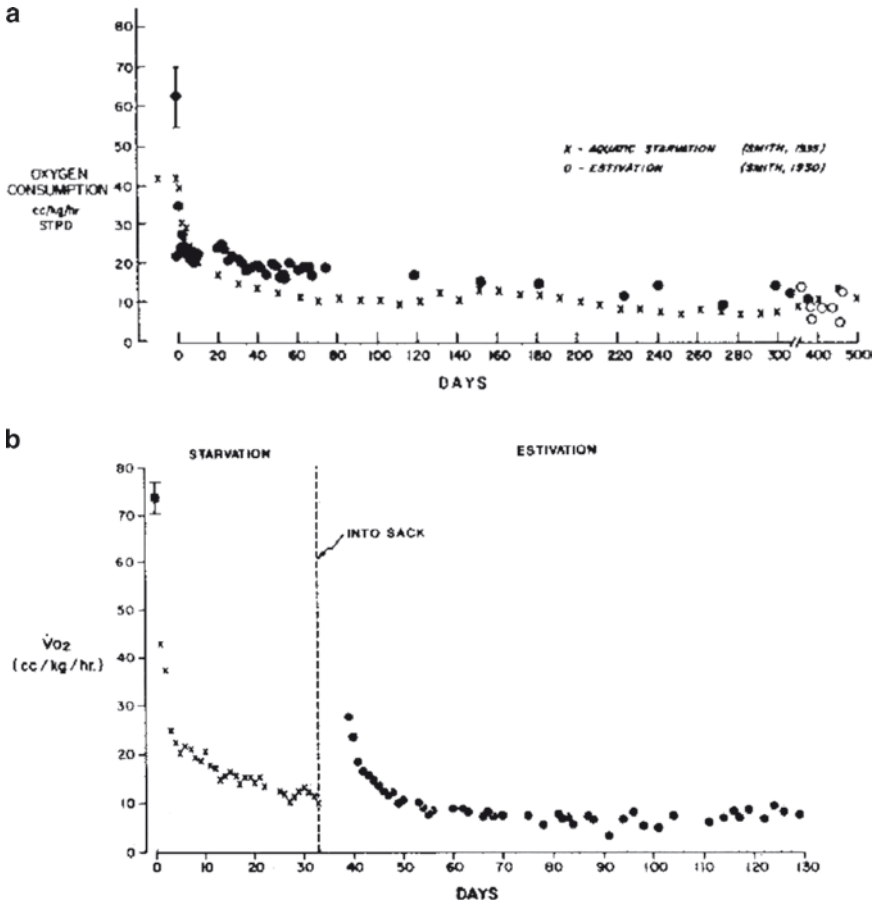

Fig. 6.3 Oxygen consumption rates of *P. aethiopicus* during starvation and aestivation. *Panel A* shows data from the early publications of Smith (1930 and 1935) showing that the reductions in O_2 consumption observed during 500 days of aestivation are primarily accounted for by decreases in O_2 consumption related to starvation. *Panel B* shows the same trend in O_2 consumption in *P. aethiopicus* during 33 days of starvation and over 130 days of aestivation in the laboratory

progressive starvation and emaciation (Fig. 6.3). Overall, it has not been easy to dissociate the physiological effects of starvation from other metabolic adjustments that may occur during aestivation. Indeed, some investigators have collected evidence in support of the contention that virtually all reductions in metabolic rate during aestivation can be attributed to starvation. Fishman (1986) demonstrated that ~33 days of starvation in active water-inhabiting *P. aethiopicus* caused a similar decrease in O_2 consumption rate as 130 days of aestivation (Table 1 and Fig. 6.3b). Similar patterns of decreases in O_2 consumption rate upon starvation were observed in *P. annectens* and *Lepidosiren* sp. (Fishman et al. 1986), but it was

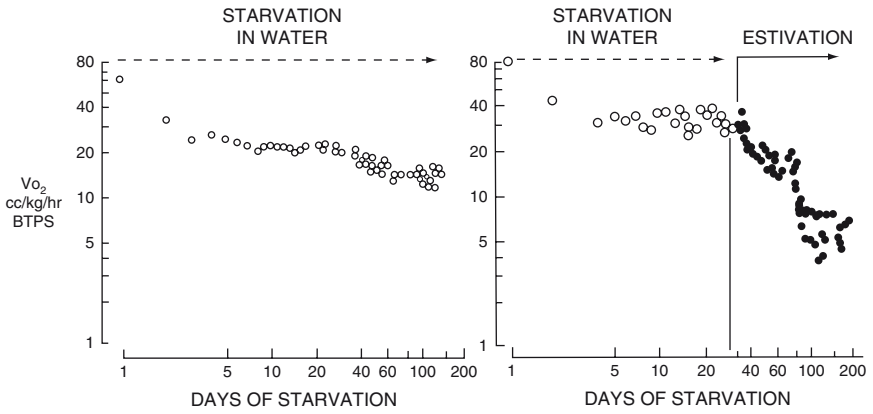

Fig. 6.4 Oxygen consumption rates of African lungfish during 100–200 days of starvation and aestivation in the laboratory. Aestivation caused a greater decrease in oxygen consumption rate than observed in animals that were starved

noted by this author that the responses to starvation were variable and this variation was probably related to differences in food consumption.

The only available evidence to suggest that aestivation involves a degree of metabolic rate suppression beyond that which can be ascribed to starvation was presented by Fishman et al. (1992); Fig. 6.4). Fishman et al. (1992) demonstrated that 100–200 days of starvation in water caused a decrease in O_2 consumption rate from ~40 to 60 ml $kg^{-1} h^{-1}$ to 12 to 17 ml $kg^{-1} h^{-1}$. In contrast, aestivation in the laboratory following a period of starvation (~25 days) resulted in substantial decrease in O_2 consumption from 25 to 40 ml $kg^{-1} h^{-1}$ after starvation to values that fluctuated between 8 and 3.5 ml $kg^{-1} h^{-1}$ between 100 and 200 days of aestivation (Fig. 6.4). These data suggest that there are other physiological or biochemical factors beyond starvation that elicit the suppression of metabolic rate. It must be noted, however, that these data were only ever presented in conference proceedings and, therefore, the methods are not available for careful scrutiny.

The cardiorespiratory responses of starvation and aestivation also appear to differ suggesting that aestivation itself involves additional modifications beyond those essential for surviving starvation. During months of starvation, there are only modest cardiovascular and respiratory changes in African lungfish, while aestivation over the same time period evokes dramatic changes in blood pressure, heart rate, and breathing (Delaney et al. 1974). During 80 days of starvation, *P. aethiopicus* showed a steady mean blood pressure of ~25 mmHg, a heart rate of ~20 beats per minute and a lung ventilation frequency of between 1 and 5 breath per hour. In contrast, during up to 9 months of aestivation, mean blood pressure decreased from ~25 mmHg to ~15 mmHg (reaching this low value by 1 month), heart rate decreased from ~25 beats per minute to 15 beats per minute (reaching this value by 2 months of aestivation) and lung breaths increases slightly during the first 2 months of aestivation, but essentially remained unchanged from control values of

between 5 and 10 breaths per hour. Reductions in cardiac contraction frequency have also been noted in the South American lungfish (*L. paradoxa*), which is thought to have a limited capacity to aestivate due to the fact that this species does not form a cocoon and aestivates in a very simple burrow (da Silva et al. 2008). Overall, however, reductions in mean blood pressure and heart rate above and beyond that observed during starvation could represent a metabolic savings, but the lack of a change in lung ventilation suggests maintenance of O_2 uptake. One possibility to explain these counterintuitive observations is that the metabolic savings accrued through reductions in cardiovascular demands could be allocated to offset the increased metabolic demands associated with enhanced costs of osmoregulation and conversion of toxic ammonia to urea.

The slender lungfish in *P. dolloi* has recently received considerable attention in terms of its metabolic and respiratory responses to aestivation. During 30–60 days of "aestivation," Perry et al. (2008) demonstrated essentially no changes in O_2 consumption rate before (0.95 mmol $O_2 Kg^{-1} h^{-1}$), during (1.21 mmol $O_2 Kg^{-1} h^{-1}$) or after (1.14 mmol$O_2 Kg^{-1} h^{-1}$) extended periods of cocooned aestivation. Due to the lack of observed decrease in O_2 consumption rate, the authors elected to describe the metabolic state as terrestrialization rather than aestivation. Breathing frequency in terrestrialized *P. dolloi* consisted of multiple rapid bouts of inspiration and expiration, but the overall lung breathing frequency was not different between *P. dolloi*

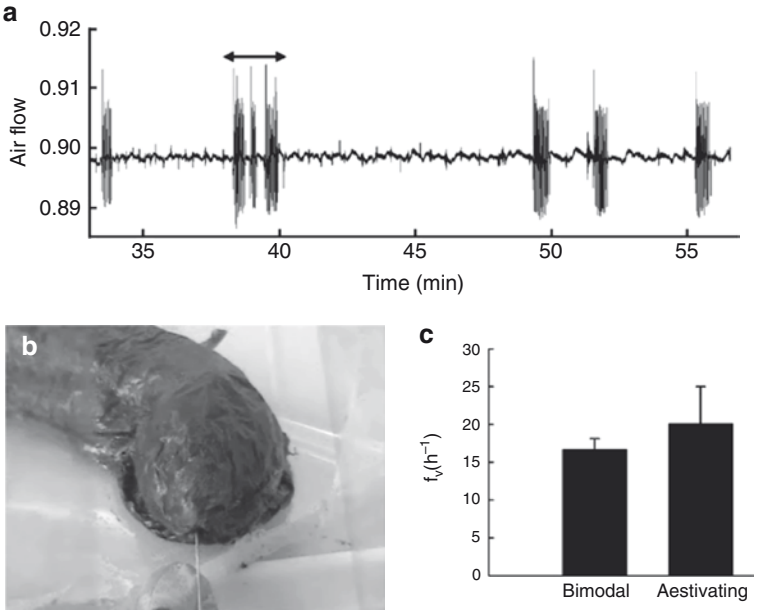

Fig. 6.5 Pulmonary breathing patterns (**a**) of *P. dolloi* during 1–3 months of terrestrialization. Measurements given in *Panel* **a**, which are obtained by measuring buccal air flow in cocooned animals, were taken (**b**) average ventilation frequency was not different between active animals breathing bimodally in water and terrestrialized animals (**c**)

in the terrestrial state and when active in water (Fig. 6.5; Perry et al. 2008). *Protopterus dolloi* appear to be unique among African lungfish in that they use the lung for both O_2 uptake and CO_2 excretion, whereas all other species of lungfish utilize the gills for CO_2 excretion (Perry et al. 2005). Whether these differences between species illustrated in Table 1 are due to fundamentally different capacities to utilize metabolic rate suppression during aestivation or simply differences in laboratory techniques, careful comparisons between species of lungfish relating aestivation capacity with the ability to undergo metabolic rate suppression could provide extremely valuable information for elucidating the importance of metabolic rate suppression during aestivation.

At the biochemical level, terrestrialization in *P. dolloi* has been characterized by a shift in metabolic pathways of ATP supply. Several months of terrestrialization in *P. dolloi* resulted in a 74–85% reduction in maximal ADP-stimulated mitochondrial respiration (state 3) as well as minimal (state 4) rates in muscle, but not in mitochondria from liver (Staples et al. 2008). This lack of change in aerobic capacity in liver was associated with an upregulation of several enzymes within the ornithine–urea cycle suggesting that aerobic capacity is maintained in the liver to support expensive urea synthesis. In the muscle however, a lack of movement during terrestrialization yields a tissue specific decrease in ATP demands which may be matched by coordinated reductions in capacity for ATP synthesis (Staples et al. 2008). Analysis of the maximal enzyme activity associated with lipid metabolism suggests that lipids may be an important fuel to support ATP production during aestivation (Frick et al. 2008b). Furthermore, there was apparent glycogen sparing during fasting and aestivation in *P. dolloi* and a lack of lactate accumulation or changes in lactate dehydrogenase (LDH) activity during both fasting and terrestrialization (Frick et al. 2008a), further supporting the notion that metabolic rate is completely supported by aerobic metabolism. Increases in glutamate dehydrogenase and aspartate aminotransferase in liver suggest some energy production from amino acid catabolism (Frick et al. 2008a). Overall, however, there is evidence from measurements of enzyme activity that aerobic ATP production persists during periods of starvation and aestivation albeit at reduced levels. The maintenance of aerobic capacity during aestivation is also supported by work performed on the South American lungfish (*L. paradoxa*) which showed lower anaerobic capacity (assessed by LDH, malate dehydrogenase, and other enzyme activities) in skeletal and heart muscle of aestivation fish compared with fish that were not aestivating (Mesquita-Saad et al. 2002). Overall, however, the activity levels of all anaerobic enzymes were high compared with other animals.

It has been proposed that measurements of metabolic rate made in the laboratory, even under the most natural simulated conditions, may not be representative of metabolic rates under natural conditions. For example, Loong et al. (2008b) compared *P. annectens* aestivating for 12–46 days in the air versus the mud. In air, no increases in tissue ammonia were observed, but tissue urea concentrations increased 1.4–2.7 fold indicating an overall increase in urea synthesis. In mud, however, there were still no changes in tissue ammonia concentrations, but 12 days of aestivation resulted in only very minor increases in urea and at 46 day of aestivation in mud,

there were no measurable increases in tissue urea concentrations compared with fish held in water. During aestivation in mud, there is a suppression of urea synthesis and ammonia production (Loong et al. 2008b). These authors suggested that fish aestivating under more natural conditions in mud might also be exposed to low environmental O_2 tension as evidenced by low measured blood pO_2 and muscle ATP content. Metabolic rate reductions are a common response to hypoxia (see section below) and may also play an important role in facilitating survival during aestivation in the wild. Hypoxia exposure during aestivation is clearly not a prerequisite for aestivation or survival of drought, because as indicated in Table 1 many species of lungfish can survive prolonged periods of desiccation under normoxic O_2 tensions. It has also been noted by the same authors that, while in the cocoon, high CO_2 tensions may help to reduce O_2 consumption rates. This is primarily evidenced by that fact that high O_2 consumption rates were measured immediately after removal from the mud cocoon and the fact that O_2 consumption rates decreased again following the potential blowing off of CO_2 (Loong et al. 2008a).

6.5 Environmental Hypoxia/Anoxia

Environmental hypoxia is a common occurrence in many aquatic environments (Diaz and Breitburg 2009) and imposes a fundamental challenge to the maintenance of metabolic energy balance. Approximately 80% of the O_2 consumed by a fish in normoxia is used as the terminal electron acceptor by the mitochondrial electron transport chain for ATP production (via oxidative phosphorylation). If environmental hypoxia leads to hypoxemia (i.e., physiological mechanisms to enhance O_2 uptake are insufficient to protect the animal from its environment and blood O_2 content is reduced), then there is the potential for an O_2 limitation at the mitochondrion, which imposes limitations on the capacity for ATP production. Under these conditions, ATP can only be generated by processes such as glycolysis yielding lactate production or through direct phosphate transfer from phosphorylated intermediates such as creatine phosphate. Although these processes of ATP generation can occur during periods of O_2 lack, the amount of ATP produced per mole of substrate consumed is approximately 15- to 30-fold lower than if mitochondrial respiration occurs.

The ability to suppress cellular ATP demand to match the limited capacity for O_2-independent ATP production has emerged as the unifying adaptive strategy ensuring hypoxia survival (Hochachka et al. 1996). Many excellent reviews have summarized the metabolic and molecular responses of fish and other lower vertebrates to hypoxia exposure (Almeida-Val et al. 2006; Bickler and Buck 2007; Nikinmaa and Rees 2005; Richards 2009). Because hypoxia exposure is known to cause activation of substrate-level phosphorylation to maintain ATP turnover, measurements of O_2 consumption will underestimate overall metabolic rate. As a result, only measurements of heat production during hypoxia exposure give an accurate estimate of the metabolic responses.

Heat production in fish during hypoxia/anoxia exposure has been assessed in several species including fish (*Carassius auratus*; Stangl and Wegener 1996;

van Ginneken et al. 2004; van Waversveld et al. 1989a; van Waversveld et al. 1989b; van Waversveld et al. 1988), crucian carp (*C. carassius*; Johansson et al. 1995), tilapia (*Oreochromis mossambiscus*; van Ginneken et al. 1999; van Ginneken et al. 1997), European eel (*Anquilla anquilla*; van Ginneken et al. 2001), zebrafish (*Brachydanio rerio*; Stangl and Wegener 1996), and in isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*; Rissanen et al. 2006) (See Table 2). Interestingly, across a very broad range of fish species, including those considered to be hypoxia tolerant (crucian carp, goldfish, and tilapia) and tissues from those considered to be intolerant (rainbow trout), all species show the capacity to decrease metabolic rate in response to hypoxia exposure. The most impressive reductions in metabolic rate, however, occur in the goldfish, tilapia, and European eel with an ~70% decrease in metabolic rate during hypoxia exposure (Table 2). Hepatocytes isolated from rainbow decreased metabolic rate to a lesser degree than seen in more hypoxia-tolerant animals such as goldfish and tilapia (whole-animal measurements), but comparison between isolated tissues and whole animals are difficult to make because of tissue-specific responses to hypoxia. Oddly, zebrafish exposed to severe hypoxia (<6% air saturation) for only 50 min showed a progressive increase in heat production, indicating an overall increase in metabolic rate during hypoxia exposure (Stangl and Wegener 1996). This increase in metabolic rate may represent increased costs associated with hypoxia-induced movement and escape behavior.

The mechanisms underlying metabolic rate suppression in fish during hypoxia/anoxia exposure are fairly well established. The reader is referred to the following reviews for analysis of the contributions of modifications to behavior (Chapman and McKenzie 2009), reproduction (Wu 2009), growth (Wang et al. 2009), physiology (Perry et al. 2009), and biochemical (Gamperl and Driedzic 2009; Richards 2009; Vornanen et al. 2009) to overall metabolic rate suppression. The cellular aspect of metabolic rate suppression in fish during hypoxia/anoxia exposure is briefly summarized below, and new research that describes how these metabolic responses are coordinated during hypoxia/anoxia exposure is highlighted.

The question of how organisms are able to reduce metabolic rate below SMR has received considerable attention over the past several decades. Original work in this field using hepatocytes isolated from the anoxia-tolerant turtle (*C. picta)*, demonstrated that a 94% suppression in metabolic rate during anoxia exposure was achieved through the dramatic downregulation of Na pumping, protein turnover, urea synthesis, and gluconeogensis (Buck and Hochachka 1993; Buck et al. 1993a; Buck et al. 1993b; Hochachka et al. 1996; Hochachka and Lutz 2001; Land et al. 1993). It is now clear that cellular mechanisms underlying metabolic rate suppression are similar across broad taxonomic groups with metabolic rate suppression involving the controlled arrest of processes involved in membrane ion movement (Buck and Hochachka 1993; Richards et al. 2007), protein synthesis (Lewis et al. 2007; Wieser and Krumschnabel 2001), RNA transcription, urea synthesis, gluconeogensis, and other anabolic pathways (Hochachka et al. 1996).

The suppression of protein synthesis has been described in both isolated hepatocytes and fish in vivo in species ranging from the crucian carp (Smith et al. 1996; Vornanen et al. 2009), goldfish (Jibb and Richards 2008), and the Amazonian oscar,

Table 6.2 Metabolic rate suppression in fish during exposure to hypoxia/anoxia

Common name	Scientific name	Whole animal or tissue	Temperature (°C)	Hypoxia	Duration	Metabolic rate suppression[a]	Reference
Goldfish	*Carassius auratus*	Whole animal	20	Anoxia	2–3 h	70%	(van Waversveld et al. 1989; van Waversveld et al. 1988)
Goldfish	*C. auratus*	Whole animal	20	10% air sat.	3 h	59%	(van Waversveld et al. 1989)
Goldfish	*C. auratus*	Whole animal	20	5% air sat.	3 h	53%	(van Waversveld et al. 1989)
Goldfish	*C. auratus*	Whole animal	20	Anoxia	3 h	70–85%	(Stangl and Wegener 1996)
Goldfish	*C. auratus*	Whole animal	20	3% air sat. progressive	5 h	55%	(van Ginneken et al. 2004)
Crucian Carp	*Carassius carassius*	Brain slices	12	Anoxia	20 h	37%	(Johansson et al. 1995)
Tilapia	*Oreochomis mossambicus*	Whole animal	20	5%	8 h	55%	(van Ginneken et al. 1997)
Tilapia	*O. mossambicus*	Whole animal	20	3% air sat. progressive	1 h	64%	(van Ginneken et al. 1999)
European eel	*Anquilla anquilla*	Whole animal	20	Anoxia	1 h	70%	(van Ginneken et al. 2001)
Rainbow trout	*Oncorhynchus mykiss*	Hepatocytes	20[a]	~4% air sat. progressive	6–12 min	46%	(Rissanen et al. 2006)
Zebrafish	*Brachydanio rerio*	Whole animal	25	6% air sat.	50 min	150%	(Stangl and Wegener 1996)

In studies with more than one level of hypoxia, the degree of metabolic rate suppression for the most severe level of hypoxia is shown

[a]Animals were acclimated to 12°C and metabolic rate was measured in isolated hepatocytes at 20°C

(*Astronotus ocellatus*; Lewis et al. 2007). In the oscar, severe hypoxia exposure (10% air saturation) caused tissue-specific decreases in protein synthesis rates that varied from 27% decreases in protein synthesis rate in brain to 60% decreases in heart. In the crucian carp, Smith et al. (1996) also demonstrated substantial decreases in protein synthesis rates in heart, liver, and muscle in response to anoxia exposure and these decreases were, in part, mediated by decreases in RNA transcription rates (Smith et al. 1999). In the goldfish, hypoxia exposure (<0.5% air saturation) caused a very rapid (within 0.5 h), ~70% decline in liver protein translation rate (assessed in cell-free isolates). These decreases in protein synthesis rates in the hypoxic goldfish were mediated through specific phosphorylation of eukaryotic elongation factor-2 (eEF2) (Jibb and Richards 2008), which halts protein elongation during translation.

Few studies have examined how other ATP-consuming processes, besides protein synthesis rates, are modified during hypoxia exposure in fish, but some modifications to ion pumping have been noted. In particular, hypoxia-induced decreases in the activity of Na^+/K^+ ATPase as observed in some studies (Bogdanova et al. 2005) could represent a substantial ATP savings, but the results are conflicting. The crucian carp does not decrease brain Na^+/K^+ ATPase activity during anoxia exposure (Hylland et al. 1997) despite increases in the inhibitory neuromodulators GABA (Nilsson 1992) and adenosine (Nilsson 1991). This lack of an effect of anoxia/hypoxia exposure on Na^+/K^+ ATPase activity in the brain of crucian carp is unlike the response observed in turtles, which suppress the activity of Na^+/K^+ ATPase. The differential responses observed in the two champions of anoxia tolerance are probably associated with the fact that crucian carp remains active during anoxia exposure, unlike the comatose turtle (Vornanen et al. 2009). Recent work by Richards et al. (2007), demonstrated a substantial decrease in gill Na^+/K^+ ATPase activity in the oscar exposed to hypoxia (~5% air saturation) and it was speculated that this decrease was achieved by a posttranslational modification to the Na^+/K^+ ATPase protein. A similar effect of hypoxia exposure was observed in isolated trout hepatocytes, where a hypoxia caused a transient downregulation of Na^+/K^+ ATPase activity (Bogdanova et al. 2005). These authors speculated that decreases in Na^+/K^+ ATPase activity in response to hypoxia may be accomplished by local changes in reactive oxygen species, but no precise mechanism was given.

Cell survival during hypoxia exposure requires a metabolic reorganization to decrease ATP demands to match the reduced capacity for ATP production and these metabolic responses must be coordinated temporally, otherwise hypoxia exposure will lead to cell death. Several signal transduction cascades have been shown to be activated in response to hypoxia exposure in mammals and other vertebrates (Storey and Storey 2004), but considerably less work has been done in fishes. In the remaining portion of this section, recent advances will be outlined that suggest that AMP-activated protein kinase (AMPK) may play a critical role in coordinating the metabolic responses to hypoxia. AMPK is a heterotrimeric protein kinase comprised of a catalytic subunit (α) and two regulatory subunits and phosphorylation of AMPK at Thr-172 on the α-subunit activates the protein

(Carling 2004). Activation of AMPK in mammals inhibits energetically expensive anabolic processes including protein synthesis (Horman et al. 2002), glycogen synthesis (Nielsen et al. 2002) and fatty acid synthesis rates (Hardie and Pan 2002). Furthermore, activation of AMPK increases skeletal muscle hexokinase activity, GLUT-4 glucose transporter expression (Holmes et al. 1999), and translocation to the membrane (Kurth-Kraczek et al. 1999), and increased phosphofructokinase-2 activity in rat cardiomyocytes (Marsin et al. 2000), all of which could enhance O_2-independent ATP production. Combined, these actions have led to AMPK being termed the cellular "energy gauge" because of its critical role in maintaining cellular energy balance.

Two recent studies have confirmed that AMPK is activated in fish in response to hypoxia exposure. Jibb and Richards (2008) demonstrated that AMPK was activated in the liver of goldfish exposed to severe hypoxia and that there was a close temporal change in $[AMP_{free}]/[ATP]$ and AMPK activity. Increases in AMPK activity in the liver were associated with an increase in the percent phosphorylation of a well-characterized target of AMPK, eukaryotic eEF2, and decreases in protein synthesis rates measured in liver cell-free extracts suggesting that a disruption of cellular energy status is important for the activation of mechanisms involved in metabolic rate suppression. AMP-activated protein kinase, however, was not activated in muscle, brain, heart, or gill during 12 h severe hypoxia exposure in goldfish suggesting a tissue-specific regulation of AMPK and metabolic responses to hypoxia (Jibb and Richards 2008). Stensløkken et al. (2008) elegantly demonstrated that AMPK, as well as another kinase system, protein kinase B (AKT) was integral to hypoxia/anoxia survival in the crucian carp. Anoxia exposure in the crucian carp elicited large increases in the phosphorylation state of AMPK in heart and brain and that blocking kinase activity with compound C resulted in an elevation of metabolic rate as measured by an increase in ethanol production (Stensløkken et al. 2008). These data clearly suggest that AMPK activity plays an important role in hypoxia/anoxia exposure in hypoxia-tolerant fish species.

Hypoxia survival in fishes necessitates a metabolic reorganization to reduce ATP consumption through a regulated metabolic rate suppression to match the limited capacity for O_2-independent ATP production. As outlined above, controlled metabolic rate suppression is essential to extend the length of time that can be supported by the limited levels of fermentable fuels. Thus, it appears reasonable to speculate that the degree of metabolic rate suppression and the quantity of stored fermentable fuel is likely strongly selected for in hypoxia-tolerant fishes.

6.6 Diapause

Diapause is a state of programmed developmental arrest, which is often associated with periods of environmental stress. Since diapuase in most species is developmentally programmed to occur at particular developmental stages, it typically precedes the onset of the environmental insult, and as a result, the organism can

exhibit developmental arrest even when environmental conditions could be conducive to carrying on normal development. Among fishes, there are numerous examples of diapause including embryonic rays (*Trygonoptera personata* and *T. muscosa*; White et al. 2002) as well as eggs of Grunion (*Leuresthes tenuis*) (Darken et al. 1998; Matsumoto and Martin 2008) and others; however, the best studied diapausing fishes are the annual killifishes (Cyprinodontiformes, Rivulidae), which will form the basis for the following discussion. Annual killifish (*Austrofundulus limnaeus*) inhabit ephemeral ponds in the desert regions of Northern South America. There ponds dry out on a seasonal basis, killing off adult and juveniles, but embryos are able to persist through drought as diapaused embryos in the sediment until the rain returns and the diapaused embryos emerge from diapause, hatch, and continue their lifecycle.

Within the annual killifish, it has been suggested that there are three distinct diapause stages (diapause I, II, and III) that may occur during development (Wourms 1972a; Wourms 1972b). Diapause I occurs early in development and is thought to be induced by unfavorable environmental conditions such as hypoxia or cold stress in *Austrofundulus myersi* (Wourms 1972b). Diapause II occurs in embryos possessing 38–40 somites, a beating heart, and the foundations of the central nervous system (Wourms 1972a). The diapause III embryo is fully developed and may hatch at any time in response to the correct environmental cues. Prolonged diapause III results in a reduction in yolk utilization and heart rate, suggesting that a dramatic metabolic rate suppression occurs when hatching is delayed (Wourms 1972a; Wourms 1972b). For *A. limnaeus*, the developmental sequence involves hatching, 24–26 days of development, followed by entry into diapause II. Diapause II is an obligatory part of development in *A. limnaeus* with predictable timing and may last for well over 100 days if hatching does not occur. Failure to hatch after this period leads to entry of the fish embryo into diapause III.

During the natural developmental progression of *A. limnaeus*, there is an initial increase in metabolic rate over the first 6–8 days of development, followed by a steady decline in metabolic rate (measured as heat dissipation) to values equivalent to values measured immediately post hatch (Fig. 6.6). These changes in heat dissipation are matched by more or less parallel increases in O_2 consumption rate, except at 2 days postfertilization (dpf) and beyond 36 dpf, which is 12 days into diapause II (Podrabsky and Hand 1999). At these points, the calorimetric/respirometic ratio, that is the ratio of heat dissipation to O_2 consumption, suggests that the metabolic rate estimated by measuring the heat dissipation is not supported solely by aerobic O_2 consumption. Oddly, no changes in anaerobic substrate utilization were observed and no anaerobic end products were measured (e.g., lactate and ethanol; Podrabsky and Hand 1999). Protein content decreases during diapause II, but amino acid catabolism cannot account for the discrepancy either. As a result, Podrabsky and Hand (1999) concluded that diapause II is characterized by a 70% decrease in oxygen consumption rates compared with the peak oxygen consumption rates observed at 8 dpf and heat dissipation rates measured at 24 dpf was depressed by 67% compared with the same peak values. Thus, entry into diapause II can be characterized by a depression of metabolic rate compared with the peak

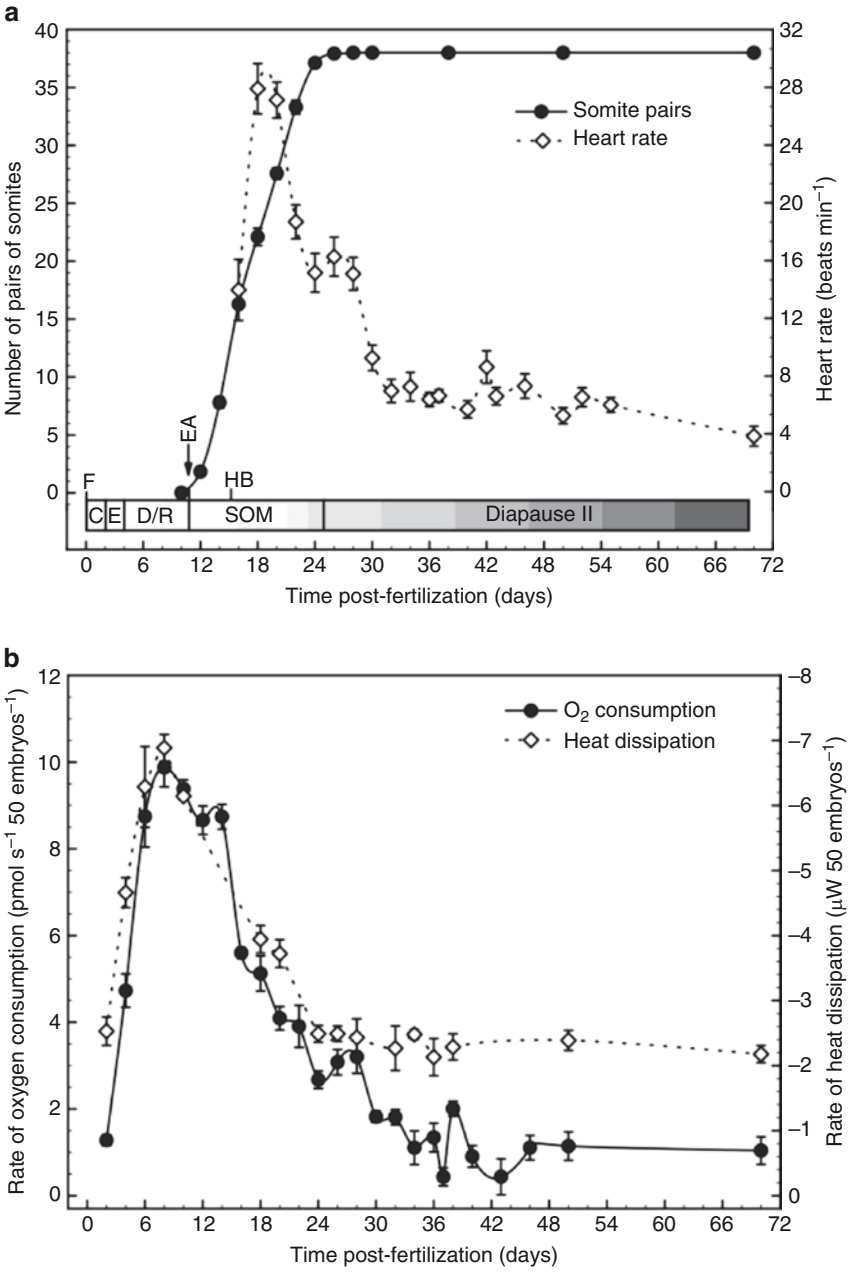

Fig. 6.6 Patterns of development in *Austrofundulus limnaeus*. The number of somites and heart rate both increase during development until *A. limnaeus* enters diapause II (**a**). Associated with entry into diapause II is a reduction in metabolic rate as estimated using both heat dissipation and O_2 consumption rates (**b**)

values observed at 8-day postfertilization. Clearly, the major component contributing to the observed metabolic rate suppression during diapause is a complete suspension of the metabolically costly accretion of structure. At the cellular level, protein synthesis rates are substantially depressed in whole embryoes during diapause II in *A. limnaeus* compared with embryos measured before entering diapause II (Podrabsky and Hand 2000). In prediapausing II embryos (8-day postfertilization), protein synthesis accounts for 36% of ATP turnover (assessed as the cycloheximide-induced reductions in O_2 consumption and heat dissipation). The contribution of protein synthesis to ATP turnover during diapause II is negligible with a 93% decrease in radiolabelled amino acid incorporation into proteins compared with those measured in embryos before entering diapause II.

A depression of metabolic rate during diapause II should mean that embryos of *A. limnaeus* in diapause should be capable of withstanding additional environmental and metabolic insults. Indeed, Podrabsky et al. (2007) demonstrated that embryos displayed anoxia tolerance early in development and a peak was observed during diapause II. Anoxia tolerance was then lost following 4 days postdiapause II. The lethal time to 50% mortality (LT_{50}) during anoxia exposure increased from ~1 day at 1 and 2 dpf to ~20 days at 4 dpf, peaking at 60 days at 32 dpf (during diapause II) (Podrabsky et al. 2007). Anoxia exposure was associated with an activation of anaerobic metabolism and an accumulation of lactate, alanine, and succinate. The results of this study suggest that the suite of characters associated with normal development and entry into diapause II in *A. limnaeus* prepares the embryos for long-term survival in anoxia even while the embryos are exposed to aerobic conditions. High anoxia tolerance was associated with an arrest of heart contractility during the first 24 h of anoxia exposure. At diapause II, there is an almost complete arrest of heart rate during anoxia exposure, which is not achieved to the same degree at any day postdiapause II (Fergusson-Kolmes and Podrabsky 2007).

Not only can diapausing embryos of *A. limnaeus* survive periods of anoxia exposure, these embryos are also able to survive desiccating conditions by reducing evaporative water loss. During diapause II, a small amount of water loss occurs from the perivitelline space even at high relative humidity, but after this initial dehydration evaporative water loss is reduced to near zero (Podrabsky et al. 2001). Dehyration causes a 36% increases in β-sheet contacts in the egg envelope, which are similar to amyloid fibrils associated with several human diseases (Podrabsky et al. 2001). In addition, Machado and Podrabsky (2007) showed that diaupausing embryos of *A. limneaus* defend their internal osmolality against hypersaline conditions (50 ppt) by maintaining a water permeability that is an order of magnitude lower than other teleost fish embryos. Ion-motive ATPases are not involved in this impressive salt/dehydration tolerance (Machado and Podrabsky 2007).

Although metabolic rate suppression appears to occur during diapuase II in *A. limnaeus*, Podrabsky and Hand (2000) pointed out that not all species that display diapause undergo metabolic rate suppression. Indeed, in the California grunion (*L. tenuis*) and some amphibian embryos, there is no metabolic rate depression associated with diapause. Adult Grunion lays their eggs in the sand at extreme high

tides and the eggs are emerged for one full tidal cycle when they are resubmerged and then hatch and proceed with development. These animals enter a state termed "delayed hatching," which is not associated with metabolic rate suppression.

6.7 Summary

The ability to modulate metabolic rate during exposure to environmental stress is an important component to enhance survival in many species of fish and other vertebrates and invertebrates. As outlined above, reductions in metabolic rate can be achieved through behavioral, physiological, and biochemical modifications that reduce overall metabolic demands. The heavy emphasis over the past several decades on the cellular and biochemical components of metabolic rate suppression has possibly overshadowed some of the more obvious and beneficial strategies for reducing whole-animal metabolic demands. Alterations to behavior and physiological adjustments related to changes in organ system function can elicit large decreases in energy consumption and are clearly important for enhancing survival of environmental stress.

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Chapter 7
Energy and Water in Aestivating Amphibians

José E. Carvalho, Carlos A. Navas, and Isabel C. Pereira

Contents

Abstract The physiological mechanisms, behavioral adjustments, and ecological associations that allow animal species to live in extreme environments have evoked the attention of many zoologists. Often, extreme environments are defined as those believed to be limiting to life in terms of water, energetic availability, and temperature. These three elements seem extreme in a number of arid and semi-arid

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settings that even so have been colonized by amphibians. Because this taxon is usually seen as the quintessential water-dependent ectotherm tetrapods, their presence in a number of semi-arid environments poses a number of intriguing questions regarding microhabitat choice and physiological plasticity, particularly regarding the ecological and physiological correlates of behaviors granting avoidance of the harshest conditions of semi-arid environments. Such avoidance states, generally associated to the concept of aestivation, are currently seen as a diverse and complex phenomena varying from species to species and involving numerous behavioral and metabolic adjustments that enhance survival during the drought. This chapter reviews the physiological ecology of anuran aestivation, mainly from the perspective of water and energy balance.

7.1 Introduction

Questions regarding energy and water balance in extreme environments such as those characterized by limited availability of water have fascinated zoologists (Lillywhite and Navas 2006). The presence of amphibians in such habitats is particularly intriguing, for it challenges broad generalizations about the ecology of this taxa, as noted by the pioneering studies of semi-arid anurans. An understanding of how amphibians live in environments with seasonal and often unpredictable rains began in the 1960s, when the spade-foot toad *Scaphiopus couchii*, a species from the California desert, became an important research model. As pointed out by Mayhew (1965), spade-foot toads colonized environments that appear to be "*entirely too hot and dry for any species of amphibian to survive successfully.*" Mayhew's amazement was well supported by environmental data, as the California locality of Glamis, which he studied, exhibits scant rain and long sequences of days with air temperatures above 40°C, and occasional high peaks upto 50°C. Mayhew (1965), as well as other authors, considered the seasonal nature of rains as one of the most important factors in the evolution of the anuran fauna in arid locations (Main et al. 1958), and aestivation emerged as a main concept in this context. Pioneering authors from the 1950s and 1960s stood out for their deep knowledge of the natural history of their study animals, which favored an integrative approach to the problem. They stated that morphological, behavioral, and physiological specialization would be expected in amphibians from the semi-arid areas and that associated physiological challenges would involve not only physiological tolerances to physical conditions, but also complex ecophysiological scenarios in which life cycles have to be completed in the context of ephemeral rains (Bentley et al. 1958; Chew 1961; Bentley 1966; McClanahan 1967). Indeed, these amphibians need to synchronize emergence and reproduction to unpredictable rain patterns and be able to complete metamorphosis in transient bodies of water (Bentley 1966) and to maintain energy and water balance during aestivation.

Later contributions lead to an improved understanding of the metabolic adjustments during aestivating and this greater understanding arrived in association with

general research on metabolic depression as a strategy to enhance tolerance to extreme environmental conditions. Accordingly, once a consistent body of literature was available, a scenario emerged in which aestivation, despite ecological differences with other states requiring downregulation of metabolism, shared some of the molecular and biochemical mechanisms of metabolic control discovered in animals that were tolerant to freezing and anoxia (Storey 2002; Boutilier and St-Pierre 2002; Tattersall and Ultsch 2008). Thus, aestivation is normally perceived as involving a special case of metabolic depression that sometimes may occur under unfavorable hydric or thermal conditions (see Withers 1993 as an example). However, given the complexity of amphibian aestivation, a full understanding of this phenomenon requires an integrative approach and studies at both organismal and mechanistic levels of organization. Such an approach must keep in mind also that ecology and behavior may modulate the extent of the physiological challenge actually experienced by aestivating individuals.

This chapter reviews the physiological ecology of amphibian aestivation, mainly from the points of view of water and energy balance in anurans. This chapter does not cover in detail other relevant aspects of amphibian aestivation that are discussed in other chapters of these book, including: (1) the preservation of muscular tissue (Chap. 8), a problem originally tackled by McClanahan who asked whether the muscle of aestivating spade-foot toads, which are exposed to increasingly higher concentrations of urea during aestivation, were tolerant to hypertonic urea solutions (McClanahan 1964); (2) metabolic regulation of gene expression and oxidative stress (Chaps. 2 and 3), which poses a problem to aestivating amphibians that must rapidly increase metabolic rates before emergence, thus increasing the production of reactive oxygen species (Grundy and Storey 1998); and the modulation of organ function during aestivation and emergence, particularly in the context of function of the digestive system (Chap. 9).

7.2 Amphibian Aestivation as an Ecological State

Aestivation appears to be physiologically diverse across amphibian taxa and such diversity is evident in the various terms used by authors when referring to aestivating anurans. Examples include McClanahan (1967), who refers to dry-season spade-foot toads *S. couchii* as overwintering or hibernating; aestivating is preferred by Bentley (1966) when citing McClanahan's earlier papers or when referring to lung-fish dormancy; and van Beurden (1980) uses the term dormant. However, the problem is not semantic. True differences in the physiological state of aestivating amphibians have been evident since the 1960s, even if not necessarily explicit in the literature published at that time. The first paradigm about aestivating ectothermic vertebrates was likely derived from lung-fish, which requires a few hours to recuperate from disturbances in the middle of aestivation, and to remain "*perfectly quiet and probably entirely devoid of muscular tone.*" (Smith 1930). In contrast, spade-foot toads aestivating in laboratory settings are responsive to stimuli such as noise,

excavate further if disturbed, and require less than 30 min to recuperate after sudden disruption of aestivation (McClanahan 1967). The authors of this chapter have observed *Pleurodema diplolistris* readily jump away when dig out in the middle of the dry season from aestivating places located at more than 1 m depth in the sand. So, the state described by Smith (1930) for aestivating fish better resembles what was later described for the Australian anuran genera *Cyclorana* (*Litoria, see* Frost et al. 2006), *Limnodynastes*, and *Neobatracus*, which also exhibit cocoons (Lee and Mecer 1967). Aestivating salamanders (*Siren intermedia*) also contrasts with the rather responsive state that characterizes *Scaphiopus* and *Pleurodema*, and their righting response time, which is about 2 min at week 2 of aestivation, increases to more than 40 min at week 12 (Gehlbach et al. 1973). Of course, temperature may play a partial role in explaining this diversity, even more given that amphibian aestivation may occur under rather cold conditions. However, the observed variation is likely to involve true differences in physiological state, especially, given the diverse strategies for survival that have been described for anuran communities in semi-arid regions across the world (Pinder et al. 1992; McClanahan et al. 1994; Abe 1995; Navas et al. 2004). Thus, the use of one single term, aestivation, seems insufficient to characterize the apparent contrasting physiological conditions of dry-season anurans in semi-arid settings. Consequently, through this chapter, we use the adjective aestivating as defining an ecological state that does not anticipate any specific physiological condition.

7.3 Physiological Challenges of Amphibian Aestivation

7.3.1 Temperature, Water and Energy in Aestivating Amphibians

The maintenance of body temperatures compatible with life was one of the first physiological challenges attributed to anurans in the semi-arid (Mayhew 1965; Bentley 1966). Attention to temperature was a logical step because the database of critical thermal maximum for amphibians available in the 1960s included mostly values below 35°C (Hutchison 1961; Heatwole et al. 1965), and such critical temperatures were ecologically realistic in semi-arid settings. Indeed, we know now that some species of anurans from semi-arid areas exhibit in the field and tolerate temperatures higher than 35°C. One example is the toad *Rhinella granulosa* (previously *Bufo granulosus*, see Frost et al. 2006) from the Brazilian semi-arid bioma called the Caatinga (Navas et al. 2007). Juveniles of this species, which are diurnal, tolerate body temperatures higher than 40°C, and this tolerance appears to have basis on biochemical adjustments that preserve muscle function at high temperatures (Navas et al. 2007). Adults of this species, in contrast, are mainly nocturnal and tend to be associated to more humid areas, and thus are exposed to more moderate field temperatures (Navas et al. 2004).

Water availability may also be critical for amphibians in the semi-arid, particularly during the dry season (Mayhew 1965; Bentley 1966; McClanahan 1967), and even

more where rain patterns are unpredictable and soils do not retain water (see Seymour 1973a). Surely, the unpredictability of the rainy season figures among the earlier factors suspected to exclude many anuran species from the driest regions within semi-arid habitats (Bragg 1945). In addition, the breeding period is considered to be the most energetically expensive period in the life cycle of amphibians, and aestivating frogs face similar energetic challenges to those experienced by other anurans, even if reproduction on a given year is uncertain. Before the rains arrive, females need to have viable eggs in a state of advanced development and males need to have proper energy stores to support the muscle function required to call intensely to attract females (see Wells 2001 and Navas et al. 2008). If rains do not arrive, reproduction must be interrupted and energy sources, normally plentiful in the rainy season, will not be available. For all these reasons, energy expenditure during aestivation needs to be sharply reduced, so that energy supplies may last over dry months that usually include one season, but from time-to-time may encompass more than 1 year.

7.3.2 *Microhabitat as a Factor Affecting Physiological Challenges During Aestivation*

Aestivation in amphibians has been traditionally associated with life underground for prolonged periods while drought persists. As evidenced by the fossil record, this behavioral strategy probably evolved early in the phylogenetic history of several groups, including amphibians (Hembree et al. 2004; Nomura et al. 2009; see also Chap. 12). However, underground aestivation may vary considerably according to the natural history of different amphibian taxa. The integrative approach to comparative physiology that was characteristic of the 1960s revealed that microhabitat selection could greatly reduce the physiological challenge of buried frogs. *Scaphiopus couchii*, for example, does not aestivate in random locations, but bury themselves in less insolated localities near patches of vegetation where temperatures tend to be more moderate, and humidity lasts longer (Mayhew 1965). This species is also selective at a landscape scale, being more common in grassland than in scrub areas, and does not occur in the chaparral (Ruibal et al. 1969). Microhabitat selection has also been verified in the burrowing behavior of *Cyclorana alboguttata*, which prefers sandy soils in laboratory experiments (Booth 2006) and in the terrestrial roaming of *Rana capito* (Blihovde 2006). However, some anuran species aestivate in microhabitats that do not decrease physiological challenge, or even that appear to increase it. A dramatic example is the reed frog *Hyperolius viridiflavus*, a species in which froglets aestivate exposed to sunshine on leaves, with no apparent protection against dehydration, high temperatures, or intense solar radiation (Kobelt and Linsenmair 1995). Finally, a number of subtle differences in the microhabitat of aestivating anurans remain to be fully interpreted in terms of eco-physiological implications. Dr. Carlos Jared (from Instituto Butantan, Brazil) pointed out to the authors during field work in North-Eastern Brazil that both *Pleurodema diplolistris* and *Proceratophrys cristiceps* buried in the sand during aestivation are surrounded by small cavities (Fig. 7.1),

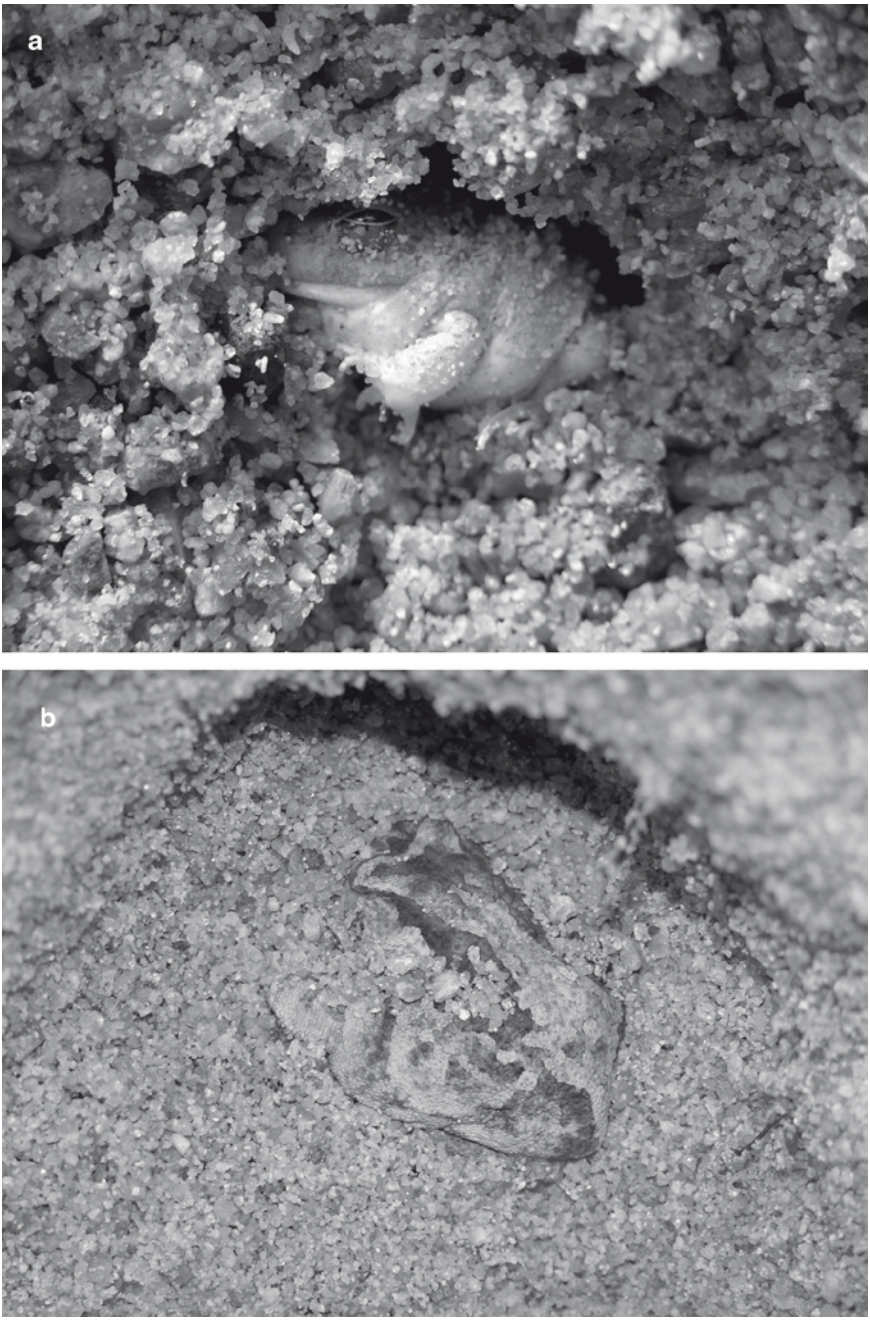

Fig. 7.1 (**a**) *Pleurodema diplolistris* (Leiuperidae) and (**b**) *Proceratophrys cristiceps* (Cycloramphidae) during aestivation in their shelters at about 0.5–1 m depth. Note the air-filled chamber above de dorsal part of the frog (Photograph by JE Carvalho)

so that only the ventral skin is in direct contact with the sand (authors, personal observation). Other anurans such as *C. alboguttata* also form a small air-filled chamber when buried, presumably by compacting chamber walls with their feet (Booth 2006). According to Booth (2006), this air-filled space could reduce the area of contact between the skin frog surface and the soil preventing osmotic loss of liquid water (see also Sect. 4.5). However, other aestivating anurans such as *Scaphiopus hammondii* are completely surrounded by sandy soil (Ruibal et al. 1969) and the above cited species from Brazil appear to aestivate under very favorable water conditions (relative humidity >70%; authors, unpublished data).

Most anurans appear to aestivate in either sandy soils or drying mud and these two substrates may impose different physiological challenges for aestivating species, particularly regarding oxygen and water availability. Buried spade-foot toads (*S. couchii*), as well as several other anurans that aestivate in sandy soils (authors, personal observations), do not leave any opening that may ventilate aestivation microhabitats. These anurans, therefore, should meet all oxygen requirements via diffusion throughout soil (Seymour 1973b). Sandy microhabitats for aestivating *S. couchii*, for example, exhibit oxygen concentrations near 20%, even at 70 cm depth (Seymour 1973b). In the Brazilian Caatinga, the oxygen content in sandy microhabitats ranges from 20.9% at 25 cm to 20.7% at 150 cm (authors, unpublished data, Fig. 7.2). This is comparable to other sandy environments, for example, those used by green sea turtles for reproduction, which also exhibit rather high oxygen concentration (Prange and Ackerman 1974). Although a more accurate approach to this issue would require analyses of oxygen diffusivity related to the metabolic rates of aestivating frogs, the general picture indicates that sandy microhabitats, as suggested by Seymour (1973b), are unlikely to be oxygen limited. Conversely, drying mud microhabitats are likely to be less permeable to oxygen (even more in fully cocooned frogs), but have not been studied in this context.

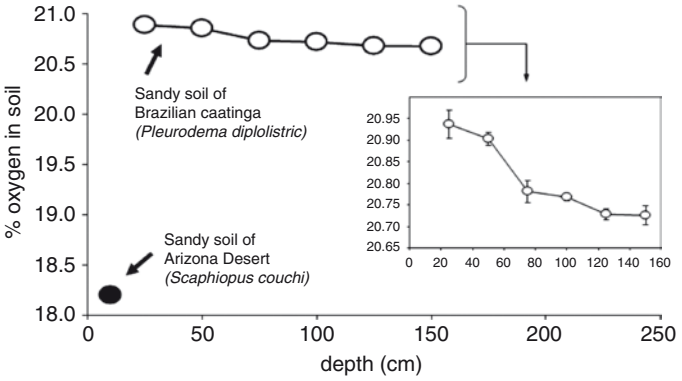

Fig. 7.2 Oxygen content (% of dry air) in different depths of soil adjacent to buried *Pleurodema diplolistris* (from Brazilian Caatinga) and *Scaphiopus couchii* (from Arizona desert; according to Seymour 1973b). The insight shows the significant decrease in O_2 content with higher depths of sandy soil of Brazilian Caatinga (authors, unpublished results)

Regarding soil water potential, Ruibal et al. (1969) performed a detailed study of the implications of microhabitat selection on aestivating spade-foot toads and analyzed the influence of sand granular structure on the ability of toads to take up water. In a series of elegant comparisons, these authors demonstrated that the availability of water to toads does not depend only on percental humidity content of the soil, but on soil moisture tension. Thus, some soils could be dehydrating despite their total water content, and spade-foot toads could extract water from soil as far as water was held at less than 446 mOsm (Ruibal et al. 1969). A related main difference in the microhabitat characteristics of aestivating frogs was noticed by McClanahan et al. (1976) when comparing general aestivation strategies of spade-foot toads in the Arizona desert with those of ceratophryd frogs from Argentina, which form a thick cocoon. The former aestivate in sand at depths usually deeper than 50 cm and the latter in muddy environments and at shallower depths. This mud hardens later in the season, leading to presumably modest water potentials (McClanahan et al. 1976). So, sandy and muddy microhabitat may offer very different conditions of oxygen and water availability for aestivating anurans. These possible differences may constitute important driving forces favoring different physiological states during aestivation among anuran species and require much additional attention.

7.3.3 Behavior, Physiological Challenge, and Reproduction

Behavioral traits may reduce physiological challenge during the underground phase of aestivating anurans. Burrowing, a most typical behavior, reduces exposure to surface conditions that may be hot and dry in semi-arid environments, but comes at a cost for it is paralleled by physiological challenges that may include water balance, energy management, and gas exchange (Pinder et al. 1992; Withers and Thompson 2000). Water may be a limited resource for amphibians aestivating in some settings and cocoon building is likely related to hydric stress (see Sect. 4.4). Energy management is essential because underground aestivation requires appropriate timing of reproduction and because series of dry years may constitute a formidable energetic challenge (see Sect. 3.1). Finally, and as discussed in the previous section, oxygen may be a limiting factor only in some environments. If microhabitats for aestivation differ among taxa and environments, so would differ the magnitude and type of physiological challenges and the expected behavioral and physiological traits that may circumvent them. So, it seems extremely difficult to hypothesize on physiological adaptation of aestivating amphibians without proper understanding of their natural history and ecology.

Aestivation as an ecological strategy to exploit semi-arid environments does require special physiological traits also in anuran larvae, which necessitate rapid development and metamorphosis given that ephemeral water sources may not last long (Main 1968; Pinder et al. 1992). Adaptive larval physiology, however, would be worthless if reproduction does not occur at the proper time of year, so that water

resources are optimized. This scenario would suggest selective advantages for aestivating amphibians able to use environmental cues to fine-tune life cycles and development strategies. Temperature and photoperiod cycles have been suggested as possibly important factors (Seymour 1973a; Pinder et al. 1992; Tracy et al. 2007), whereas the most intuitive clue, rain, is of dubious value. Anurans from semi-arid settings may be active and closer to the surface even before the first rains arrive, as illustrated by *S. couchii,* which move up the soil, and even feed, before the first heavy rains (Bragg 1965; Ruibal et al. 1969). However, they fully emerge for reproduction only when rains begin and water pools are available (Bragg 1965; Ruibal et al. 1969; McClanahan 1972). Because aestivation in many amphibians occurs under unpredictable conditions, it is very possible that endogenous rhythms combined with environmental cues are important. Studies on intrinsic aestivation rhythms have been neglected (this statement by Pinder et al. 1992 remains valid today), even if endogenous rhythms driving seasonal patterns of metabolic depression are evident in many terrestrial hibernators. Examples include mammals (Boyer and Barnes 1999), birds (Wang and Wollowyk 1988), lizards (Rismiller and Heldmaier 1988; Souza et al. 2004), and anurans (Pinder et al. 1992; Bícego-Nahas et al. 2001; Tracy et al 2007).

7.3.4 Aestivation, Development, and Life History

It is worth noting that in some species alternate life history strategies may be possible to take advantage of the short rainy season. An excellent example was presented by Lampert and Linsenmair (2002), who indicated that male and female West African frogs (*Hyperolius nitidulus*) born on a given rainy season may exhibit growth rates fast enough to enable reproduction in the same rainy season. This life-history strategy diverges from the typical one-generation-per-year alternative, and might contribute to the extension of the geographical range of this species on unpredictable environments (Lampert and Linsenmair 2002). The work conducted by Shalan et al. (2004) with *Neobatrachus surtor* corroborates related ideas, because elevated levels of plasma testosterone after aestivation correlate with renewal rate of reproductive cells, a process that is essential to prepare frogs for breeding.

7.4 Morpho-Physiological Solutions

7.4.1 Metabolic Depression as a Hallmark Strategy

Some early contributions already suggested that reduced metabolism in aestivating lungfish could be ecologically relevant in terms of energy savings (Smith 1930). Smith (1930) explained this pattern in terms of emaciation, not metabolic regulation, an understandable misconception given the status of physiological sciences at

that time. However, seminal later contributions relating metabolic rates to body mass (Kleiber 1947; Zeuthen 1953) boosted the field of animal energetics and favored a view of metabolic rate as a variable susceptible of regulation. Davison, for example, noticed that in four anuran species respiratory rates and frequency of buccopharyngeal movements relate to body mass, and match predictions of higher relative values for smaller individuals (Davison 1955). Studies such as Davison's attempted to verify whether interspecific patterns such as Kleiber's applied also for ontogenetic variation, and were relevant in pointing out that metabolic rates vary significantly during ontogeny. The idea that metabolic rates could be modulated stimulated the investigation of seasonal patterns as well. An excellent early review by Fromm and Johnson (1955) unambiguously supports the idea, contemptuous at the date, that true seasonal changes in metabolic rates with lower winter values, are a characteristic of anurans (Fromm and Johnson 1955). In the 1970s, students studying amphibian aestivation were fully aware that these animals could not survive prolonged periods of inactivity on stored reserves unless they reduced significantly their energy expenses and by this decade the scientific community had a clear idea that vertebrates could downregulate metabolism during dormant states, even if the mechanisms involved were not completely understood (Guppy and Withers 1999; but see also Guppy 2004).

Metabolic depression occurs in a variety of Australian species that aestivate and may be a quite general phenomenon in aestivating anurans (Withers 1993) and urodeles, even if the type and magnitude of such depression vary among species. McClanahan (1967) discussed the energetic implications of reduced metabolism for aestivating spade-foot toads and estimated that given the rather cool temperatures of the overwintering sites chosen by *S. couchii* (about 15°C), buried toads would have just about enough fat to survive over 10 months. Safety margins proved to be greater (van Beurden 1980) because McClanahan's calculations were based on resting metabolic rates for he could not circumvent technical problems associated with measuring this variable in aestivating toads. The first measures of metabolic rates in undisturbed dormant toads involved *S. couchii* and *S. hammondii*, which exhibit values near 20% of those measured in resting toads (Seymour 1973a). Seymour (1973b) stated that, given the temporal pattern and scale of reduction in metabolic rate during dormancy, the phenomenon could not be explained in terms of starvation or inactivity. Similarly, the process could not be analogous to the rapid changes observed previously for amphibians exposed to low oxygen tension. Instead, he proposed that metabolic rates were regulated hormonally, perhaps through thyroid activity (Seymour 1973a). The idea of metabolic downregulation in aestivating anurans was soon extended to *Cyclorana* and *Neobatrachus* (Seymour 1973a; van Beurden 1980; Withers 1993; Withers and Thompson 2000), and to the salamander *S. intermedia*, which exhibits a gradual decrease in oxygen consumption during aestivation (Gehlbach et al. 1973).

The ecological significance of metabolic depression during amphibian aestivation seems undisputable and the energetic model developed by van Beurden (1980), based on measures of metabolic rates and body composition, shows that some frogs, given their energetic reserves, could remain in a dormant state during several years. In addition, many seasonal animals still possess considerable amounts of fat in

abdominal bodies after a season of inactivity (Pinder et al. 1992). A careful analysis on *S. couchii* estimated that aestivating toads use about 36% of the lipid and 28% of the total energy reserves available at the start of dormancy, whereas the congeneric *S. multiplicatus* uses 62 and 51%, respectively (Jones 1980). So, providing energy to maintain frogs alive during one, or even more seasons of dormancy, seems a solvable problem given the extent of metabolic depression (see Sect. 4.3) and the magnitude of energetic reserves that can be accumulated during the rainy season.

7.4.2 Mechanisms Underlying Metabolic Depression

An initial understanding of mechanisms allowing for reduced energy fluxes in dormant animals was possible in the 1980s (see Hochachka and Guppy 1987), but initially associated to hibernation. Aestivating amphibians were intriguing because expected hypometabolic states could occur at the warmest time of year. When this is the case, the process of metabolic depression has to compensate a presumed tendency to increase metabolism as a consequence of higher body temperatures (Abe 1995; Glass et al. 1997). Currently, it is clear that metabolic depression during aestivation is paralleled by temperature-independent downregulation of many physiological functions (see an example regarding cardiac output in *Bufo paracnemis* in Glass et al. 1997).

Another stimulating aspect of anuran aestivation dealt with oxygen availability (as discussed in Sect. 3.2). The best understood mechanism of metabolic arrest was linked to anoxia in aquatic environments. For example, in amphibians that hibernate during winter months under the frozen water surface, a decrease in the resting rates of mitochondrial respiration occurs through considerable reorganization of cellular function in hypoxic conditions (St-Pierre and Boutilier 2001; Boutilier and St-Pierre 2002). However, terrestrial amphibians may achieve significant metabolic depression under apparently normoxic conditions. In the toad *S. couchii*, a broad process of metabolic reorganization takes place during aestivation and affects several tissues, as indicated by changes in maximal activity of intermediary metabolism enzymes (Cowan et al. 2000). Flanigan et al. (1990) proposed that some anurans would undergo nonhypoxic metabolic depression and that such process would require downregulation of both glycolytic and aerobic energy pathways. Such metabolic depression should be intrinsic to tissue and not directly mediated by external factors, not even hormonal action. This hypothesis was corroborated by the pronounced depression of metabolism in the isolated muscle of aestivating frogs *Neobatrachus pelobatoides* that prevailed several hours after tissue collection (Flanigan et al. 1991). The idea that amphibian metabolic depression is intrinsic to tissue was further supported by a parallel reduction in protein synthesis in isolated liver (Fuery et al. 1998). In fact, downregulation of protein synthesis in aestivating anurans seems coordinated by suppressing of transcription and translation, as well as of signal transducing in the cells (Cowan et al. 2000; Pakay et al. 2003; Storey and Storey 2004).

More recently, a number of mechanisms associated with metabolic depression have been identified, and some are relevant for amphibians (see Chap. 2 and Hochachka and Guppy 1987). One key aspect of the regulation of metabolic rate is reversible protein phosphorylation, particularly in the context of carbohydrate catabolic pathways such as glycolysis (Storey 1990). The higher presence of dephosphorylated forms of the glycolytic enzymes pyruvate kinase and phosphofructokinase in skeletal muscles of *S. couchii* during aestivation suggest a depression in the glycolytic pathway (Cowan and Storey 1999). In turn, reduced activity of glycolytic enzymes is apparently paired with a coordinated suppression of other metabolic processes, such that the whole regulation system keeps an overall balance between the rates of ATP generation and consumption (Cowan and Storey 1999; Storey and Storey 2004). However, not all process are downregulated, as pointed out by studies on enzymes related to urea synthesis (urea accumulation enhances the osmotic potential of aestivating amphibians, see Sect. 4.5). In aestivating *S. couchii*, urea levels increases 14-fold, presumably via upregulation of urea-synthesizing enzymes in the liver (Grundy and Storey 1998; Storey et al. 1999). One specific example is the liver carbamyl phosphate synthetase, which is twice as active in dormant than in active *S. couchii* (Jones 1980).

Reversible protein phosphorylation is also important in the context of membrane ion channels, especially Na^+–K^+ATPase, which contributes for most of the energy cycling during normal life. In mammalian skeletal muscle, Na^+–K^+ ATPase and Ca^{2+}ATPase is strongly reduced during hibernation as a result of protein phosphorylation (Hand and Hardewig 1996; Guppy and Withers 1999; Storey and Storey 2004). Similarly, in aquatic amphibians, the activity of the skeletal muscle Na^+–K^+ATPase decreases by 30% (normoxic) to 50% (hypoxic) after 2 months of hibernation, and is accompanied by reduction in the flux of Na^+ and K^+ across the sarcolemma (Donohoe et al. 2000; Tattersall and Ultsch 2008). This is an underexplored aspect of physiology in terrestrial amphibians and the limited data available are controversial. For example, Flanigan and Guppy (1997) reported that the total activity of Na^+–K^+ATPase isolated from muscle, ventricle, kidney, and brain of the frog *Neobatrachus kunapalari* remains constant during aestivation, suggesting that this protein is somehow well stabilized even during metabolic depression (see also Guppy and Withers 1999).

The mechanism responsible for modulating neural channels and receptors in aestivating amphibians may also involve reversible protein phosphorylation. This speculation is rooted in the findings on fish (Bickler et al. 2001) and reptiles (Lutz and Nilsson 1997a, 1997b) and would be particularly relevant for the most inactive species, such as those forming cocoons (see Sect. 4.4). In addition, if anurans that aestivate in dry mud soils face some level of hypoxia (see Sect. 3.2), low oxygen tension would require cardiovascular changes to secure the supply of oxygenated blood to the brain (Pinder et al. 1992). Under such conditions, local tissue adjustments produced in response to shifts in energy balance should be consistent with the local need for metabolic activity, which may be highly depressed. For example, during anoxic hypometabolism induced in freshwater turtles, the increase in blood flow to the brain is controlled by locally produced adenosine, while ion channel activity is arrested and neural GABA action is upregulated (Lutz and Nilsson 1997a, 1997b).

The net result is an active decrease in the synaptic transmission and overall suppression of neural activity.

7.4.3 *Energy Substrate Cycling during Aestivation*

In aestivating amphibians, metabolic rates usually reach less than 30% of the resting values, but the magnitude of metabolic depression differs considerably among species (see Sect. 4.1 and Table 1). Interestingly, many aestivating anurans exhibit resting metabolic rates during the active season that are comparatively lower than those of nonaestivating species (Withers 1993; Withers and Thompson 2000). This pattern would suggest that aestivating anurans indeed live on the slow lane from the metabolic point of view, and that reduced energy expenditure influence patterns of energetic substrate utilization throughout their full life cycles (Storey 2002). Most authors accept that lipids stored in liver, abdominal fat bodies, and muscles are the main energetic substrate utilized to fuel metabolism for aestivating anurans (see review in Pinder et al. 1992). However, carbohydrates are also important because brain and kidneys are more dependent on glucose to support basal activity, even at slow rates (Hochachka and Guppy 1987; see also Donohoe et al. 1998). So, it is not surprising that vertebrate ectotherms that exhibit seasonal metabolic depression accumulate large amounts of carbohydrates in the neural glia cells (Hochachka and Somero 1984; Lutz and Nilsson 1997a; Partata and Marques 1994; Souza et al. 2004). If hypoxia is an issue (see Sect. 4.2), the oxygen-independent pathways and carbohydrates stores might be crucial to the maintenance of the energetic homeostasis. Proteins are alternative sources of energy for some amphibians during starvation that occurs in the inactive period, but even in such cases, proteins remain unimportant in comparison with other energetic substrates (van Beurden 1980). After 9–10 months of aestivation, adults of *S. couchii* and *S. multiplicatus* use about 17 and 36% of their protein content, respectively, a low percentage when compared with the depletion rate of lipids in the abdominal fat bodies (Jones 1980). Protein catabolism seems more important for urea formation (see Sect. 4.5) than for energy balance.

Energy cycling requires a dramatic shift at arousal, which is a costly energetic event itself. During arousing, almost all physiological functions demand energy at rates comparable to those of pre-dormant periods or even higher and much of the endogenous reserves are depleted during this period. The natural history of species may also generate notable complications; for example, some anuran species eat only after breeding (Wells 2007), and species such as *P. diplolistris* (from Brazilian Caatinga) must perform very intense calling before and during amplexus, as males must beat legs energetically to build foam nests for eggs deposition just after the rains arrive (Fig. 7.3). During arousal, brain metabolism might be supported by glucose, both intracellular and that provided by the liver, as described for anoxic fish (Lutz and Nilsson 1997a), dormant lizards (Souza et al. 2004), turtles (Partata and Marques 1994), and hibernating mammals (Nestler 1991). In addition, the intense oxidation of lipids in the liver may also produce ketone bodies (β-hydroxybutirate and acetoacetate)

Table 7.1 Behavioral and metabolic aspects of aestivating species of amphibians

Family/species	Cocoon	Depth burrowing (cm)	VO_2 activity (ml $O_2 g^{-1} h^{-1}$)	VO_2 resting (ml $O_2 g^{-1} h^{-1}$)	VO_2 aestivating (ml O_2 $g^{-1} h^{-1}$)	Aerobic metabolic arrest (% of depression)	Temp (°C)	Reference
Myobatrachidae								
Arenophryne rotunda	No	80						Tyler et al. 1980; Roberts 1984
Myobatrachus gouldii	No	100						Philipp 1958; Roberts 1981; Withers and Roberts 1993
Uperoleia micromeles	No	100						Paltridge and Southgate 2001
Uperoleia mjobergi	No	122						Slater and Main 1963
Brevicipitidae								
Breviceps adspersus	No	200						Rose 1962
Breviceps eyrei	No	122–152						Poynton 1964
Bufonidae								
Bufo (Anaxyrus) alvarius	No	56–80	0.216	0.034			30	Secor 2005; Seymour 1973b
Ceratophryidae								
Ceratophrys aurita	Yes			0.048	0.033	1.45	25	Bastos and Abe 1998
Ceratophrys ornata	Yes	15	0.405	0.036			30	McClanahan et al. 1976; Secor 2005
Lepidobatrachus llanensis	Yes	Surface		0.097	0.015	6.47	25	McClanahan et al. 1983
Hylidae								
Cyclorana alboguttata	Yes							Booth 2006
Cyclorana australis	Yes	3–13.5	0.158		0.095		24	Tracy et al. 2007; Withers and Thompson 2000
Cyclorana cultripes	Yes	25–36	0.160		0.049		24	Main 1965; Withers and Thompson 2000
Cyclorana maini	Yes		1.494	0.067	0.025	2.66	25	Withers 1993

Cyclorana platycephala	Yes	30	0.000	0.047	0.009	5.40	25	Spencer 1896; Withers 1993
Pternohyla fodiens	Yes							Ruibal and Hillman 1981
Smilisca baudinii	Yes							McDiarmid and Foster 1987
Leiuperidae								
Pleurodema diplolistris	No	20–150	1.756	0.231	0.120	1.93	30	authors, unpublished data
Limnodynastidae								
Heleioporus albopunctatus	No	66–84	0.371	0.040				Bentley et al. 1958; Main et al. 1959; Withers 1993
Heleioporus eyrei	No	10–30						Main 1965
Limnodynastes spenceri	No	30		0.084				Parker 1940; Withers 1993
Neobatrachus aquilonius	Yes	30–126						Carteledge et al. 2006
Neobatrachus centralis	Yes			0.090	0.021	4.39	25	Withers 1993
Neobatrachus fusvus	Yes			0.097	0.033	2.92	25	Withers 1993
Neobatrachus kunapalari	Yes		0.708	0.072	0.016	4.53	25	Withers 1993
Neobatrachus pelobatoites	Yes		0.821	0.130	0.019	6.99	25	Withers 1993
Neobatrachus pictus	Yes							Fenner 2007
Neobatrachus sutor	Yes		1.341	0.063	0.014	4.47	25	Withers 1993
Neobatrachus wilsmorei	Yes			0.081	0.012	6.69	25	Withers 1993
Notaden nichollsi	No	120–240		0.072				Slater and Main 1963; Main 1965; Cartledge et al. 2006
Microhylidae								
Ramanella montana								Krishna et al. 2004
Pyxicephalidae								
Pyxicephalus adpersus	Yes	3–4	0.251	0.037	0.008	4.46	30/25	Loveridge 1976;Secor 2005; Withers 1993
Pyxicephalus sp	Yes	122–152						Poynton 1964
Tomopterna delalandii	No	100–120						Meek 1897

(continued)

Table 7.1 (continued)

Family/species	Cocoon	Depth burrowing (cm)	VO_2 activity (ml $O_2 g^{-1} h^{-1}$)	VO_2 resting (ml $O_2 g^{-1} h^{-1}$)	VO_2 aestivating (ml $O_2 g^{-1} h^{-1}$)	Aerobic metabolic arrest (% of depression)	Temp (°C)	Reference
Rhinophrynidae								
Rhinophrynus dorsalis	No	70–150						Foster and McDiarmid 1983
Scaphiopodidae								
Scaphiopus bombifrons	No	50–80		0.1392			15	Seymour 1973b; Hutchison et al. 1968
Scaphiopus couchii	No	30–90		0.074	0.018	4.18	24	McClanahan 1967; Shoemarker et al. 1969; Seymour 1973a
Scaphiopus hammondii	No	35–90		0.085	0.014	6.23	15.2	Jones 1980; Seymour 1973b
Scaphiopus multiplicatus	No							Jones 1980
Sirenidae (Salamander)								
Pseudobranchus striatus				0.076	0.022	3.45		Etheridge 1990; Gatten et al. 1992

VO_2: oxygen consumption rate expressed as volume of O_2 consumed in time per gram of body mass (in STPD)
Aerobic metabolic depression was calculated based on VO_2 during aestivation in relation to resting

that can be used by brain as alternative sources of acetyl-CoA for Krebs cycle (Hochachka and Somero 1984, 2002; Cowan et al. 2000). The control of use of substrates during arousal in aestivating anurans is still a promising area for investigation.

7.4.4 Cocoons as Morpho-Physiological Strategies

Very early reports already suggest that salamanders experience morpho-physiological changes associated with droughts (Viosca 1924), but a formal approach to this question came later, perhaps, stimulated by findings on the lung-fish *Protopterus*. Mayhew (1965) reported changes in the characteristics of the skin in aestivating frogs and McClanahan, commenting on spade-foot toads induced to aestivate in captivity, stated that a "*Dark, supposedly keratinized skin covered the toads when they were removed from the cylinders, and this material started to build up within 1 month after submergence*" (McClanahan 1967). Later descriptions pointed out to differences among amphibian cocoons. In *Scaphiopus*, the cocoon is directly derived from the *stratum corneum*, whereas that of salamanders was initially interpreted as the product glandular secretion (Reno et al. 1972), although dermal elements were identified later (McClanahan et al. 1976). Overall, the cocoon of *Siren* resembles that of ceratophryd frogs and is different to those of spade-foot toads (McClanahan et al. 1976). *Cyclorana*, *Limnodynastes*, and *Neobatracus* present cocoons that are morphologically similar to the *stratum corneum* of the epidermis, and are not built as the multilayer structure that characterizes *S. couchii* (Lee and Mecer 1967). These cocoons were evident only a few days after the onset of aestivation, and covered all body orifices with the exception of the narins (Withers 1995). As aestivation progressed, the number of layers increased (Ruibal et al. 1969; Withers 1995, 1998; Thompson et al. 2005; Booth 2006; Cartledge et al. 2006, 2008). The cocoons have acid mucopolysaccharides in between layers that seem to work as a cement, possibly decreasing the water permeability (Bayomy et al. 2002). Some frogs may aestivate aboveground, for example, in tree cavities, as deduced by the observation of a seemingly aestivating and cocooned *Smilisca baudinii*, a tree-frog species from Mexico and Central America (McDiarmid and Foster 1987). Cocoons probably evolved mainly in the context of water preservation (Toledo and Jared 1993; Withers 1995; Withers and Thompson 2000), but they may also serve as a physical barrier against parasites or other unwanted hosts that may attack animals when metabolic rate is depressed.

Behavioral and morphological specializations are integrated with burrowing habits and cocoon formation in terrestrial anurans. Changes in the digging behavior of anurans have been associated with burrowing speed and efficiency in different soils (Emerson 1976; but see also Nomura et al. 2009), whereas ability to build cocoons is given by the dermal and epidermal cellular activity as described in the previous section. No studies have quantified the energetic cost of burrowing or cocooning in aestivating anurans, probably because of obvious methodological difficulties, but it is possible that energy investment on cocoon formation is

modest (Wells 2007). For example, different species of *Cyclorana* from Australia vary in the magnitude of metabolic depression during aestivation; however, species from subhumid to arid environments have similar cocoons, so that cocooning strategy may be a conserved trait (Withers 1995, 1998; Withers and Thompson 2000).

As a final comment, a lipid skin barrier reduces evaporative water loss in genera such as *Phyllomedusa*, *Chiromantis*, *Hyperolius,* and *Litoria* (*Cyclorana,* see Frost et al. 2006) (Withers et al. 1982; Jørgensen 1997; Withers et al. 2005; Young et al. 2005), but in genera such as *Phyllomedusa*, this trait is also present in species from mesic settings (Lillywhite 2006). *Litoria alboguttata* spends most of their life in a dormant state, usually with cocoon formation (Withers and Thompson 2000). However, little is known about how tree-frogs from semi-arid settings aestivate and about the possible importance of skin lipid production for anuran aestivation.

7.4.5 Water Balance

7.4.5.1 Urea

Some initial studies focused on tolerance to desiccation as a variable to study possible adaptation of anurans to semi-arid conditions. This variable proved to be quite similar across Australian anuran species from mesic and semi-arid areas (Bentley et al. 1958). Another variable, rate of water uptake, lead to different results, as anurans from semi-arid environments could have enhanced ability to obtain water from the soil. For example, within the genus *Neobatrachus*, which occupies areas of different aridity in Australia, species from the driest regions take up water more rapidly in comparison with those of less extreme habitats (Bentley et al. 1958). Such relationships were not evident in *Heleioporus* (Bentley et al. 1958), but physiological diversity was anyhow evident. It follows that, if water uptake is a factor in the evolution of semi-arid anurans, enhanced ability to extract water from the soil would be advantageous during the dry season. This line of reasoning led to the supposition that, during the dry season, some anurans could increase the concentration of plasma solutes beyond expectations from simple dehydration (Ruibal et al. 1969; Shoemaker et al. 1969). This physiological process would cause the lowering of water potential, and therefore could increase their ability to take up water from soils with low matric water potentials (McClanahan 1964; McClanahan 1972; Booth 2006). This ability was noted in a number of unrelated species of semi-arid anurans, not only in North America and Australia, but also in South America (Abe and Garcia 1990; Abe 1995) and Africa (Schmuck and Linsenmair 1997). The whole picture suggests convergent evolution and favors the hypothesis that accumulation of urea via upregulation of the synthesis pathway (see Sect. 4.2) enhances transfer of water from the soil to the animal (Pinder et al. 1992; Abe 1995; Schmuck and Linsenmair 1997). The spade-foot toad *S. couchii* can increase plasma urea up to 200–300 mM, as to account for a considerable part of the high plasma concentration

(as high as 600 mOsm l^{-1}) exhibited by emerging toads (McClanahan 1967; Cowan and Storey 2002). In the cocoon-forming species *Cyclorana platycephala*, plasma osmolality reaches maximal levels by the 9th month of aestivation at 487 mOsm kg^{-1}, whereas urine osmolality continues to increase till the 15th month of aestivation (Cartledge et al. 2008). At that late state of aestivation, plasma and urine concentrations become isosmotic (Cartledge et al. 2008).

The accumulation of urea in the plasma of aestivating anurans seems clearly advantageous in terms of water balance, but it could be toxic at high levels by altering protein function. So, tolerance to urea or counteracting of possibly injurious effects would be expected in aestivating amphibians that accumulate urea. Counteracting intracellular organic solutes, such as methylamines, may accumulate intracellularly and counteract possible effects of urea, as observed in elasmobranches (Withers and Guppy 1996; Muir et al. 2007). Reed frogs (*Hyperolius*) accumulate free aminoacids in the gastrocnemious muscle, a process that may protect against osmotic stress (Schmuck and Linsenmair 1997). However, reduced sensitivity to urea accumulation more than counteracting solutes may characterize many aestivating anurans (Withers and Guppy 1996; Fuery et al. 1997). Cowan and Storey (2002) tested the effects of both urea and an inorganic solute (KCl) on the maximal activity of 25 enzymes of intermediary metabolism of liver and muscle of *S. couchii*, and compared the results with those of nonaestivating anuran species and rats. Urea caused a general and consistent inhibitory effect on enzyme activity in the rat and in nonaestivating anurans, but the enzymes of spade-foot toads were almost unaffected, or even activated in muscle tissue. A similar effect was also observed for antioxidant enzymes in these animals, which are largely insensitive to urea accumulation (Grundy and Storey 1998). It seems that in aestivating anurans, the accumulation of urea contributes to minimize cell volume reduction and its consequences on the activity of metabolic enzymes (Cowan and Storey 2002; but see also Hochachka and Somero 2002). In addition, Muir et al. (2007, 2008) found that urea induces a decrease in aerobic metabolic rate in dehydrated frogs (*Rana sylvatica*), and that the metabolic rates of liver, stomach, and skeletal muscle are depressed after urea treatment at low temperatures.

7.4.5.2 Water Stores and Uptake

Desert-adapted anurans such as *S. couchii* (McClanahan, 1967), *Cyclorana australis, C. platycephala*, and *Neobatrachus aquilonius* (Cartledge et al. 2006, 2008; Tracy et al. 2007) regulate body water using the content stored in their bladders. This water may be absorbed previous to aestivation or from the soil while the difference between the water potential of the animal and that of the surrounding soil is favorable (McClanahan et al. 1976; Booth 2006). Water uptake probably decreases as soils become dry with the progress of the dry season and at this point further responses may take place; for example, further burrowing, cocoon-formation, and others. Such shifts in strategy or behavioral modifications imply that frogs are capable of sensing a change in physiological state, in environmental conditions, or

both. Whether aestivating toads can perceive changes in water potential of soil is still uncertain, but some evidence suggests that osmoreceptors located in the central nervous systems may be sensible to changes in extracellular osmolality. Responses may be mediated through endocrine function, particularly the hormone arginine vasotocin (AVT) produced by the neurohypophysis that affects the permeability of skin and bladder in amphibians (Bentley 1966). This hormone may contribute to the water balance in aestivating anurans (Cartledge et al. 2006, 2008) and seems responsible also for the control on blood flow between body core and ventral regions (Tracy and Rubink 1978; see also Tracy et al. 2007). These physiological adjustments, combined with behavioral shifts, can be important in the overall responses related to water uptake and conservation in aestivation amphibians.

7.4.6 Skeletal Muscle and Gastrointestinal Adjustments

Given that aestivation involves a long period of reduced activity and absence of feeding, it is expected that some organs and physiological systems shift to "low-activity states" in order to reduce maintenance costs. In this context, the skeletal muscle and the gastrointestinal tract are of particular relevance because they are of limited use during aestivation and normally involve a significant toll of energy budgets. However, these organs exhibit considerable morpho-physiological plasticity in many vertebrates, including anurans, and are exposed to seasonal and acclimatory modifications (Hudson and Franklin 2002b). Many aspects of metabolic control on muscle biochemistry are amply discussed in Chap. 8, so, here, this chapter only points out that aestivating anurans do not exhibit skeletal muscle atrophy or decreased functional performance, even after long periods of inactivity (Hudson and Franklin 2002a, 2002b, 2003; Hudson et al. 2004; Symonds et al. 2007). These intriguing aspects of muscle physiology of aestivators seem related to the upexpression of genes that silence other genes involved in several bioenergetic pathways in frog muscle (Hudson et al. 2008). Moreover, some biochemical traits concerning the control on antioxidative stress and oxidative phosphorylation, which may be responsible for muscle atrophy, seem also object of metabolic regulation during aestivation (Hudson et al. 2006, 2008).

Given that anuran aestivation is usually associated with starvation and disuse of the gastrointestinal tract, physiological and morphological changes would be expected for such structure during the transition from active to dormant state and vice versa. A complete review of the adjustments in gastrointestinal tract of amphibians, and other groups, is provided by Secor and Lignot in Chap. 9, so this section will present only a few considerations about energetics because of the importance of energetic balance in the seasonal cycle of aestivating anurans. In *C. alboguttata*, the mass of both the stomach and the small intestine is reduced during aestivation, a process that is concomitant with a reduction of food passage rates through the digestive tract (Cramp and Franklin 2003). Under experimental treatment, these frog species experience increased retention of food in the small intestine, a finding

suggesting that, despite changes in organs masses, nutrient assimilation following prolonged food deprivation during aestivation may be indeed enhanced (Cramp and Franklin 2003). So, in nature, the last meal before aestivation might be properly processed even if overall metabolic activity is being turned down. With the progression of metabolic arrest, *C. alboguttata* undergoes significant cellular and subcellular downregulation of morphological traits that result also in changes on the morphophysiology of the small intestine, including length, longitudinal fold heights, enterocyte cross-sectional area, microvillus height and density, and rearrangements of mitochondria and nuclei (Cramp et al. 2005; Cramp and Franklin 2003). However, at the onset of arousal, when the metabolic state is being restored, several of those morphological shifts are promptly reversed, ensuring that the gastrointestinal tract becomes capable of functioning. The morphological changes occurring during frog aestivation are

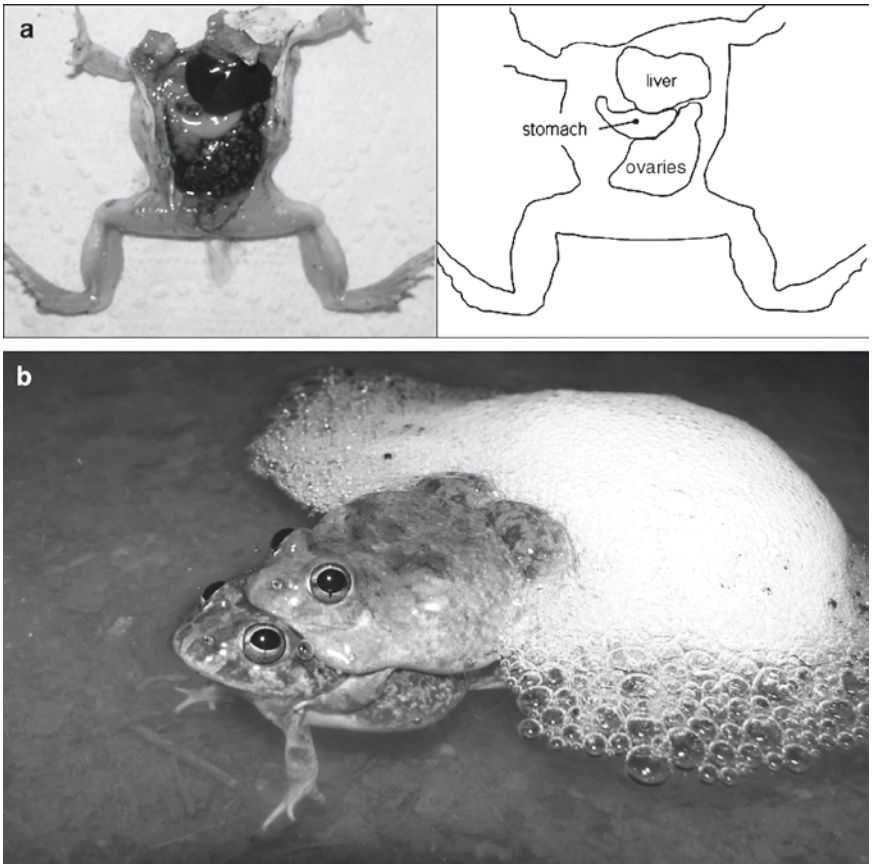

Fig. 7.3 (**a**) Ovaries in an adult female of *Pleurodema diplolistris* (Leiuperidae) taken at about 1-m depth in the sandy soil of Brazilian Caatinga after 7–8 months of aestivation. (**b**) A couple of *P. diplolistris* in amplexus during rainy season, when male build nest foam for eggs deposition. (Photographs by JE Carvalho and IC Pereira)

transitory and rapidly reversible (Cramp et al. 2005) and complete gut reorganization occurs when first after-aestivation meal is ingested (Cramp and Franklin 2003; Cramp et al. 2005; Secor 2005). Furthermore, metabolic and functional postprandial responses to feeding are significantly less intense in nonaestivating anuran species when compared with aestivating species (Secor 2005).

7.5 Emergence

Some semi-arid environments inhabited by anurans are likely to exhibit dry years or sequences of years with insufficient rain to grant successful reproduction (McClanahan 1967; van Beurden 1980), so environmental cues linked to rain may be ambiguous. Even so, aestivating anurans must emerge timely in order to reproduce. This scenario favors the hypothesis that intrinsic mechanisms are fundamental, a premise supported by some field observations and experimental work. Females of some species show evidence of egg maturation in progress much before any environmental cue allows to determine whether or not a given year will be rainy. The authors of this chapter have seen this in female *P. diplolistris* from semi-arid Brazilian Caatinga, which exhibit developed ovaries after 7–8 months of aestivation (Fig. 7.3). In addition, aestivating spade-foot toads show, as one of the most conspicuous changes in gene expression during aestivation, upregulation of riboflavin-binding protein, which has been identified as linked to maturation of eggs in some species (Storey et al. 1999).

Probably, some specific environmental cues help to fine-tune emergence in aestivating amphibians, but the nature of such cues are unclear, particularly, in the case of anurans. The actual onset of heavy rains seems secondary. *Scaphiopus* toads emerge from aestivation in two steps, one before heavy rains in which they reduce depth significantly and may even reach the surface (Bragg 1965). This contention was supported by the later finding that spade-foot toads start to emerge from artificial borrows after 8 months, even when kept at constant temperature and in the dark (McClanahan 1972). In addition, Ruibal and collaborators noticed that experimental local floods do not elicit reproductive behavior and that emergence may be coupled with extremely light rainfall. In addition, they found food in the stomachs of toads before the evening of the first seasonal rain, which suggests that at least some toads are active before the onset of the rainy season (Ruibal et al. 1969). The timing of emergence and the regulation of reproduction in unpredictable environments remains as an understudied topic of anuran biology.

7.6 Conclusions

The understanding of amphibian aestivation encompasses an initial and very productive period of classical physiological ecology that set the basis of what we know now regarding organismal physiology. This period was followed by later research

primarily on mechanisms and focused on events at a subcellular level. These two approaches have plenty of room for further interaction; such integration would help to better understand the observed patterns, and the area would benefit from modern approaches in evolutionary physiology. The field has also the potential to make more accurate predictions by considering interspecific diversity in amphibian aestivation modes. Given the possibilities of reducing physiological challenge through ecological associations and behavior, a variety of context-specific predictions can be made regarding physiological adaptation and physiological mechanisms. Bringing more species to the literature seems, also, essential. Despite the merits of building on previous knowledge, a more complete understanding of amphibian aestivation requires to include a varied sample of aestivation strategies and ecological associations. Otherwise, we will only learn more about a few model species and may incur in the risk of overgeneralization.

Many questions regarding anuran aestivation remain opened for productive research, some of which have been presented in this chapter. Surprisingly, we do not have a full understanding of very basic issues, such as the implication of substrate type as a factor modifying physiological challenge during aestivation in anurans. The role of hypoxia in amphibian aestivation, for example, cannot be established unambiguously with the data available. Energy management, particularly, where rains are unpredictable and dry years common, also remains understudied. Failed reproduction may be a factor excluding anurans from the least predictable areas, but if not, remarkable physiological mechanisms are there to be discovered. Also, the timing of emergence remains underexplored, as does the physiology of anural larvae, some of which produce tiny froglets that metamorph over just a few weeks. Finally, the magnitude and nature of metabolic arrest in anurans remains to be studied in context that acknowledges evident ecological diversity. Recent works also signals to the expected enhanced aridization of semi-arid environments due to global climate change, and understanding the consequences for amphibians in such habitats is a concern among herpetologists.

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Chapter 8
Effects of Aestivation on Skeletal Muscle Performance

Rob S. James

Contents

Abstract Fitness, ecology, and behaviour of vertebrates are dependent upon locomotor performance. Locomotor performance can be constrained by underlying intrinsic skeletal muscle properties. Skeletal muscle is a highly plastic tissue undergoing phenotypic change in response to alteration in environment. Clinical and experimental models of muscle disuse cause decreases in skeletal muscle size and mechanical performance. However, in natural models of skeletal muscle disuse, both atrophy and changes in mechanical properties are more limited. Aestivation in frogs can cause decreases in muscle cross-sectional area and changes in some enzyme activities, with effects varying among muscles. However, long-term aestivation causes limited changes in muscle mechanics during simulated sprint or endurance type activities. Therefore, at least in frogs, there is maintenance of skeletal muscle performance during prolonged periods of aestivation, allowing avoidance of harsh environmental conditions without compromising the locomotor capacity to perform fitness-related activities when favourable environmental conditions return.

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8.1 Introduction

Fitness, ecology, and behaviour of vertebrates are dependent upon locomotor performance (Calsbeek and Irschick 2007; Huey et al. 1984; Jayne and Bennett 1990a; Miles 2004; Navas et al. 2006). Fitness can be dependent on sprint running or jumping performance (as used in escape responses or prey capture) or on endurance activities (such as migration and amplexus). Although locomotor performance is heritable (Le Galliard et al. 2004; Jayne and Bennett 1990b; Tsuji et al. 1989), there is considerable within-generation variability as a result of environmental effects and gene–environment interactions (Le Galliard et al. 2004; Rupert 2003). Locomotor performance can be constrained by underlying intrinsic muscle properties such as muscle activation and relaxation rates, maximal shortening velocity, maximal force, and power production, especially during sprint (burst) performance (Bennett et al. 1989; James et al. 2007; Johnston and Temple 2002; Marsh 1994; Navas et al. 2006). Therefore, for animals undergoing aestivation, it is important to preserve skeletal muscle performance to maintain locomotor performance for fitness-dependent behaviours (especially, as many of these animals have a small temporal window of opportunity for food acquisition and breeding).

Skeletal muscle is a highly plastic tissue undergoing phenotypic change in response to alteration in environment. Such "environmental" cues for phenotypic change include alterations in mechanical load, activity, resting fibre length, temperature, circulating hormonal concentrations (including insulin, growth hormone, insulin-like growth factor 1), growth, development, nutrition, and disease (Pette and Staron 2001; Goldspink 2002; Rennie et al. 2004; Boonyarom and Inui 2006). Phenotypic changes in skeletal muscle can include changes in muscle size (atrophy or hypertrophy) and fibre type. Classic examples of the dramatic effects of environment on skeletal muscle phenotype include cross-innervation studies that involve swapping the nerve supply innervating a relatively fast muscle, such as flexor digitorum longus, with that innervating a relatively slow muscle, such as soleus (Buller et al. 1960). Such experiments resulted in the mechanical properties, such as twitch activation and relaxation times, of the relatively slow muscle becoming more like that of the relatively fast muscle and vice versa.

8.2 Skeletal Muscle Disuse

8.2.1 Clinical and Experimental Models of Muscle Disuse

Clinical and experimental studies on nonhibernating mammals demonstrate that enforced muscle disuse (reduced muscle activity during limb immobilisation, microgravity, bed rest, etc.) leads to decreases in both muscle size (atrophy) and mechanical performance (Musacchia et al. 1988; Fitts et al. 2000; Hudson and Franklin 2002a; Boonyarom and Inui 2006; Powers et al. 2007). For instance,

84 days of bed rest in humans led to a 17% decrease in quadriceps volume and a 43% decrease in maximal isometric squat strength (Trappe et al. 2004). Underlying these changes were decreases in muscle fibre diameter, maximal isometric force, and maximal unloaded shortening velocity in both type I and IIB fibres, along with an overall shift towards faster muscle fibre types. Muscle disuse atrophy also affects sarcoplasmic reticulum calcium uptake rates, with 10 days of plaster cast immobilisation causing an 80% decrease in calcium uptake rate in human quadriceps muscle (Thom et al. 2001), although such effects on other animals are more varied (Musacchia et al. 1988; Thom et al. 2001). Therefore, during disuse atrophy, absolute mechanical performance is compromised by alterations in the intrinsic mechanical properties of the muscle as well as by the effects of decreased muscle size. Muscle atrophy results from both an increase in protein degradation as well as a decrease in protein synthesis (Musacchia et al. 1988; Hudson and Franklin 2002a; Boonyarom and Inui 2006; Powers et al. 2007). The key proteolytic pathways in skeletal muscle include the proteosome system, lysosomal proteases, calpains and caspase-3 (Powers et al. 2007). Recent evidence in the literature indicates that reactive oxygen species can cause direct oxidative injury in muscle, but can also play a role in signalling pathways contributing to skeletal muscle atrophy (Powers et al. 2007). Accumulation of oxidative injury may, therefore, be due to a shift in the balance between reactive oxygen species and production of antioxidants or an insufficient rate of repair affected by rate of protein synthesis. Muscle atrophy is more pronounced when muscles are immobilised in a shortened position (Tabary et al. 1972; Williams and Goldspink 1973), in muscles of slower fibre type (as these muscles are normally used for a greater proportion of time; Musacchia et al. 1988) in animals of smaller body mass, and in animals with higher mass-specific metabolic rate (Hudson and Franklin 2002a).

8.2.2 Natural Models of Muscle Disuse

Many animals undertake prolonged periods of dormancy to survive extreme environmental conditions, e.g., aestivation or hibernation. While responses to seasonal cold temperatures are termed hibernation, responses to seasonal dry periods are termed aestivation (Pinder et al. 1992). These periods of dormancy allow many animals to extend their geographical range, often at the cost of being "active" for only small time periods per year or every few years. Once these animals become active, they will rapidly undertake activities that are fitness-related such as those associated with breeding and food acquisition, placing large demands on their skeletal muscle. Therefore, animals that undergo a period of dormancy would be disadvantaged if they underwent the same levels of muscle disuse atrophy and loss of mechanical performance observed in the clinical and experimental models of atrophy outlined in Sect. 2.1.

During hibernation, animals undergo less muscle atrophy and smaller changes in mechanical properties of muscle than is observed in clinical and experimental

models of atrophy (Hudson and Franklin 2002a; Carey et al. 2003; Shavlakadze and Grounds 2006). For example, 4 months of hibernation in black bears, *Ursus americanus*, caused a significant decrease (less than 15%) in muscle protein concentration, but no change in fibre cross-sectional area in both gastrocnemius and biceps femoris muscles (Tinker et al. 1998; due to balancing of protein synthesis and degradation during hibernation Lohuis et al. 2007), with only a 23% loss of tibialis anterior muscle force (Harlow et al. 2001). There was a 10% increase in the percentage of muscle occupied by fast twitch fibres in biceps femoris and an approximately 30% decrease in citrate synthase activity with hibernation in biceps femoris muscle, but no such changes in gastrocnemius. Three to four months of cold water submerged hibernation in the common frog, *Rana temporaria*, had no effect on the isometric stress, force–velocity (F–V) relationships (maximum shortening velocity, peak power output, and curvature of the F–V relationship) or work loop power output-cycle frequency curves of sartorius (locomotory) and external oblique (calling) muscle (West et al. 2006). The maintenance of these mechanical properties also suggests that fibre type was maintained in these muscles in *R. temporaria*, although it should be noted that this type of hibernation does involve some infrequent short bursts of activity. Hibernation in golden hamsters had no significant effect on twitch times or myosin ATPase staining patterns in extensor digitorum longus (EDL), soleus, and diaphragm muscle ($n=4$), although succinate dehydrogenase staining patterns suggested increased activity of mitochondrial enzymes in the EDL and diaphragm during hibernation (Vyskočil and Gutmann 1977).

To survive prolonged periods of dormancy, animals generally also need to accumulate metabolic fuel reserves prior to dormancy (often as lipid), undergo global metabolic rate depression during dormancy to conserve fuel reserves and switch from carbohydrate to lipid oxidation as the primary metabolic fuel source (Carey et al. 2003; Pinder et al. 1992; Storey and Storey 2007; Chapter 2). Aligned to these changes are use of reversible phosphorylation of enzymes and other proteins to rapidly reduce the metabolic rates and the increased levels of antioxidant defences to reduce tissue damage. Clearly, these general changes might also affect skeletal muscle performance.

8.3 Effects of Aestivation on Skeletal Muscle Morphology

The aforementioned work on hibernating species suggests that natural models of disuse incur fewer changes in skeletal muscle physiology than would be predicted from clinical models. The same appears to be true for aestivating species. For example, 9 months of aestivation had no effect on muscle mass, total muscle cross-sectional area, fibre number, or fibre type distribution in iliofibularis or sartorius muscles in the green-striped burrowing frog, *Cyclorana alboguttata* (Symonds et al. 2007). In the same species, 6 months of aestivation also had no effect on total cruralis muscle cross-sectional area (Hudson et al. 2006), while 3 months of aestivation had no effect on muscle mass of gastrocnemius, sartorius, semimembranosus, or gracilis major (Hudson and Franklin 2002b). However, aestivation caused different effects on muscle fibre cross-sectional area in different muscles, with 6 months of aestivation in cruralis

muscle leading to a significant (28%) decrease in cross-sectional area of oxidative fibres (Hudson et al. 2006), 9 months of aestivation leading to a significant decrease in cross-sectional area of both glycolytic (40%) and oxidative (44%) fibres in iliofibularis muscle, but no such effects in sartorius muscle (Symonds et al. 2007).

There was no change in connective tissue morphology in cruralis muscle after 5 months of aestivation in the green-striped burrowing frog, *C. alboguttata*, however, there was reduced expression of enzymes involved in connective tissue remodelling, tissue inhibitor of metalloproteinase 2 and metalloproteinase 2 (Hudson et al. 2007). In contrast, experimental limb immobilisation in laboratory rats induces increased connective tissue volume relative to skeletal muscle fibre volume during concomitant skeletal muscle atrophy (Oki et al. 1995).

Four months of aestivation had no effect on the three-dimensional structure, tortuosity, or diameter of capillaries in semimembranosus muscle in the green-striped burrowing frog, *C. alboguttata* (Hudson and Franklin 2003). In contrast, experimental limb immobilization causes decreased capillary tortuosity in rat soleus muscle which may lead to haemorrhaging on remobilisation (Oki et al. 1998).

8.4 Effects of Aestivation on Skeletal Muscle Metabolism

Aestivation is associated with metabolic depression (Pinder et al. 1992; Chapter 1), exemplified by an 85% decrease in rate of resting oxygen consumption following 12 weeks of aestivation in the Humming frog, *Neobatrachus pelobatoides* (Flanigan et al. 1990). These reductions in total oxygen consumption by the animal are accompanied by reduced transcription and activity of many metabolic enzymes (Flanigan et al. 1990).

Significant reductions in transcription of NADH ubiquinone oxidoreductase subunit 1 (71%) and ATP synthase (67%) occurred over 6 months of aestivation in gracilis muscle of the green-striped burrowing frog, *C. alboguttata* (Hudson et al. 2006), indicating reduced mitochondrial activity during aestivation. However, after a shorter time period, of 10 weeks of aestivation, there were no significant changes in aldolase and glyceraldehydes 3 phosphate activity in the Humming frog, *N. pelobatoides* (Flanigan et al. 1990).

Ten days of aestivation caused significant decreases in activity, but no change in amount of enzyme protein, of sarcoendoplasmic reticulum calcium ATPase and Na^+/K^+-ATPase in desert snail, *Otala lactea*, foot muscle (Ramnanan and Storey 2006; Ramnanan and Storey 2008). These aestivation-induced changes were due to greater levels of posttranslational phosphorylation of these ATPase enzymes in aestivated snails. It is thought that reversible phosphorylation of tyrosine residues on proteins may play a role in control of dormancy during aestivation with dephosphorylation of enzymes acting to decrease the enzyme activity (Storey and Storey 1990; Cowan and Storey 2001; Chapter 2). Two months of aestivation in spadefoot toads, *Scaphiopus couchii*, caused reduced (28%) protein tyrosine kinase activity in leg muscle which was accompanied by apparent changes in enzyme subtype indicative of dephosphorylation (Cowan and Storey 2001).

Levels of both water-soluble and membrane-bound antioxidants were maintained over 6 months of aestivation in gracilis muscle of the green-striped burrowing frog, *C. alboguttata* (Hudson et al. 2006). However, as there is a decrease in metabolism during aestivation, this maintenance of antioxidants represented an increase in total antioxidant power during aestivation. This period of aestivation did, however, have differential effects on the transcription of different antioxidants as transcription of superoxide dismutase 2 was significantly decreased (67%; matching the decrease in mitochondrial activity during aestivation), whereas transcription of catalase and glutathione peroxidise 4 was maintained. Transcription of uncoupling protein 2 was maintained, probably due to its suspected role in reducing mitochondrial leakage of reactive oxygen species. Both reduced and oxidised glutathione levels were higher in leg muscle from active spadefoot toads, *S. couchii*, than in those that had undergone 2 months of aestivation (Grundy and Storey 1998). However, the ratio of these forms of glutathione were maintained suggesting no change in susceptibility of the muscle to oxidative damage with aestivation. No significant changes in activity of glutathione-*S*-transferase, glutathione reductase, glutathione peroxidase, or catalase occurred with aestivation in spadefoot toads; in contrast, superoxide dismutase levels significantly increased with aestivation.

8.5 Effects of Aestivation on Properties of the Neuromuscular Junction

There were no changes in neuromuscular junction morphology, miniature endplate potential, or resting membrane potential in iliofibularis muscle after 6 months of aestivation in the green-striped burrowing frog, *C. alboguttata* (Hudson et al. 2005). However, 6 or 15 months of aestivation did lead to a significant reduction in endplate potential and a significant increase in nerve stimulations in aestivated muscle that lead to failure to evoke an endplate potential (Hudson et al. 2005; Lavidis et al. 2008). The 15 months of aestivation treatment did not lead to any dramatic changes in expression of vesicle docking proteins, suggesting that the inhibition of neuromuscular function is at the physiological level (Lavidis et al. 2008).

8.6 Effects of Aestivation on Skeletal Muscle Mechanics and Relationship with Locomotor Performance

Very few studies have considered the effects of aestivation on skeletal muscle mechanics or locomotor performance and all of these have concentrated on one particular frog species. Long-term aestivation caused limited changes in the skeletal muscle mechanics of the green-striped burrowing frog, *C. alboguttata* (Hudson and Franklin 2002b; Hudson et al. 2006; Symonds et al. 2007), when tested in vitro. The maximal isometric twitch and tetanus force produced per muscle cross-sectional

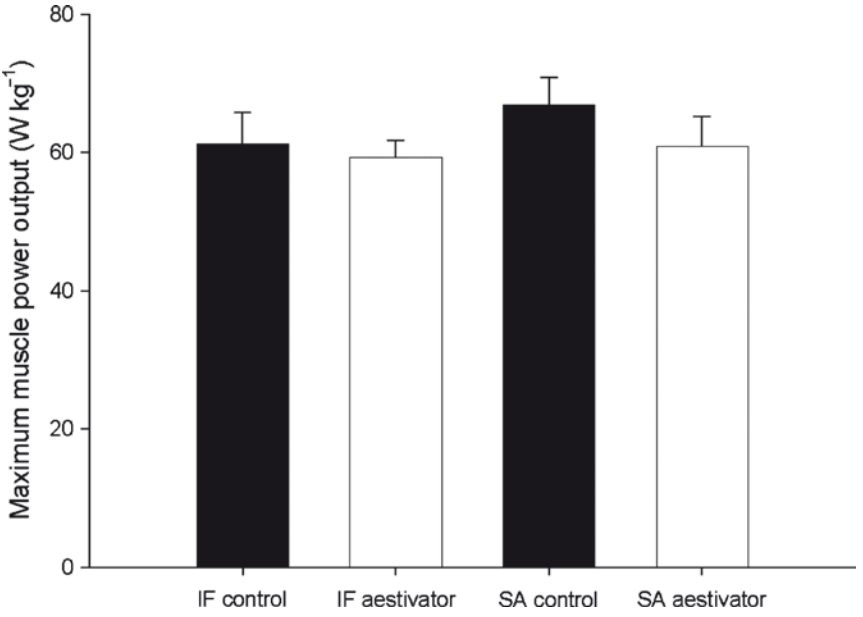

Fig. 8.1 Effect of 9 months aestivation on skeletal muscle power output in the green striped burrowing frog *Cyclorana alboguttata*. Power output is normalised to muscle mass. *IF* iliofibularis muscle, *SA* sartorius muscle, Data represent mean+SEM; control $N=6$, aestivator $N=5$. Based on data in Symonds et al. 2007

area remained unchanged in gastrocnemius muscle after either 3 months or 9 months of aestivation (Hudson and Franklin 2002b; Hudson et al. 2006) and in iliofibularis and sartorius muscles after 9 months of aestivation (Symonds et al. 2007). Maximum power output, determined by the work loop technique (Fig. 8.1), and the power output – cycle frequency curve remained unchanged in iliofibularis and sartorius muscles after 9 months aestivation (Symonds et al. 2007). After 3 months or 9 months of aestivation there were no changes in the latency period, twitch force rise or relaxation times in gastrocnemius muscle (Hudson and Franklin 2002b; Hudson et al. 2006). However, after 9 months of aestivation twitch force rise and relaxation times were significantly slower in iliofibularis muscle and tended towards being slower in sartorius muscle when compared with controls (Symonds et al. 2007). Fatigue resistance was not affected by aestivation when assessed either during isometric tests of gastrocnemius muscle force after 3 months of aestivation (Hudson and Franklin 2002b) or when assessed via work loop tests of iliofibularis and sartorius muscle power output after 9 months of aestivation (Symonds et al. 2007). However, 9 months of aestivation lead to a significant decrease in fatigue resistance in gastrocnemius muscle when tested isometrically (Hudson et al. 2006). The relative maintenance of skeletal muscle mechanics during aestivation allows these frogs to maintain maximal swimming performance over at least 3 months of aestivation (Hudson and Franklin 2002b).

8.7 Discussion

Aestivation is used to avoid unfavourable conditions. This is particularly important in ectotherms as they are unable to regulate temperature and remain active, other than by behavioural means, and are, particularly, challenged by environments with low water availability. From the evidence reviewed above, it appears that during aestivation, skeletal muscle undergoes less phenotypic and mechanical changes than would be predicted from clinical or experimental (nonnatural) studies of disuse. However, it would be interesting to better and more widely understand the phenotypic flexibility and performance of skeletal muscle in animals that aestivate. Do those species or populations living in semi-arid environments differ in skeletal muscle physiology from closely related species or populations in more humid environments? Can species or populations living in semi-arid environments use behavioural strategies and phenotypic changes to enable them to delay or avoid the need for aestivation or reduce the time spent in aestivation? For example, *Rhinella granulosa* (previously named *Bufo granulosus*) from the semi-arid Caatinga region of Brazil exhibits a very high critical thermal maximum for amphibians and may be active at field temperatures bordering 40°C (Navas et al. 2007). *Rhinella granulosa* also inhabit the relatively cool and humid Atlantic Forest. When plantaris longus muscle mechanics were compared between individuals from the two populations, there were very few differences between them; however, there was a large variability in twitch relaxation times at 35°C in muscle from individuals from the Atlantic Forest (James and Navas 2008). This high variability in relaxation rate, perhaps, suggests that calcium reuptake or sequestering in these individuals from Atlantic Forest is somehow affected by this relatively high temperature. Overall, the data from a number of previous studies suggests that activity of *R. granulosa* in the Caatinga region is largely maintained via other physiological and morphological adaptations to high temperature and low humidity such as increased thermal stability of the enzyme citrate synthase (to maintain muscle performance at higher than normal temperatures; Navas et al. 2007), decreased ventral skin thickness, and increased capillary density near the skin (possibly to increase opportunistic water uptake; Navas et al. 2004). Such findings begin to give us some idea of the limits in adaptations, to skeletal muscle and other systems that species in semi-arid environments can make to extend their geographic and temporal range before avoidance strategies, such as aestivation, are needed. Future studies might further investigate such populational differences between different environments to determine whether skeletal muscle adaptation is useful as a tool to cope with semi-arid environments. In anurans, thermal acclimation of muscle performance has only once been demonstrated in adult animals (Wilson et al. 2000), as it is thought that thermal adaptations in ectotherms requires a more stable thermal cue than that which occurs in environments with large daily fluctuations in temperature (Temple and Johnston 1998). However, it is feasible that skeletal muscle in animals from a semi-arid environment could be better adapted to lowered hydration state; however, no studies have tested for this. Systematic study of animal activity during aestivation

could also prove to be important; i.e., are animals such as frogs totally immobile throughout aestivation or are there infrequent periods of muscle activity as often occurs in hibernating animals? Even brief infrequent periods of activity can help to decrease the stimulus for disuse atrophy, but again little is known about the natural behaviour of many animals in semi-arid environments to determine the patterns of activity used. Therefore, further studies of natural activity patterns, locomotory performance of whole animals, and mechanical performance of key locomotory muscles before, during, and after aestivation in a range of animals will help to more widely determine the physiological and ecological implications of aestivation.

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Chapter 9
Morphological Plasticity of Vertebrate Aestivation

Stephen M. Secor and Jean-Herve Lignot

Contents

Abstract Aestivation or daily torpor is an adaptive tactic to survive hot and dry periods of low food availability, and has been documented for species of lungfishes, teleost fishes, amphibians, reptiles, birds, and mammals. Among these species, aestivation is characterized by inactivity and fasting, and for lungfishes and amphibians the formation of a cocoon around the body to retard water loss. While metabolic and physiological changes to aestivation have been well examined, few studies have explored the morphological responses of organs and tissues to aestivation. Predictably, inactive tissues such as skeletal muscles and those of the gastrointestinal

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tract would regress during aestivation, and thus aid in the reduction of metabolic rate. African lungfishes experience changes in the structure of their skin, gills, lungs, and heart during aestivation. For anurans, the group most thoroughly examined for morphological responses, aestivation generates significant decreases in gut mass and modification of the intestinal epithelium. Intestinal mucosal thickness, enterocyte size, and microvillus length of anurans are characteristically reduced during aestivation. We can surmise from laboratory studies on fasting reptiles, birds, and mammals that they likewise experience atrophy of their digestive tissues during torpor or aestivation. Aestivation-induced loss of tissue structure may be matched with a loss of cellular function generating an integrative decrease in tissue performance and metabolism. Ample opportunity exists to remedy the paucity of studies on the morphological plasticity of organs and tissues to aestivation and examine how such responses dictate tissue function during and immediately following aestivation.

9.1 Introduction

Aestivation is a natural history strategy of dormancy to survive periodic or seasonal episodes of drought, high temperatures, and low food availability (Pinder et al. 1992; Storey 2001). Among vertebrates, aestivation or a brief period of dry-season dormancy (i.e., daily torpor) has been reported for fishes, amphibians, reptiles, birds, and mammals (Geiser 1988; Graham 1997; Gregory 1982; Pinder et al. 1992). Described as "a behavioral strategy accompanied by physiological adjustments" (Seidel 1978), aestivation is characterized by three features; inactivity, fasting, and for many species a decrease in basal metabolism (Guppy and Withers 1999; Pinder et al. 1992). The depression of metabolic rate serves to lengthen the duration that endogenous body stores can support the animal during nonfeeding dormancy (Guppy and Withers 1999; Storey 2001).

An expected phenomenon stemming from the disuse of organs and tissues during aestivation is cellular atrophy and reduction in performance. For example, immobilized skeletal muscle will atrophy and lose contractile strength (Booth 1982; Nordstrom et al. 1995). Likewise, gastrointestinal epithelium will reduce mass and function during extended periods of fasting or when nutrition is provided intravenously (Meurling and Roos 1981; Secor 2005a). Laboratory studies on the 13-lined ground squirrel (*Spermophilus tridecemlineatus*) found that after a minimum of 6 weeks of hibernation, the small intestine had experienced a 75% reduction in mass and a 36% reduction in villus height (Carey 1990). Other organs associated with digestion (e.g., liver and pancreas) will likewise experience atrophy during extended periods of fasting (Secor and Diamond 2000). Skin structure will, undoubtedly, be altered during aestivation for fishes and amphibians that form a cocoon around their body of dried mucus and/or epithelium (Lee and Mercer 1967). Hence, aestivating animals predictably experience morphological changes of their skin, skeletal muscles, gastrointestinal tract, and other organs involved in digestion.

Our aim in this chapter is to provide a comprehensive review of the morphological responses of tissues and organs during aestivation for vertebrates. Our coverage includes published papers and abstracts as well as unpublished findings from our own laboratories. For many species, we also provide brief comments on their aestivation behavior, ecology, and physiological responses. It is surprising the paucity of vertebrate studies that have examined changes in the mass and structure of organs and tissues during aestivation. The organisms that have received the most attention are anurans that aestivate during the dry season (Cramp and Franklin 2003; Cramp and Franklin 2005; Cramp et al. 2005; Secor 2005b). For groups with little information on morphological responses to aestivation, we provide results stemming from laboratory studies of fasting to predict how tissues might respond to aestivation. Evident from this review is the need to further explore the morphological as well as the functional plasticity of tissues during aestivation and to examine how they are integrated to determine tissue performance during aestivation.

9.2 Fishes

Among fishes, seasonal aestivation has been documented for five of the six species of lungfishes, and members of the teleost genera *Lepidogalaxias* (Salmoniformes), *Neochanna* (Salmoniformes), *Galaxias* (Salmoniformes), *Pseudapocryptes* (Perciformes), *Monopterus* (Synbranchiformes), and *Synbranchus* (Synbranchiformes). In addition, members of the teleost genera *Clarias* (Siluriformes), *Periophthalmus* (Salmoniformes), *Macrognathus* (Salmoniformes), and *Channa* (Channiformes) have been found out of water in burrows during the dry season suggesting that they also undergo seasonal episodes of aestivation (Graham 1997). The vast majority of studies on aestivation responses for fishes have been conducted on lungfishes.

9.2.1 Lungfishes

The extant lungfishes (Dipnoi) are members of a relic lineage of Sarcopterygian fishes that arose during the Devonian in marine habitats, and today are represented by six freshwater species. The Australian lungfish, *Neoceratodus forsteri*, inhabits the Brisbane, Mary, and Burnett rivers of northeastern Australia (Kemp 1987). This lungfish possesses well-developed gills on all branchial arches and a single lung (Grigg 1965). It is considered a facultative air breather that is heavily dependent on gill respiration and is not known to aestivate (Grigg 1965; Lenfant et al. 1966). The South American lungfish, *Lepidosiren paradoxia*, inhabits the Amazon and Parana river basins, and the four African lungfishes, *Protopterus aethiopicus*, *P. amphibious*, *P. annectens*, and *P. dolloi*, occupy a variety of aquatic habitats throughout central Africa (Graham 1997; Greenwood 1987). The South American and African lungfishes possess rudimentary gills and well-developed paired lungs

(Graham 1997). They are obligate air breathers, relying largely on lung respiration, and will aestivate in subterranean burrows during the dry season (Graham 1997; Greenwood 1987).

Of the five species of lungfishes that aestivate, it is the aestivation of *P. annectens* that is best described (Greenwood 1987). Sparked by the drop in water level, this lungfish excavates a vertical burrow into the mud and positions itself in a U-shaped fashion with head and tail facing upward. This lungfish then secretes copious amounts of mucus from epithelial mucous glands which upon mixing with soil forms a hardened cocoon that surrounds the body with a single opening at the mouth for breathing. The other three African lungfishes and the South American lungfish have been observed to aestivate within burrows, but it is questionable whether they typically do so within a cocoon (Greenwood 1987). Once dormant, lungfish experience decreases in gas exchange, cardiac performance, and ammonia production, all integrated responses that serve to reduce their energy expenditure and maintain homeostasis (DeLaney et al. 1974; Fishman et al. 1986; Janssens and Cohen 1968; Lomholt 1993).

For more than a century, anatomists have described the structure and histology of the visceral organs of lungfishes (Coujard and Coujard-Champy 1947; Klein 1864; Parker 1892; Rafn and Wingstrand 1981). Beginning with Homer Smith's study in 1930, there has been, in addition, a wealth of physiological studies conducted on lungfishes (Smith 1930; Burggren and Johansen 1986; Perry et al. 2008). It is, therefore, surprising that few studies have explored the morphological changes that lungfishes experience during aestivation. Sturla et al. (2002) did examine the cytomorphological changes of the skin, gills, and lungs of *P. annectens* following induced aestivation within the laboratory. In that study, lungfish were placed in a tank of clay, sand, and water, and the water was allowed to evaporate. The lungfish responded by excavating burrows, positioning themselves in a U-shape fashion, and becoming encased in a cocoon of dried mucus, clay, and sand.

With the onset of aestivation, the skin of *P. annectens* transforms from a sponge-like appearance with elevated microplicae and microridges to a relatively smooth and flattened surface covered by mucus (Figs. 9.1a,b; Sturla et al. 2002). The skin epithelium is normally composed of columnar cells interspersed by numerous mucous cells and with aestivation the columnar cells become compressed and there is reduction in mucous cells (Figs. 9.1c,d; Sturla et al. 2002). Smith and Coates (1937) presented a similar description of the aestivation-induced changes of the skin for *P. annectens*. The gills of free-swimming *P. annectens* bear the typical arrangement of well-separated secondary lamellae that allows the easy passage of water (Sturla et al. 2002). During aestivation, the secondary lamellae become stuck together and the interlamellar spaces are filled with mucus (Figs. 9.1e,f). The seemingly nonfunctional lungs of free-swimming *P. annectens* appear collapsed, possess few blood vessels which were almost devoid of blood cells, and lack any apparent compartmentalization. In contrast, the lungs of aestivating individuals are swollen, extensively vascularized, and possess ridges and pillars that formed numerous small pockets which increase respiratory surface area (Sturla et al. 2002).

For the lungfish, *P. dolloi*, laboratory-induced aestivation generates modest changes to the structure of the ventricular myocardium (Icardo et al. 2008).

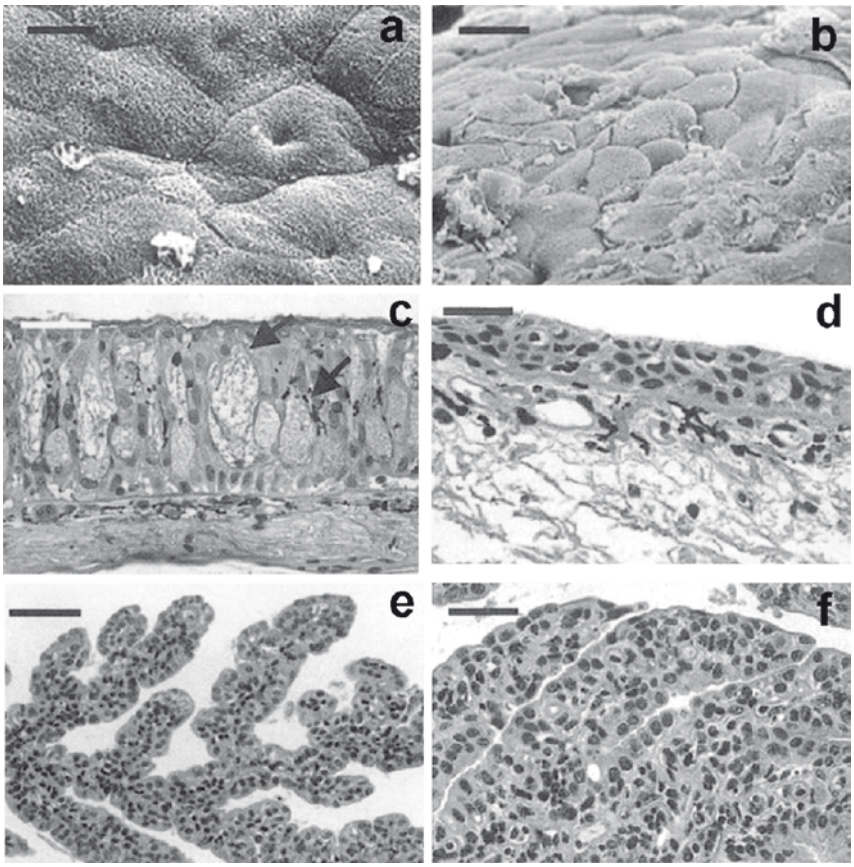

Fig. 9.1 Scanning electron (**a** and **b**) and light (**c** and **d**) microscopy images of the skin surface of aquatic (**a** and **c**) and aestivating (**b** and **d**) *Protopterus annectens*. Note during aestivation the flattening of the epithelium and loss of mucous cells (present in **c** and noted with *arrows*). Light microscopy images of the gill secondary lamellae demonstrate well separated lamellae in the aquatic condition (**e**) and compacted lamellae during aestivation (**f**). Scale bars: 3.5μm (**a**); 5.5 μm (**b**); 80 μm (**c**); 75 μm (**d**); 125 μm (**e**); 80 μm (**f**). Images are from Sturla et al. (2002) and are reprinted with the permission of John Wiley and Sons, Inc

Noted for the myocytes was a 56% increase in mean cellular area and a doubling of sarcomere length. The mitochondria develop a lighter matrix during aestivation and decrease in number, though individually increase in size by 140%. It is hypothesized that the lungfish's cardiac mitochondria fuse together during aestivation. For the ventricular septum, there is an increase during aestivation in the number of necrotic myocytes that are characterized by a completely vacuolated cytoplasm (Icardo et al. 2008). The subendocardium of aestivating lungfish possesses a greater amount of collagen and more monocytes (to clear cellular debris) than observed for free-swimming individuals. Many of these changes were reversed within 6 days

after aestivating lungfishes were returned to water (Icardo et al. 2008). Interestingly, several of the cellular changes observed during aestivation for *P. dolloi* suggest the maintenance of elevated metabolic activity. Hypothetically, such activity is necessary during aestivation to maintain circulatory and ventilatory performance and to produce urea (Icardo et al. 2008; Perry et al. 2008).

9.2.2 Teleost Fish

The salamander fish, *Lepidogalaxias salamandroides*, is a small teleost fish that inhabits tributaries of the Shannon River of southwestern Australia (Graham 1997). During the 3–4 month dry season, this fish aestivates in mud burrows or under leaves. Although possessing a gas bladder, it is assumed that gas exchange during aestivation for *L. salamandroides* is primarily cutaneous (Berra et al. 1989). *Monopterus alba* of southeastern Asia can survive periods of drought by burrowing into the mud, though it does not form a cocoon nor experience a decrease in metabolic rate (Liem 1967). Six months of fasting had little effect on the morphology of their stomach and intestine, with the exception of a pronounced decrease in the number of goblet cells for the hindgut (Liem 1967). The South American swamp eel, *Synbranchus marmoratus*, is found in a variety of aquatic habitats throughout much of South America. When such habitats dry up during the dry season, individuals burrow into the substrate and aestivate without forming a cocoon (Bicudo and Johansen 1979). During this time their metabolic rate is significantly depressed and they respire across the epithelium that lines their mouth, pharynx, and branchial chambers (Bicudo and Johansen 1979). In the laboratory, *S. marmoratus* have survived for 6–9 months in a burrow without water (Bicudo and Johansen 1979).

Although no study has explored the morphological responses of any teleost fish with aestivation, we can assume that they experience similar changes to their skin and gills as observed for lungfishes. Additionally, there would be changes in intestinal mass and morphology as observed from studies on fasted fish. Two and four weeks of fasting resulted in a 40 and 60% decrease in intestinal mass for the shark *Squalus acanthias*, and the teleost, *Salmo gairdneri*, respectively (Bogé et al. 1981; Wood et al. 2007). For *S. gairdneri*, the decrease in intestinal mass is disproportionate between the mucosa (decreasing by 71%) and muscularis/serosa (decreasing by 37%) layers (Bogé et al. 1981).

9.3 Amphibians

Although the general perception is that amphibians inhabit moist tropical, subtropical, or temperate regions of the world, there are numerous species that inhabit more arid environments (e.g., deserts or grasslands) that experience

seasonal episodes of drought (Pinder et al. 1992). In such habitats when faced with the dual challenges of no standing water and no food, the characteristic adaptive response of amphibians is to burrow into the soil or mud and enter a state of dormancy. This adaptive tactic includes the formation of a cocoon around the body to reduce the rate of desiccation and the depression of metabolic rate to increase the duration of survival on endogenous body stores (Pinder et al. 1992; Guppy and Withers 1999). Depending on locality and habitat, amphibians may routinely spend as much as 10 months of the year in aestivation (Pinder et al. 1992). In an extreme case, it has been suggested that the Australian hylid *Cyclorana platycephala* can survive five consecutive years of aestivation during continuous multiyear droughts (van Beurden 1980).

Amphibian aestivation has been previously reviewed by Mayhew (1968) and Pinder et al. (1992). Aestivation among salamanders has been reported for the genera *Ambystoma* (Ambystomatidae), *Amphiuma* (Amphiumidae), *Pseudobranchus* (Sirenidae), and *Siren* (Sirenidae). For anurans, aestivation has been documented for members of the genera *Breviceps* (Microhylidae), *Bufo* (Bufonidae), *Ceratophrys* (Leptodactylidae), *Cyclorana* (Hylidae), *Heleioporus* (Limnodynastidae), *Hyla* (Hylidae), *Lepidobatrachus* (Leptodactylidae), *Leptodactylus* (Leptodactylidae), *Neobatrachus* (Limnodynastidae), *Notaden* (Limnodynastidae); *Physalaemus* (Leptodactylidae), *Pseudophryne* (Myobatrachidae), *Pyxicephalus* (Ranidae), *Scaphiopus* (Pelobatidae), and *Xenopus* (Pipidae) (Pinder et al. 1992; Glass et al. 1997). There are no reported instances of aestivation for caecilians (Pinder et al. 1992).

9.3.1 Salamanders

Following the drying of their pond habitat in the Chihuahuan desert of New Mexico, USA, recently transformed subadult tiger salamanders (*Ambystoma tigrinum*) were excavated from cracks and crevices in the dried mud at depths of 15–30 cm (Webb 1969). Ponds in this region remain dry for 1–2 months during the summer, thereby, requiring salamanders to remain below the surface until the next heavy rainfall. Although no morphological information was presented in that report, we have found in a laboratory study that a 1-month fast reduces small intestinal wet mass for larva *A. tigrinum* by 40% (S. Secor unpublished data).

The three extant species of *Amphiuma* inhabit ponds, swamps, and streams of the Coastal Plain region of the southeastern United States (Petranka 1998). Both the two-toed amphiuma (*A. means*) and the three-toed amphiuma (*A. tridactylum*) have been reported to burrow as deep as 1 m with the drying of their habitat and aestivate for as long as 2 years (Knepton 1954; Petranka 1998). No study has described the morphological changes experienced during aestivation for these large aquatic salamanders.

Sirens (*Pseudobranchus* and *Siren*) are long slender aquatic salamanders that possess gills and inhabit both stationary (marshes, swamps, and ponds) and moving (streams and rivers) bodies of water of the southeastern United States

(Petranka 1998). With the drying of their habitat, the lesser siren, *Siren intermedia*, burrows into the mud or enters existing crayfish burrows and in similar fashion to the African lungfish, forms a cocoon of dried mucus that completely covers their body except the mouth (Reno et al. 1972; Gehlbach et al. 1973). They will remain cocooned for 4–8 months until the next heavy rainfall (Gehlbach et al. 1973). In contrast to *S. intermedia*, the greater siren, *S. lacertina*, is reported to form a cocoon of dried epithelium with the onset of aestivation (Etheridge 1990).

Following 16 weeks of laboratory-induced aestivation, individuals of *S. intermedia* lost approximately 25% of their body mass, primarily from the posterior portion of their body (Gehlbach et al. 1973). Body diameter had decreased by 25–37% and there was a 58% reduction in body fat. At this time their gills had atrophied, noted by the 50% decrease in gill ramus length, and their heart rate and oxygen consumption had decreased by 50 and 80%, respectively (Gehlbach et al. 1973). Similarly, *S. lacertina* experiences gill atrophy and metabolic depression during laboratory-induced aestivation (Etheridge 1990).

9.3.2 Anurans

Although second to lungfishes in the number of physiological studies conducted on aestivation, more attention had been directed in anuran studies on the morphological responses associated with aestivation. Morphological as well as physiological responses to aestivation have been explored for aestivating anurans of Africa (*Pyxicephalus*), Australia (*Cyclorana*), North America (*Scaphiopus*), and South America (*Ceratophrys*). Of these anurans, the most detailed published account of the morphological changes occurring during aestivation has been for the green-stripped burrowing frog (*Cyclorana alboguttata*) of Australia (Cramp and Franklin 2003; Cramp and Franklin 2005; Cramp et al. 2005; Hudson and Franklin 2002). Using these studies as a model, we have used published (Secor 2005) and unpublished findings to additionally describe the morphological responses to fasting and aestivation for *Ceratophrys ornata*, *Pyxicephalus adspersus*, and *Scaphiopus holbrooki* (Fig. 9.2).

9.3.2.1 *C. alboguttata*

C. alboguttata is a medium-size frog of eastern Queensland (northeastern Australia) whose habitat includes both grasslands and woodlands that maintain temporary pools of water (Cogger 1992; Fig. 9.2a). With the drying of these pools, *C. alboguttata* burrows into the substrate, forms a cocoon of shed layers of epithelium and mucus, and remains dormant for up to 10 months (Fig. 9.2e).

In the before mentioned studies, *C. alboguttata* were collected in the field, placed in plastic containers holding water-saturated soil, and after burrowing themselves into the soil were left undisturbed to aestivate for 3 or 9 months.

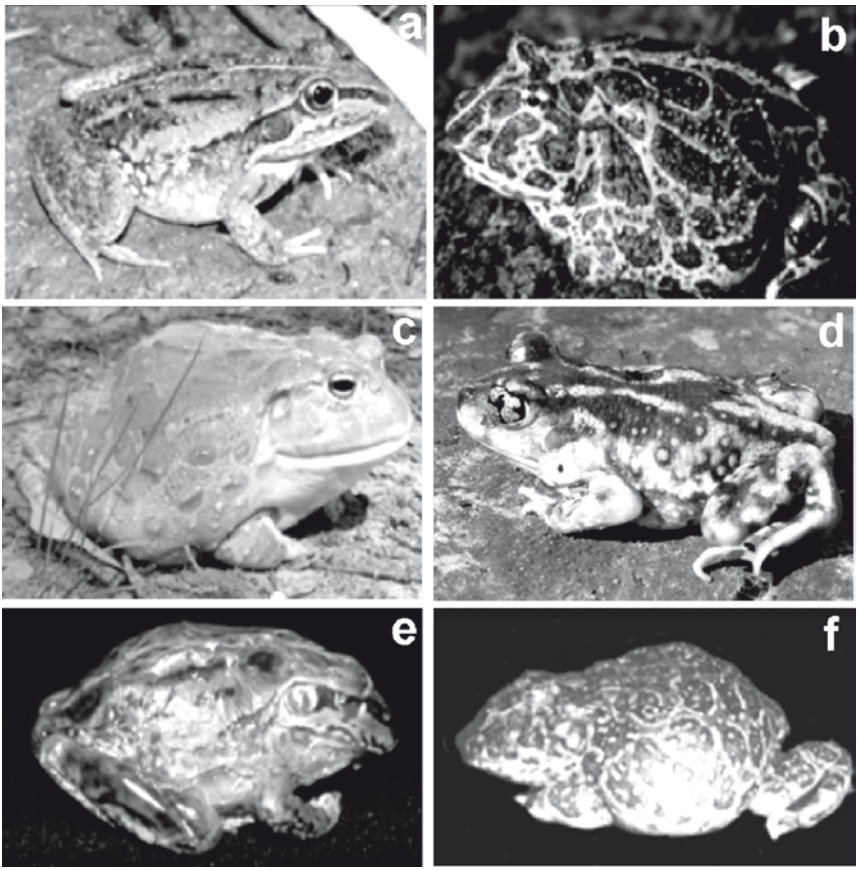

Fig. 9.2 Morphological responses of the gastrointestinal tract to fasting and aestivation have been examined for the anurans *Cyclorana alboguttata* (**a**), *Ceratophrys ornata* (**b**), *Pyxicephalus adspersus* (**c**), and *Scaphiopus holbrooki* (**d**), each of which aestivate annually for up to 10 months. During aestivation, anurans surround themselves in a cocoon made from layers of dried epithelium as illustrated for *C. alboguttata* (**e**) and *C. ornata* (**f**). Photographs of **a** and **e** are by E.A. Meyer and of **b** by N. Alt and used with permission. Photographs of **c**, **d**, and **f** are by S. Secor

Compared with frogs recently fed, frogs that had been fasted for 1 week exhibited no change in the masses of their stomach, small intestine, large intestine, or liver, and no change in small intestinal length (Cramp et al. 2005). Laboratory-induced aestivation for 3 and 9 months lead to significant decreases in the wet masses of the stomach, small intestine, and large intestine; each declining by 50, 77, and 50%, respectively, after 9 months of dormancy (Fig. 9.3). After 9 months of aestivation, intestinal length and width had significantly declined by 39 and 43%, respectively (Fig. 9.4). Interestingly, even after 3–9 months of aestivation, *C. alboguttata* experiences no loss in skeletal muscle mass, long bone structure, or capillary or

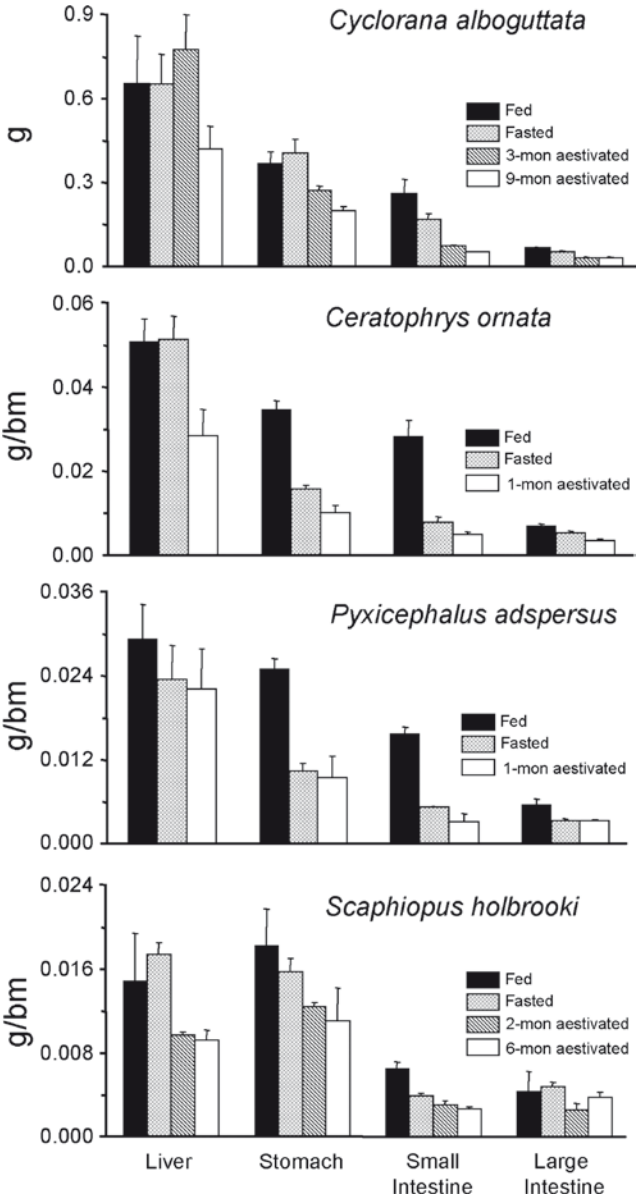

Fig. 9.3 Wet mass (g) and wet mass normalized to body mass (g bm^{-1}) of the liver, stomach, small intestine, and large intestine of *Cyclorana alboguttata*, *Ceratophrys ornata*, *Pyxicephalus adspersus*, and *Scaphiopus holbrooki* during digestion (fed), fasting (fasted), and aestivation. Duration of aestivation was 1 month for *C. ornata* and *P. adspersus*, 2 and 6 months for *S. holbrooki*, and 3 and 9 months for *C. alboguttata*. In almost all cases, organs experienced a significant decrease in mass from active digestion to aestivation. Values are presented as means and error bars represent ±1 SEM. Figures were drawn from data presented in Cramp et al. (2005), Secor (2005), and unpublished data of the authors

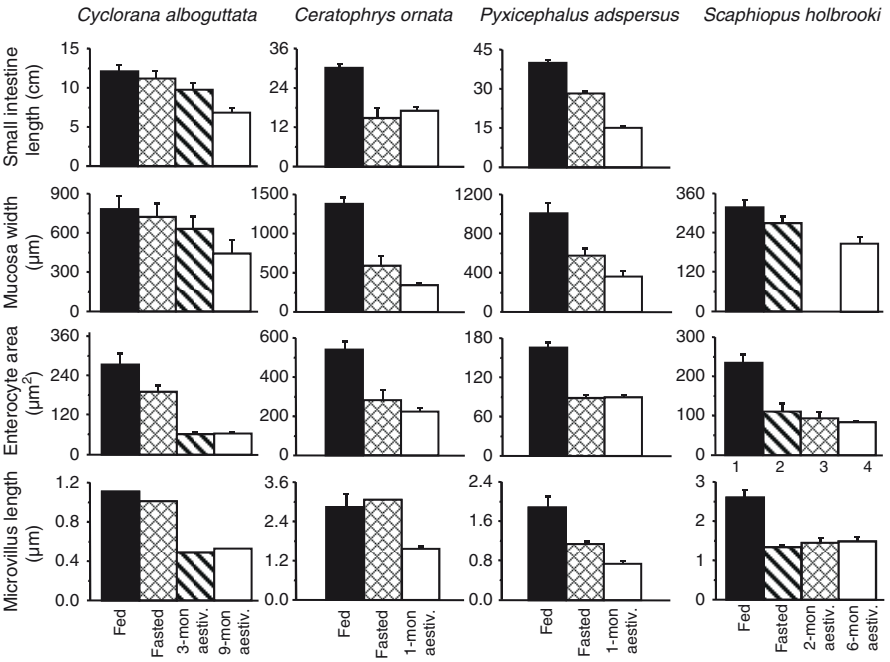

Fig. 9.4 Small intestine length, small intestine mucosa width, enterocyte transectional area, and microvillus length of the anurans *Cyclorana alboguttata*, *Ceratophrys ornata*, *Pyxicephalus adspersus*, and *Scaphiopus holbrooki* during digestion (fed), fasting (fasted), and various durations of aestivation. Measures of intestinal morphology decreased with fasting and/or aestivation for all four anuran species. Values are presented as means and error bars represent ±1 SEM. Figures were drawn from data presented in Cramp et al. (2005), Secor (2005), and unpublished data of the authors

neuromuscular junction architecture (Hudson and Franklin 2002; Hudson and Franklin 2003; Hudson et al. 2004; Hudson et al. 2005; Symonds et al. 2007).

Histological examination of the small intestine revealed significant decreases after 3 and 9 months of aestivation in mucosal width (by as much as 67%) and enterocyte transectional area (by 53%; Figs. 9.4 and 9.5). At the ultrastructural level of the intestine, aestivating frogs possess shorter microvilli (Figs. 9.4 and 9.6). During aestivation, enterocyte mitochondria are largely concentrated beneath the terminal web, are more spherical in shape, with many possessing a disrupted inner membrane. Additional changes of enterocytes with aestivation include more deeply invaginated nuclei and fewer golgi and smooth and rough endoplasmic reticulum.

In a companion study, *C. alboguttata* were fed after 3 months of aestivation (Cramp and Franklin 2005). Within 2 days following their postaestivation meal, small intestinal length, and wet masses of the stomach, small intestine, and large intestine had been restored to levels of fed control frogs. Similarly, villus height, enterocyte transectional area, microvillus length and density of fed frogs had returned to fed control levels within 2–6 days after feeding. While feeding restored the appearance of enterocyte organelles, it also resulted in a large accumulation of lipid droplets within enterocytes.

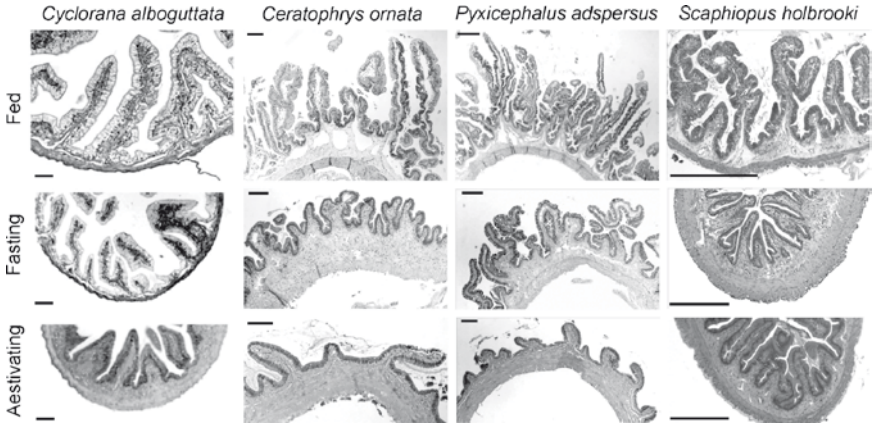

Fig. 9.5 Light microscopy images of the intestinal mucosa of digesting (fed), fasting, and aestivating *Cyclorana alboguttata*, *Ceratophrys ornata*, *Pyxicephalus adspersus*, and *Scaphiopus holbrooki*. Note the reduction in villus length and, hence, mucosal thickness with fasting and aestivation. All scale bars = 250 μm. Images for *C. alboguttata* are from Cramp et al. (2005) and reprinted with the permission of the authors and Wiley-Blackwell. The other images are those of the authors

9.3.2.2 *Ceratophrys ornata*

Ceratophrys ornata, the Argentine horned frog, inhabits the forests of Argentina, Paraguay, Uruguay, and southern Brazil (Obst et al. 1988; Fig. 9.2b). With the start of the dry season, this frog buries itself and becomes encased in a cocoon of dried epithelium (McClanahan et al. 1976; Fig. 9.2f). The following summarizes published and unpublished accounts of the morphological changes experienced by this frog with fasting and after 1 month of laboratory-induced aestivation (Secor 2005b; S. Secor unpublished data). For *C. ornata*, a 2-week fast had no significant effects on the wet masses of the heart, liver, spleen, or lungs. In contrast, such a fast resulted in 25–55% decreases in the wet masses of the stomach, pancreas, large intestine, and kidneys (Fig. 9.3). One month of aestivation did not further impact the wet masses of the heart, spleen, and lungs; however, wet masses of the liver, stomach, pancreas, large intestine, and kidneys were further reduced significantly by 20–45% (Fig. 9.3).

The small intestine of *C. ornata* experiences a dramatic reduction in mass with fasting (72% decrease) and again with aestivation (37% from fasting levels; Fig. 9.3). The length of the small intestine decreases by 50% with fasting, but does not change further after 1 month of aestivation (Fig. 9.4). While serosa/muscularis thickness is not affected by either fasting or aestivation, mucosa thickness decreases by 57% with fasting, and again by 43% with aestivation (Figs. 9.4 and 9.5). Enterocyte transectional area decreases by 48% after a 2-week fast, though it does not further decrease following a month of aestivation (Fig. 9.4). Fasting has no effect on microvillus length, whereas 1 month of aestivation reduces microvillus length by 45% (Figs. 9.4 and 9.6).

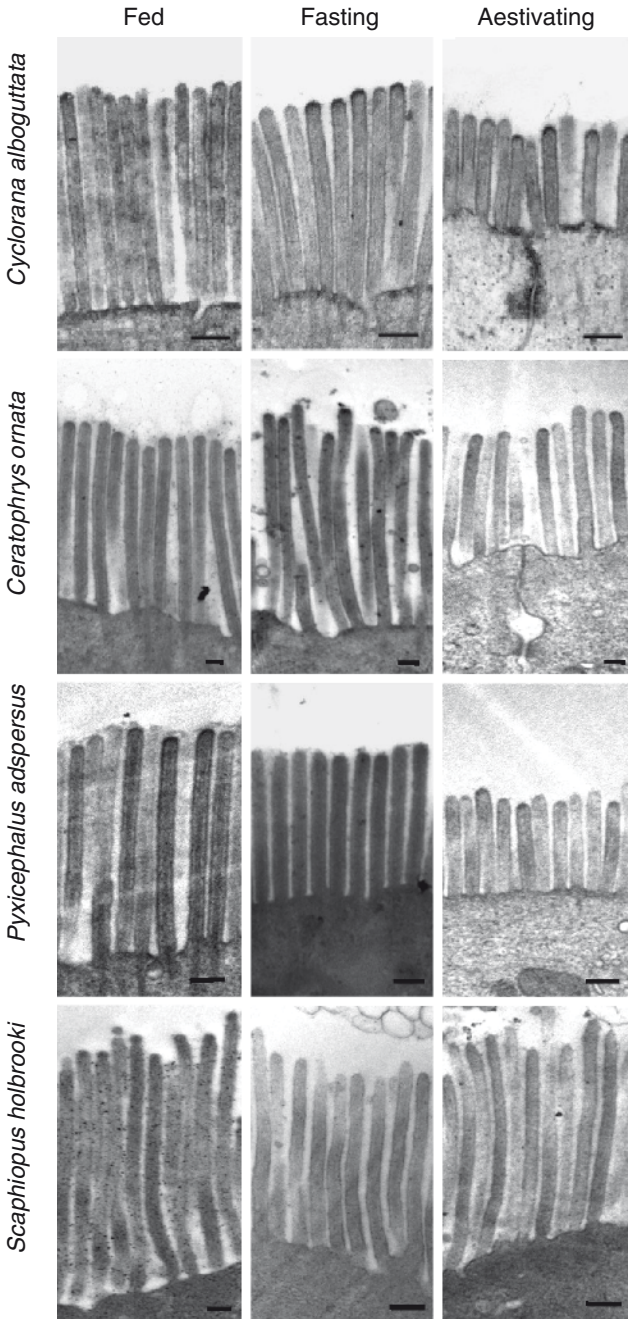

Fig. 9.6 Transmission electron microscopy images of the intestinal microvilli of digesting (fed), fasting and aestivating *Cyclorana alboguttata*, *Ceratophrys ornata*, *Pyxicephalus adspersus*, and *Scaphiopus holbrooki*. Fasting generated a significant decrease in microvillus length for *P. adspersus* and *S. holbrooki*, whereas aestivation significantly reduced microvillus length for *C. alboguttata* and *C. ornata*. All scale bars = 0.2 μm. Images for *C. alboguttata* are from Cramp et al. (2005) and reprinted with the permission of the authors and Wiley-Blackwell. The other images are those of the authors

9.3.2.3 *Pyxicephalus adspersus*

The African bullfrog, *Pyxicephalus adspersus*, inhabits open savannas of subSaharan Africa and like *C. ornata* aestivates underground for up to 10 months of the year surrounded by a cocoon of compacted layers of dried epithelium (Loveridge and Withers 1981; Fig. 9.2c). Morphological responses to fasting and aestivation for *P. adspersus* are summarized from published and unpublished accounts of frogs fasted for 2 weeks and those that were induced to aestivate in the laboratory for 1 month (Secor 2005b; S. Secor, unpublished data). A 2-week fast has no significant effects on the wet masses of the heart, liver, spleen, and lungs of *P. adspersus* compared with when actively digesting, fasting results in a 30–60% decrease in the wet masses of the stomach, pancreas, large intestine, and kidneys (Fig. 9.3). One month of aestivation further reduces the mass of the pancreas by 42%, but has no effect on the masses of the lungs, heart, liver, stomach, spleen, large intestine, and kidneys (Fig. 9.3).

The small intestine of *P. adspersus* declines in mass with fasting (by 68%), and continues to decrease in mass during aestivation (by 40%, Fig. 9.3). Fasting decreases intestinal length by 30% and aestivation further reduces length by another 45% (Fig. 9.4). Neither fasting nor aestivation has significant effects on the thickness of the intestinal serosa/muscularis layer. In contrast, the mucosa layer decreases by 43% with fasting, and from fasting levels decreases by an additional 38% with aestivation (Figs. 9.4 and 9.5). Enterocyte transectional area decreases by 46% with fasting, though does not continue to change after 1 month of aestivation (Fig. 9.4). The intestinal microvilli of *P. adspersus* are reduced in length by 40% with fasting, and further again by 35% with aestivation (Figs. 9.4 and 9.6).

9.3.2.4 *Scaphiopus holbrooki*

The eastern spadefoot toad, *S. holbrooki*, inhabits forested lowlands of the Atlantic coast and southeastern United States (Conant and Collins 1991; Fig. 9.2d). During dry periods, they bury themselves into the soil and remain dormant for months. The following summarizes an unpublished laboratory study on the morphological responses of *S. holbrooki* to fasting and aestivation. Measurements were taken from *S. holbrooki* 2 days after ingesting a cricket meal, after 2 weeks of being fasted, and after being dormant and undisturbed for 2 or 6 months. Dormancy was induced by allowing toads to bury into a large container filled with a sand/clay mixture. The soil was then allowed to dry at room temperature and humidity.

Following 2 weeks of fasting, only the small intestine had experienced a significant decrease (by 54%) in wet mass (Fig. 9.3). In this study, none of the toads excavated after 2 or 6 months of dormancy were encased in a cocoon. Most appeared well hydrated and several possessed fluid within their bladders. Two months of dormancy resulted in significant decreases in the wet masses of the liver (by 44%), stomach (by 21%), small intestine (by 23%), large intestine (by 47%), and kidneys (by 51%) (Fig. 9.3). An additional 4 months of dormancy only resulted in a further

reduction in the mass (by 13%) of the small intestine (Fig. 9.3). There was no change with dormancy in the wet masses of the lungs, heart, pancreas, or spleen.

The thickness of *S. holbrooki* intestinal mucosa decreased by 15% following 2 weeks of fasting and an additional 23% after 6 months of dormancy (Figs. 9.4 and 9.5). The decrease in enterocyte height and width after the 2-week fast generated a 53% reduction in enterocyte transectional area, which had declined by an additional 25% six months later (Fig. 9.4). Fasting had a significant impact on microvilli, decreasing its length by 45%. An additional 2 or 6 months of fasting had no further effects on microvillus length (Figs. 9.4 and 9.6).

9.3.3 Other Anurans

In southern Africa, members of the genus *Breviceps* aestivate during the dry season, occasionally within termite nests (Roots 2006). In Brazil, *Bufo marinus* and *B. paracnemis* will spend the dry season within shallow burrows, but do not form a cocoon (Glass et al. 1997). In the laboratory, a 2-week fast is enough to significantly reduce the wet mass of the small intestine of *B. marinus* by 42% (Secor 2005b). In addition to *C. alboguttata*, the Australian anurans *C. platycephalus*, *C. australis*, *Limnodynates spenceri*, and *Neobatrachus pictus* completely surrounded themselves with a cocoon of dried epithelium during aestivation (Lee and Mercer 1967). *Lepidobatrachus llanensis*, an aquatic anuran of the Chaco region of southern South America, forms a cocoon of multiple layers of flatten epidermal cells while aestivating during the warm dry winters (McClanahan et al. 1976). At the onset of a drought, African clawed frogs (*Xenopus laevis*) will burrow into mud and remain dormant for 2–3 months (Balinsky et al. 1967). Even in water, a 3-week fast is sufficient to reduce the wet masses of *X. laevis* stomach and small intestine by 45–50% (S. Secor unpublished data).

9.4 Reptiles

For reptiles, aestivation (or aestivation-like behavior) has been reported for species of turtles, lizards, snakes, and crocodilians, but not for any amphibaenids (Abe 1995; Gregory 1982; Winne et al. 2006). At the start of the dry season, individuals will bury into the mud or move into crevices, burrows, or under leaf litter. They remain relatively inactive and do not feed until the next significant rainfall. In contrast to lungfishes and amphibians, aestivating reptiles do not surround themselves in a cocoon of dried skin and/or mucus. While lungfish and amphibians significantly depress their metabolic rate during aestivation, this is not considered a general trait among aestivating reptiles (Christian et al. 1996; Guppy and Withers 1999; Ligon and Peterson 2002).

9.4.1 Turtles

For turtles, aestivation behavior has been documented for members of the genera *Chelodina* (Chelidae), *Chelydra* (Chelydridae), *Gopherus* (Testudinidae), *Kinixys* (Testudinidae), and *Kinosternum* (Kinosternidae) (Ernst et al. 1994; Kennett and Christian 1994; Hailey and Coulson 1996; Peterson and Stone 2000; Voigt and Johnson 1976). With the drying of its wetland habitat in Australia, *Chelodina rugosa* buries into the mud which hardens to form a chamber slightly larger than the turtle (Kennett and Christian 1994). The conspecific *C. longicollis* buries into the leaf litter and remains inactive for several months (Roe and Georges 2008). In southwestern United States, *Kinosternum flavescens* is reported to survive underground for as long as 2 years during extended periods of drought (Rose 1980). In the laboratory without water for 3 months, dormant *K. flavescens* and *K. sonoriense* lose about 27% of their body mass, largely due to evaporative water loss (Peterson and Stone 2000; Seidel 1978).

Although no study has explored the morphological responses to aestivation for turtles, we can deduce potential changes in organ mass during aestivation from the study of Secor and Diamond (1999). In that study, organ mass and intestinal performance were compared between fasted (1 month since last meal) and fed *Chelydra serpentina*, *Sternotherus odoratus* (Kinosternidae), and *Trachemys scripta* (Emydidae). Compared with digesting individuals, a 1-month fast resulted in a significant reduction (by 37%) in stomach mass for *S. odoratus*, and near significant ($P \leq 0.1$) decrease (by ~40%) in small intestinal mass for *C. serpentina* and *T. scripta* (Fig. 9.7). The 1-month fast did not significantly impact enterocyte dimension for any of these three species (Secor and Diamond 1999).

9.4.2 Lizards and Snakes

Lizards of the genera *Heloderma* (Helodermatidae), *Sauromalus* (Iguanidae), *Sceloporus* (Phrynosomatidae), *Trachydosaurus* (Scincidae), *Tubinambis* (Teidae), and *Varanus* (Varanidae) have been reported to enter a state of dormancy during the dry season (Abe 1995; Beck 2005; Bustard 1970; Nagy 1973; Wilhoft 1958). In southeastern Brazil, *Tubinambis merianae* will spend 4–5 months underground and is inactive during the autumn and winter months (Abe 1995). In Namibia, the monitor lizard, *Varanus albigularis*, retreats to refugia (burrows or trees) from September to December. Although alert and active, they do not leave their refugia in search of food (Philips 1995).

Laboratory studies have generally found that with fasting, lizards experience decreases in the masses of their digestive organs. A 1 month fast for the Gila monster (*Heloderma suspectum*) resulted in a 32–36% decrease in the masses of the pancreas, small intestine, and kidneys, and a 50% decrease in enterocyte volume (Christel et al. 2007; Fig. 9.7). Chuckwallas (*Sauromalus obesus*) fasted for 10 days to 2 months lost, on average, 25% of the mass of their gastrointestinal tract (Tracy and Diamond 2005; Fig. 9.7). For other lizard genera known to aestivate, a 2–4 week fast reduces small intestinal wet mass by 17–34% (S. Secor unpublished data; Fig. 9.7).

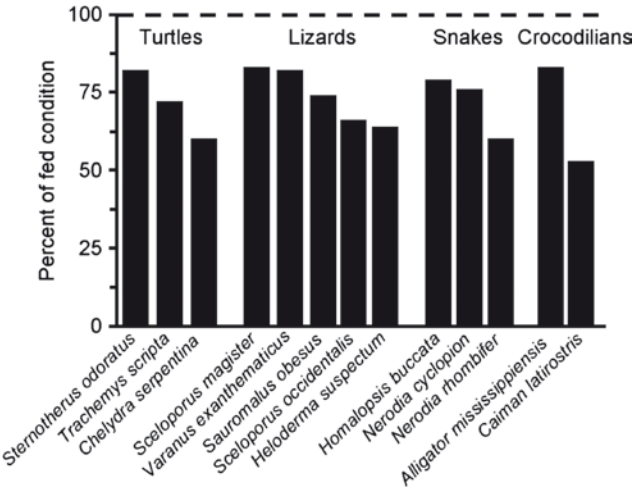

Fig. 9.7 Small intestinal mass of fasted individuals as a percentage of intestinal mass during active digestion for several species of turtles, lizards, semi-aquatic snakes, and crocodilians. Among these reptiles, fasting results in a 17–47% reduction in small intestinal mass. Since several of the species and genera that are presented are reported to aestivate, we predict similar changes in small intestinal mass during aestivation. Data originate from Christel et al. (2007), Secor and Diamond (1999), Starck et al. (2007), Tracy and Diamond (2005), and unpublished observations of S.M. Secor

While there is extensive literature on the overwinter dormancy (i.e., hibernation) of snakes, little has been reported regarding snake aestivation (Gregory 1982). Snake species most apt to aestivate are small semiaquatic species that are less inclined to migrate to other aquatic habitats when their original habitat dries up. One such species, the black swamp snake, *Seminatrix pygaea*, is reported to survive a 2.5-year drought during which no standing water was present in its habitat (Winne et al. 2006). Because no snakes were found under cover on the surface during this drought, it was presumed that they were dormant beneath the dried mud (Winne et al. 2006). In response to 1 month of fasting within the laboratory, the similarly semi-aquatic diamondback watersnake (*Nerodia rhombifer*) experiences 38–44% reduction in small intestinal mass, intestinal mucosa width, and enterocyte volume (C. Cox and S. Secor unpublished data; Fig. 9.7). More modest decreases in small intestinal mass after 1 month of fasting have been observed for other species of semi-aquatic snakes (S. Secor unpublished data; Fig. 9.7).

9.4.3 Crocodilians

Aestivation has been anecdotally reported for various species of crocodilians (Guggisberg 1972; Taplin 1988). The freshwater crocodile, *Crocodylus johnstoni*, of northern Australia will annually spend 3–4 months inactive in underground refugia, during which they will lose 16% of their body mass (Christian et al. 1996).

In a laboratory study on neonate and juvenile American alligators (*Alligator mississippiensis*), a month fast resulted in a significant decrease in mass (by 10–30%) for only the stomach (S. Secor unpublished data). Intestinal mass, enterocyte dimension, or microvillus length were not significantly impacted by the 1-month fast (S. Secor unpublished data; Fig. 9.7). In contrast, a 3-month fast reduced the wet mass of the liver and small intestine of the broad-nose caiman (*Caiman latirostris*) by 44 and 47%, respectively (Starck et al. 2007; Fig. 9.7).

9.5 Birds and Mammals

The term "aestivation" has been used to label periods of dormancy, irrespective of duration, for both birds and mammals during conditions of moderate ambient temperature (Hudson and Bartholomew 1964; MacMillen 1965). Shaw (1925) reported from the observed lack of activity that Columbian ground squirrels (*Citellus columbianus*) aestivate for several days during summer periods of high temperatures and low rainfall. MacMillen (1964), in observing the disappearance of individual cactus mice (*Peromyscus eremicus)* for 6–8 weeks during the dry summer and their reappearance later concluded that the mice were aestivating within their burrows during that time. When deprived of food in the laboratory, *P. eremicus* will enter into torpor within 12 h and experience decreases in both metabolic rate and body temperature (MacMillen 1965). Since laboratory torpor seldom lasted more than a day, it was concluded that during midsummer *P. eremicus* alternates between arousal and torpor states while remaining within burrows and feeding upon cached food (MacMillen 1965). Daily torpor and accompanying physiological responses (e.g., reduced metabolic rate and body temperature) has been described for species of hummingbirds, swifts, poor-wills, marsupials, shrews, bats, rodents, and the badger (Geiser 1988; Pearson 1960). As evident from examples of more extended periods of dormancy, the onset of daily cycles of torpor is apparently triggered by the seasonal shortages of food and water (Hudson and Bartholomew 1964).

The morphological changes that occur during daily periods of torpor would expectedly be modest due to their short time span. In a laboratory study on the edible dormouse (*Glis glis*), daily torpor bouts ranged between 3 and 21 h in duration (Wilz and Heldmaier 2000). While longer bouts of dormancy may occur for birds and mammals in the wild, they are much shorter than bouts experienced by aestivating fishes, amphibians, and reptiles. Unfortunately, no study has addressed the extent by which tissues of birds and mammals respond structurally to daily torpor or to longer periods of dry-season dormancy. Predictably, the magnitude of the morphological response is dependent upon the duration of fasting even if it is for a few days.

If the assumption is made that dry-season torpor for birds and mammals includes bouts of fasting ranging from 24 to 120 h, then laboratory studies on fasted birds and mammals could provide a predicted profile of torpor responses. In a relatively quiet laboratory setting, blackcaps (*Sylvia atricapilla*, Passeriformes) that have been fasted for 48 h experience significant reduction (by 18–45%) in the masses

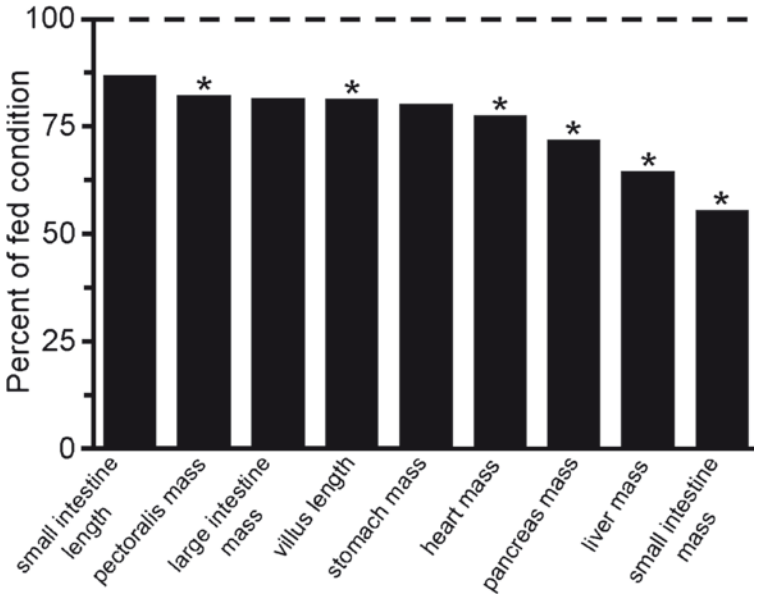

Fig. 9.8 Measures of intestinal morphology and organ masses of 2-day fasted blackcaps (*Sylvia atricapilla*) as a percentage of measurements from fed birds. The 2-day fast resulted in 13–45% reduction in tissue morphology and organ mass, with significant changes noted by an asterisk above the bars. Figure drawn from data presented in Karasov et al. (2004)

of their small intestine, liver, pancreas, heart, and pectoralis muscle (Karasov et al. 2004; Fig. 9.8). These same birds experienced a 20% decrease in intestinal villus length, but no change in the thickness of the serosa/muscularis layer. The intestinal epithelium of fasted birds compared with fed birds exhibited noticeable apoptosis and the shedding of enterocytes into the intestinal lumen (Karasov et al. 2004; Fig. 9.9).

There is a strong body of literature on the fasting responses of laboratory rats (*Rattus norvegicus*). Rats fasted for up to 5 days experience a daily loss of body mass (Fig. 9.10). Independent of the decrease in body mass during food deprivation, rats experience an additional decrease in the masses of the liver, small intestine, and spleen (Chatamra et al. 1984; Goodman and Ruderman 1980; Ju and Nasset 1959; Fig. 9.10). Heart, kidneys, and skeletal muscle of fasting rats do not decrease in mass at the same pace as total body mass, and thus increase in mass, relative to body mass (Chatamra et al. 1984; Goodman and Ruderman 1980). Histological examination of the small intestine of fasted rats have revealed a 28 and 36% decrease in villus length after 3 and 5 days of fasting, respectively (Clarke 1975; Dunel-Erb et al. 2001). The decrease in villus length with fasting is due in part to the narrowing of enterocytes (Dunel-Erb et al. 2001). The villus of fasted rats exhibited a protracted lamina propria, thereby, depriving the tip of the villi of lymphatic and blood supply (Dunel-Erb et al. 2001; Fig. 9.11).

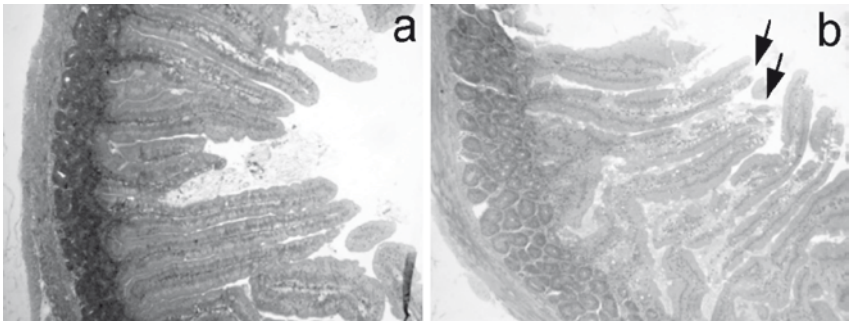

Fig. 9.9 Light microscopy images of the intestinal mucosa of feeding (**a**) and 2-day fasted (**b**) blackcaps (*Sylvia atricapilla*). Two days of fasting results in the shedding of enterocytes from the tip of the villi (noted by *arrows*). Images are from Karasov et al. (2004) and used with permission of the corresponding author

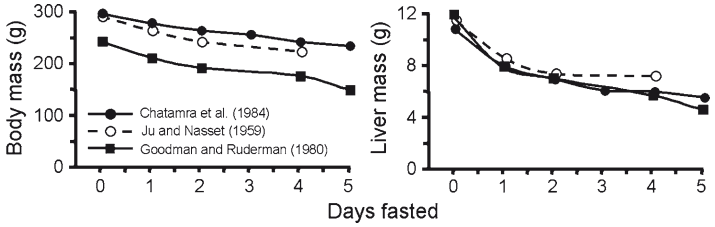

Fig. 9.10 Decrease in body mass and liver mass as a function of days fasted for the rat as reported by Chatamra et al. (1984), Goodman and Ruderman (1980), and Ju and Nasset (1959). A torpor mammal that has not significantly reduced metabolic rate may experience similar decreases in body and organ masses

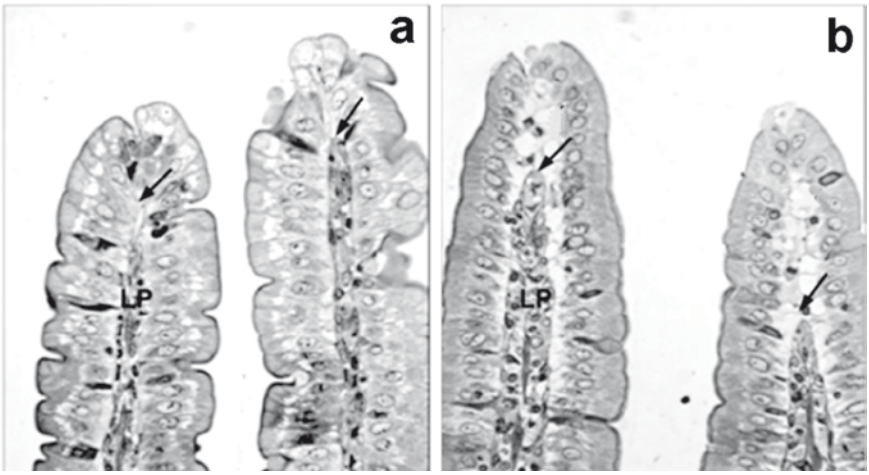

Fig. 9.11 Small intestinal villi from the jejunal region of a fed rat (**a**) and a 5-day fasted rat (**b**). The lamina propria (LP) extends to the tip of the villus for the fed rat, but is retracted from the tip for the fasted rat (noted by arrows). Figure from Dunel-Erb et al. (2001) and used with permission of the corresponding author

Interestingly, rat intestinal microvilli are observed to become thinner and longer with fasting (Dunel-Erb et al. 2001; Waheed and Gupta 1997).

9.6 Integration of Form and Function During Aestivation

The physiological responses to aestivation represent an integration of structural and functional changes occurring at the tissue and cellular levels. In this chapter, we have summarized observed and predicted morphological changes to periods of dormancy induced by hot and dry conditions. Each of these changes can impact cellular, tissue, and whole-animal performance. For example, it has been well recognized that aestivation or torpor is characterized by a depression in whole-animal metabolism (Geiser 1988; Guppy and Withers 1999). Intermingled with cellular mechanisms that reduce tissue metabolism and hence whole-animal energy expenditure (Guppy and Withers 1999; Storey 2002), are structural modifications (e.g., atrophy or the sequestering of membrane pumps) that may aid in reducing the metabolic flux of cells.

A quandary with this line of logic is the apparent disassociation between changes in mass and metabolic rate for some tissues of aestivating anurans. For the aestivating *Neobatrachus pelobatoides,* the metabolic rate of skeletal muscle decreases by 70%, although for the closely related *C. alboguttata*, skeletal muscle experiences no significant change in mass during aestivation (Flanigan et al. 1991; Hudson and Franklin 2002; Symonds et al. 2007). The small intestine of *C. alboguttata* decreases by 77% with aestivation, whereas its mass-specific metabolic rate does not change, the same being true for *N. pelobatoides* (Cramp and Franklin 2008; Flanigan et al. 1991). For both species, the metabolic rate of the small intestine does decrease with aestivation, due to mass loss, but not at the same pace as other tissues. Hence, compared with active frogs, the contribution of intestinal metabolic rate to whole-animal metabolism more than doubles during aestivation (Cramp and Franklin 2008; Flanigan et al. 1991).

The interplay between structure and function during aestivation has best been described for the anuran small intestine. As previously noted for the anurans *C. alboguttata*, *C. ornata*, and *P. adspersus*, laboratory-induced aestivation generates 37–77% decreases in small intestinal mass and 35–50% decreases in microvillus length from the fasted state (Figs. 9.3 and 9.6). For *P. adspersus*, aestivation also results in significant decreases in mass-specific rates of intestinal uptake of the nutrients L-leucine and D-glucose (S. Secor unpublished data). *Ceratophrys ornata* experiences significant declines in uptake rates of L-proline and D-glucose with aestivation (S. Secor unpublished data), whereas *C. alboguttata* experiences no significant reduction in intestinal L-proline uptake rates from fasting to aestivation (Cramp and Franklin 2008). The decrease in small intestinal mass together with any further decline in mass-specific nutrient uptake contributes to 40–90% reduction in the small intestine's total capacity to absorb nutrients with aestivation (Secor 2005). For *C. ornata* and *P. adspersus*, the 58 and 84% respective decreases in carrier-mediated D-glucose uptake may be explained in part by decreases in the functional surface area of enterocytes due to the respective 45 and 35% decrease in microvillus length.

9.7 Conclusion

Two sources of variation that dictate the magnitude of morphological plasticity of organs to aestivation are the duration of dormancy and tissue type. Although there is a clear dichotomy in the duration of aestivation between ectotherms (months and years) and endotherms (days), the longer the episode of dormancy then, predictably, the greater the change in organ structure and function. This phenomenon has been observed for aestivating anurans and for fasting rodents (Chatamra et al. 1984; Cramp et al. 2005; Goodman and Ruderman 1980). Tissues also differ in how they respond to aestivation. A seemingly universal phenomenon of fasting, and hence aestivation, is a decrease in small intestinal mass. But the small intestine itself differs in tissue response, the absorptive epithelium experiences significant atrophy, whereas the smooth muscle/serosa layer remains structurally intact throughout aestivation (Cramp et al. 2005; Secor 2005b). With the studies currently in hand, aestivation does not reduce the mass of the heart, lungs, kidneys, and skeletal muscles (Cramp et al. 2005; Hudson and Franklin 2002; S. Secor unpublished data). The preservation of these tissues during aestivation is hypothesized to ensure adequate performance immediately upon emergence from aestivation (Hudson and Franklin 2002). Even for those tissues that do regress in structure and function during aestivation and fasting, their plasticity is apparent from the rapid upregulation in their morphology and function with refeeding (Cramp and Franklin 2003; Cramp and Franklin 2005; Dunel-Erb et al. 2001; Karasov et al. 2004).

We noted earlier the paucity of published studies describing the morphological responses to aestivation. With the exception of the African lungfish, several anuran species, and a couple of Australian reptiles, there is an absence of detailed ecological accounts of species during aestivation. Many of the accounts of aestivation are anecdotal and provide little information regarding the behavior and physiology of the animal. One impression from reports of aestivation and studies documenting seasonal activity of animals is that, undoubtedly, there are more species that aestivate than currently recognized. This may be, especially, true for many species of amphibians and reptiles that essentially "disappear" for months and even years before reemerging under favorable conditions (Pechmann et al. 1991). Hence, there is ample opportunity to explore the behavior and ecology of aestivation among vertebrates, and to integrate those findings with the examination of the morphological and physiological responses of organs and tissues to aestivation.

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Chapter 10
Water Management by Dormant Insects: Comparisons Between Dehydration Resistance During Summer Aestivation and Winter Diapause

Joshua B. Benoit

Contents

Abstract During summer in temperate regions and tropical dry seasons insects are exposed to extended periods with little available water. To counter this dehydration stress, insects have two options. They can either remain active by utilizing mechanisms to function under severe water stress and high temperatures, or they can escape from the stressful environment by exploiting an aestivation mechanism. During aestivation, insects undergo a variety of molecular and biochemical changes to arrest development, reduce metabolism, tolerate high temperatures, and increase their ability to maintain

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water balance. In this review, I provide a synopsis of known and possible mechanisms utilized by insects to reduce the stress of dehydration during aestivation. Comparative observations of aestivating and diapausing insects are also discussed to assess similarities and differences in the methods used by insects to increase dehydration resistance between these two types of dormancies. Adaptations that alter moisture requirements during diapause (low metabolic rate, increases in osmolytes, shifts in cuticular hydrocarbons, cell membrane restructing) are likely similar to those utilized at the induction and during the maintenance phase of aestivation. Few studies have been conducted on the physiology, particularly the biochemistry and molecular regulation, of aestivating insects, indicating that much more research is needed to fully assess water balance characteristics of insects during aestivation. Whether an insect is in diapause or aestivation, behavioral, biochemical, and physiological adaptations are essential for suppressing water loss and enhancing survival in a desiccated state.

10.1 Introduction

Most insects are capable of developing and reproducing during short periods each year when conditions are appropriate for their growth (Denlinger 1986, 2002). When outside of these optimal conditions, insects have an amazing ability to resist and adapt to fluctuations in their environment for short periods, but when these periods of stress are longer, large scale biological changes are needed (Wolda 1988; Denlinger 2002; Danks 2006). The two most stressful times for most insects are winter (freezing temperatures, lack of water due to its presence as ice, and the absence of food resources) and during the peak of summer or during tropical dry seasons (high temperatures, lack of water due to periods of drought, and inadequate quantities of food). A few insects avoid such periods by migrating to favorable locations, but most species are too small, too slow, or too specialized to engage in long distance flights to new habitats. Thus, most species have mechanisms to tolerate extremely long resting periods under stressful conditions, responses characterized as changes in behavior, reductions in metabolism, prevention of temperature stress, and the maintenance of water balance.

In this chapter, I provide a comparative synopsis of water balance characteristics that occur when insects undergo summer aestivation to tolerate heat and drought and winter diapause to survive low temperature and lack of water. Particularly, I will address behavioral, biochemical, and molecular responses that are involved in water management. I discuss four categories of response: (1) suppressing water loss, including cuticle changes, reductions in metabolic rates and loss of water through regulation of spiracular valves, reducing moisture loss in waste products, and behaviorally by aggregating in favorable microhabitats; (2) increasing the internal water pool by liquid ingestion, water vapor absorption, and/or metabolic water production; (3) reducing the stress of dehydration by the upregulation of stress-related proteins (e.g., heat shock proteins (HSPs)), increasing protective solutes (e.g., trehalose), and partitioning water unevenly into metabolically active tissues;

and (4) exploiting water loss to become anhydrobiotic. Additionally, I provide a brief discussion for the role of water as a cue to initiate and break dormancy.

10.2 Maintenance of Water Balance

Development, distribution, and survival are dependent on the insect's ability to maintain water balance. Simply stated, water balance can be summarized by Wharton's (1985) general equation:

$$m = m_S - m_T$$

Where water mass (m) is controlled by the movement of water into the insect (m_S) and movement of water out of the insect (m_T). Thus, if $m_S > m_T$, the water pool within the insect increases and if $m_T > m_S$, the water pool decreases. The net transpiration rate or water loss rate (m_T) usually relates to where the insect resides, those living in arid environments or environments that lack water for extended periods tend to have low water loss rates (Hadley 1994; Gibbs 2002a). Few insects can tolerate either dehydration or overhydration for extended periods. To use chironomids as examples, the Antarctic midge, *Belgica antarctica*, when held at hydrating conditions ($m_S > m_T$) for extended periods eventually succumbs to severe overhydration (Lopez-Martinez et al. 2009). At the opposite extreme, the sleeping midge, *Polypedilum vanderplanki*, when dehydrated slowly ($m_T > m_S$, Kikawada et al. 2005; Watanabe 2006) can reduce its water pool to exceptionally low levels, but even in this extreme example a small percent (2–3%) of body water is essential to allow the midge to recover following anhydrobiosis. For an insect to survive and develop within a particular environment, water balance ($m_S = m_T$) must be achieved.

10.3 Mechanisms Utilized by Insects to Suppress Transpiration

10.3.1 Cuticular Changes

Readers not familiar with the structure of the insect cuticle can refer to comprehensive descriptions by Hepburn (1985) and Hadley (1994). Briefly, the epicuticle, a thin layer at the external surface of the insect, serves as the interface between the insect and the environment, and underneath is the thick procuticle which represents nearly 95% of the cuticle. The procuticle is divided into three sections, the exocuticle, mesocuticle, and endocuticle, all consisting predominantly of a chitin–protein complex. The exocuticle hardens through sclerotization to provide support to the cuticle, the endocuticle remains soft and highly flexible, and the mesocuticle represents a transition between the endocuticle and exocuticle. Though the epicuticle is significantly thinner than the procuticle, it is responsible for regulating most of the

water flux through the cuticle. The cuticulin layer (or outer epicuticle) and the inner epicuticle layer (or dense layer) account for most of the epicuticle, and both are impregnated with lipids secreted by dermal glands. These lipids, which are mainly hydrocarbons, serve to waterproof the cuticle. The outer epicuticle is coated with a wax layer that is secreted through channels from cells below the procuticle. Lastly, the wax layer for some insect species is protected from the environment by an external cement layer composed of lipoproteins. Slight variations in the organization, structure, and composition of the cuticle can significantly alter the waterproofing capacity of the insect cuticle.

Water lost through the cuticle accounts for a significant portion of the total water lost by insects (Hadley 1994; Chown and Nicolson 2004). Commonly, species that reside in moist habitats have highly permeable cuticles and those that live in more arid areas have cuticles resistant to water flux (Edney 1977; Hadley 1994; Gibbs 1998; Bradley et al. 1999; Gibbs and Matzkin 2001). Three possible changes in the cuticle occur to alter water loss: (1) procuticle restructuring, (2) reorganization or changes in the epicuticle, particularly the lipids; and/or (3) the presence of a chorion or pupal case.

Changes in the procuticle have been well documented, but the involvement for this portion of the cuticle in reducing water loss has not been thoroughly studied (Hadley 1994). Some studies have suggested that the structure of the endocuticle may significantly alter the water movement across the cuticle (Machin and Lampert 1987; Hadley 1994). Increases in scleritization of the cuticle could increase the density of the procuticle, thus improving water retention (Benoit et al. 2005). There is also recent speculation that cuticular proteins are involved in water regulation, but this has not yet been documented conclusively. Currently, no studies have documented changes in the procuticle during aestivation or diapause.

The best-known suppressor of water loss is cuticular lipids located on the epicuticle, particularly the wax layer (Blomquist et al. 1987; Hadley 1981; Lockey 1988; de Renobales et al. 1991; Hadley 1994; Gibbs 1998; Gibbs 2002b). The composition of the epicuticular lipids varies significantly between species, but the dominant constituents are nonpolar, hydrophobic compounds such as hydrocarbons and their derivatives (Blomquist et al. 1987; Hadley 1994; Gibbs 1998; Gibbs 2002b). It has been well-documented that disturbing the epicuticular lipids alters water loss rates of insects and other terrestrial arthropods (Noble-Nesbitt 1991; Hadley 1994), thus their importance as a hydro-insulator is well established. Increases in the amount of cuticular hydrocarbons results in lower cuticular water loss rates (Hood and Tschinkel 1990; Yoder and Denlinger 1991; Hadley 1994; Benoit and Denlinger 2007; Benoit et al. 2008a), and such increases in cuticular lipids have been well documented during diapause for many insects (Yoder and Denlinger 1991; Benoit and Denlinger 2007). In addition to changes in the amount of cuticular lipids, the composition can also change with long-chained, saturated lipids with few methyl side chains acting as a more effective water barrier (Hadley 1994; Gibbs 1998; Benoit et al. 2007a). In most cases, the differences in the cuticular hydrocarbons are the consequence of rearing or storing insects under different conditions. For example, summer-acclimated beetles have more long-chain hydrocarbons than winter-acclimated

beetles, and cuticular lipids change during thermal acclimation in grasshoppers (Hadley 1977, 1994; Gibbs and Mousseau 1994). Thus, it is likely that aestivating insects have modified profiles of epicuticular lipids, but I am not aware of any experiments that have explicitly tested this hypothesis.

Other characteristics that involve the relationship between lipids and water loss are activation energy (E_a) and critical transition temperature (CTT) (Hadley 1994). These factors define how temperature influences water loss and cuticle changes (Toolson 1978; Rourke and Gibbs 1999; Gibbs 2002b; Yoder et al. 2005a,b). For many years it was assumed that the CTT involved a transition or melting of lipids that increased water loss rates (Wharton 1985; Gibbs 1998; Hadley 1994). Recent observations on CTT indicate that it does not correlate with increased water loss for many insects, is far above the lethal temperature and may be influenced by factors in addition to lipids; in combination these factors indicate that the CTTs may be an artifact that has no ecological relevance (Gibbs 2002b, Yoder et al. 2005a,b). Thus, the relevance of the CTT and E_a are in limbo, but trends have been observed. Insects with a low E_a are more resistant to water loss with increasing temperatures, and those with a CTT are actively suppressing water loss until a particular temperature is reached (Yoder et al. 2005b; Benoit et al. 2008a).

Additional water-proofing barriers can also occur, particularly during diapause and aestivation. The two most common examples are found in insects that are dormant as eggs or pupae. Insects that undergo dormancy as pharate first-instar larvae within the chorion of the egg are extremely resistant to dehydration. This is likely due to the presence of two water proofing barriers: the egg chorion and the larval integument. This high resistance to water loss has been demonstrated in many insect eggs (Krysan et al. 1977; Yoder et al. 2004; Roberts 2004; Benoit et al. 2007b) and is particularly striking in mosquito eggs that undergo diapause (Sota and Mogi 1992). For fly pupae, the puparium serves a similar function as the egg chorion; it provides an additional layer that helps to suppress water loss. Also, it is important to note that the inside of the puparium or chorion may be coated with additional lipids, providing even more resistance to water loss (Yoder et al. 1992).

10.3.2 Respiratory Water Loss and Metabolism Reduction

Along with water lost through the cuticle, respiration represents a secondary route usually accounting for 5–20% of the total water loss for most insect (Hadley 1994; Chown 2002). In comparison to hydrophilic species, insects residing in arid regions usually lose a higher proportion of water through respiration, up to 70%, than through their highly water-proofed cuticle (Hadley 1994; Chown 2002). During respiration, water is lost rapidly when the spiracles are open due to the humidity gradient within the tracheal system, which is very high, and the external environment, which is very low, thus causing an outward water flux (Hadley 1994). Water loss through the spiracles can be suppressed by opening within an internal cavity (Ahearn 1970; Benoit et al. 2005); such is the case for beetles with fused-elytra or

elytra that rarely open, such as in the tenebrionid, *Eleodes armata* (Ahearn 1970; Cloudsley-Thompsen 2001) and the spider beetle, *Mezium affine* (Benoit et al. 2005). Another mechanism is to locate the spiracles closely together. This method increases the local humidity around the spiracle cluster, which in turn suppresses water loss through interference (Pugh et al. 1988). Both of these structural arrangements retard water flux out of the spiracles.

The most prominent mechanism to reduce respiratory water loss is the most simple, closing the spiracles. By closing or even partially blocking the spiracles, water loss, particularly at low relative humidities, is reduced (Bursell 1957; Hadley 1994; Chown 2002). Discontinuous gas exchange (DSC) can also reduce water loss through the spiracles by limiting gaseous diffusion to short periods when carbon dioxide accumulates to high levels (Lighton et al. 1993; Hadley 1994). Recent research has questioned the ability of DSC to reduce water loss, indicating that more studies are needed to determine exactly how DSC relates to water loss suppression (Sláma 1999; Chown 2002). Some of the arguments against DSC are that it is abandoned during periods of water stress, respiratory water loss only accounts for a small portion of water loss and that ability of DSC to limit water loss is dependent on increasing the PCO_2 to the highest levels, which does not occur naturally (Chown 2002). Arguments for DSC are other factors such as hemolymph pH and oxygen demand may be more critical under dehydrating conditions than water retention due to changes associated with declining hemolymph volume, thus respiration increases during dehydration (Chown 2002). Even so, without spiracle control water loss rates would be higher (Bursell 1957; Lighton 1996; Sláma 1999; Chown 2002). One of the hallmarks of dormancy is the suppression of metabolic rate, which allow spiracles to remain closed for extended periods (Hadley 1994; Storey 2002; Denlinger 2002). In particular, flight is a period when water is lost rapidly through respiration (Lehmann 2001; Chown and Nicolson 2004), and flight is usually reduced during adult diapause (Denlinger 1986, 2002).

10.3.3 Excretory System

The elimination of waste is another conduit for the loss of water. The alimentary canal is responsible for the internal regulation of salt and water levels. The regions of the alimentary canal that regulate a majority of the fluid levels are the Malphigian tubules and the hindgut, divided into the ileum and rectum. Many studies have focused on the role of the alimentary canal on water and osmotic regulation, as reviewed by Bradley (1985) and Chown and Nicolson (2004). Briefly, water, along with organic molecules, particularly urine and ions, are absorbed from the hemolymph into the upper portion of the Malphigian tubules, and the urine is then actively concentrated in the lower portion of the tubules. The hindgut, particularly the rectum, acts as the primary site for the readsorption of water and select solutes. Insects that are dehydrated or reside in dry regions generate extremely hyperosmotic rectal products as a result of efficient water reabsorption, while aquatic species, those

living in moist regions, or those feeding on water-diluted fluid have hyposmotic rectal products. In some larval Coleoptera, Diptera, and Lepidoptera, Malphigian tubules are not free floating, but instead the distal ends of the tubules are physically attached to the rectum; this arrangement known as the cryptonephridial system allows the feces to be dried extremely efficiently and in some species is associated with rectal water vapor uptake (Hadley 1994).

Secretion and absorption by the Malphigian tubules and hindgut reabsorption are regulated by neuropeptide hormones: diuretic hormones cause the secretion of water into the alimentary canal, thus increasing the net water loss, while antidiuretic hormones act in the opposite direction to retain water. Many of the neuropeptides involved in water balance are summarized in papers by Coast et al. (2002), Riehle et al. (2002), Gäde (2004), and Coast (2006). Some key examples are calcitonin-like peptides, corticotropin-releasing factor related peptides, insects kinins, and cardioacceleratory peptides, which all function as diuretic hormones, and chloride transport-stimulating hormone and an ion-transport process peptide that serve as antidiuretics (Coast 2006). The presence and absence of these hormones regulate urine production and subsequent reaborption of the lost water. Studies involving aestivation and diuretic/antidiuretic hormones are currently lacking, but I suspect such hormones, particularly antidiuretics, may be involved in regulating water loss during dormancy.

10.3.4 Nitrogenous Waste

Uric acid is the dominant nitrogenous waste product produced by terrestrial arthropods, but guanine and other closely-related nitrogenous products are used by ticks and spiders (Hadley 1994; Benoit et al. 2008b). Why use uric acid rather than urea or ammonia? Ammonia is highly toxic and highly soluble, thus requiring insects to immediately expel this waste product, a response that would require large quantities of water; this metabolic end product is thus usually restricted to aquatic insects. Although urea is significantly less toxic than ammonia, it still has to be eliminated in solution. Increases in urea have been linked to increased temperature resistance (Storey 2004), thus accumulation of this molecule may be useful under certain environmental situations. Uric acid is the least toxic of the potential waste products for insects, and due to its low solubility, excretion of a nearly dry waste product is possible. Also, due to its low toxicity, uric acid can be accumulated within the body of the insect (storage excretion), a situation that completely prevents a loss of water by defecation. This is especially important for insects that aestivate as eggs or pupae since defecation does not normally occur during these stages. Uric acid accumulation occurs in adult insects as well, and has been documented in a few types of bugs, including the shield bug, *Parastrachia japonensis*, during diapause (Kashima et al. 2006). Some insects contain microbes that generate uricase, an enzyme that breaks down uric acid, thus allowing uric acid to be recycled (Sasaki et al. 1996; Kashima et al. 2006). The reduced metabolism associated with

dormancy also means that less nitrogenous waste is produced, thus insects in dormancy generally defecate less or not at all and if they do defecate they utilize uric acid, two features that maximize water conservation.

10.3.5 Behavioral Changes

Since aestivation is characterized by heat and water deprivation, changes in insect behavior are frequently involved in the response. The simplest response is to move into a more favorable region or to hide in protective harborages until favorable conditions return (Denlinger 1986). Long-distance migrations occurs in only a few insect species (e.g., Monarch butterflies), thus retreating to a near-by protected refugia is the most common response. Within these protective sites the relative humidity is usually higher and the temperature lower, both of which reduce water loss. The protective capacity of these sites can be enhanced by construction of a nest or by lining their microhabitat with silk or wax (Roubik and Michener 1980; Denlinger 1986; Kikawada et al. 2005). In many cases, plants can be altered by insect (e.g., galls) to generate small protective harborages (Chown and Nicolson 2004). Aggregation is also a frequent behavior response associated with dormancy. Formation of an aggregation increases the local humidity, suppressing water loss for members of the group (Yoder et al. 1993; Benoit et al. 2005, 2007b, 2007c). For example, adults of the tropical beetle, *Stenotarsus rotundus*, form aggregations of up to 70,000 individuals at the base of a palm tree in Panama (Wolda and Denlinger 1984; Tanaka 2000). As the group size increases, metabolic rate drops and water conservation is enhanced (Yoder et al. 1993). These benefits gained by forming an aggregation are likely adaptive advantages behind the frequent occurrences of aggregations during periods of aestivation.

10.4 Increases in the Water Pool during Aestivation and Diapause

10.4.1 Ingestion of Water

Ingestion of water represents the main route used by terrestrial insect to replenish their water content (Hadley 1994). Many insects simply drink free standing water to rehydrate, and this is usually regulated by hemolymph volume (Chown and Nicolson 2004). For fluid-feeding insects, as with those that live in saline environments, presence of excess ions (K^+ in plant phloem and Na^+ in vertebrate blood and salt water) ingested needs to be removed (Wharton 1985; Hadley 1994). For blood feeders such as the bed bug, *Cimex lectularius*, blood represents the only source of water between long periods of quiescence (Benoit et al. 2007c). Moisture contained within solid food is sufficient to replace water stores for certain

insects. For example, food is the only water source for the spider beetle, *M. affine*, a species that survives on the water present in dry stored grain throughout its entire development (Benoit et al. 2005). Some insects retain access to free water during diapause if their habitat temperature remains above freezing and their dormant stage is capable of movement; e.g., females of the northern house mosquito, *Culex pipiens* (Benoit and Denlinger 2007). But, for many other overwintering species or aestivating species, water may be inaccessible and certain stages such as eggs and pupae do not have the capacity to imbibe water. Thus dormancy in such stages eliminates drinking as an option. Other insects, particularly tenebrionid beetles, ingest massive amounts of water that may push their water content over 70% before entering extremely dry periods (Hadley 1994; Zachariassen and Pedersen 2002).

10.4.2 Metabolic Water Production

Metabolism of food resources generates water and this water source is immediately transferred to the water pool of the insect (Edney 1977; Hadley 1994). The amount of water released in this manner is dependent on the biomolecules utilized: fat releases the most water (1.07 ml g^{-1}), followed by carbohydrates (0.56 ml g^{-1}) and proteins (0.40 ml g^{-1}). Even though fat metabolism releases the most water, water is also utilized in the metabolism of fat, thus metabolism of sugar, particularly when stored as glycogen, is the most efficient form of metabolism for releasing water (Loveridge and Bursell 1975; Hadley 1994; Chown and Nicolson 2004). The contribution of metabolic water is rather small for most insects, but for species with extremely low water loss rates and those in flight the amount is proportionally higher. This proportional increase has been noted in a few species of desert-dwelling and dry-adapted beetles. For two tenebrionids (*E. armata* and *Cryptoglossa verrucosa*) and a spider beetle (*M. affine*), water generated by metabolism contributed substantially to their water pool during their prolonged exposure to dehydrating conditions (Cooper 1985; Benoit et al. 2005). Metabolic water production can be increased only if ATP accumulation can be reduced (Hadley 1994). This can be achieved by decoupling within the mitochondria to prevent the conversion of ADP to ATP, but this has been demonstrated only in wax moth larvae (*Galleria mellonella*, Jinda and Sehnal 1990) and is not considered by Hadley (1994) to be true for most insects. During periods of aestivation, water is unavailable for extended periods, thus I assume utilization of metabolic water is especially important at this time, yet there are actually few examples documenting the use of metabolic water by terrestrial arthropods during dormancy periods (Dautel 1999; Hadley 1994; Danks 2000). Most likely the paucity of examples reflects the fact that this question has not been adequately addressed. Also, since it is more efficient to retain water than generate water metabolically, increasing metabolism solely to generate water would deplete food reserves that are critically needed to survive extended dormant periods (Danks 2000). Thus, I suspect that water generated during aestivation is likely a small but vital byproduct of metabolism that contributes to the water pool during dry periods.

10.4.3 Water Vapor Absorption

Quite a few arthropods, including acarines, beetles, collembolans, fleas, grasshoppers, walking sticks, flies, lice and isopods, are able to absorb water from subsaturated air (<99% RH, Hadley 1994). A relatively complete list of arthropods capable of absorbing water vapor from the air is presented by Edney (1977), with later examples listed by Hadley (1994). Water vapor absorption is particularly important for dormant stages such as eggs, pupae and other stages that lack the ability to ingest free water (Danks 2000). Examples include diapausing flesh fly (*S. crassipalpis*) pupae (Yoder and Denlinger 1991), walking stick (*Extatosoma tiaratum*) eggs (Yoder and Denlinger 1992) and larvae of the ectoparasitoid, *Nasonia vitripennis* (Yoder et al. 1994). Although *S. crassipalpis* and *N. vitripennis* are both examples of species with temperate zone diapause, the example of the walking stick diapause is a subtropical example with an aestivation diapause, and indeed this species is able to absorb water vapor at relative humidities as low as 30% RH, the lowest reported critical equilibrium humidity (CEH, lowest threshold where water movement into and out of the insect is equal). Water vapor absorption is either active (requiring energy input) or passive (Hadley 1994; Bayley and Holmstrup 1999; Danks 2000). It is important to note that passive absorption occurs at all relative humidities and requires no energy, but it cannot counter water loss unless the relative humidity is at saturation (100% RH, Hadley 1994; Chown and Nicolson 2004). Potential sites utilized by insects for active water vapor absorption are the mouth and anus, and rarely water may be absorbed directly through the cuticle (Hadley 1994; Bayley and Holmstrup 1999). Oral and anal sites utilize hyperosmotic or hygroscopic secretions to absorb water (Knülle 1984; Hadley 1994). In the case of Collembola, absorption through the cuticle involves the accumulation of osmolytes (Bayley and Holmstrup 1999). Thus far, the mechanisms of water vapor absorption have been most extensively studied for nondormant insects, but it is likely that similar mechanisms observed will be evident in aestivating or diapausing insects. Larvae, particularly beetle and lepidopteron larvae, utilize rectal uptake mechanisms. (Edney 1977; Hadley 1994). Adult insects predominantly use oral mechanisms (Hadley 1994). Exactly how eggs and pupae absorb water vapor is currently unknown. Possibly pharate larvae and adults ingest or absorb water that has condensed on the inside of the chorion or puparium, but this remains speculation. Thus, the only aestivating insect known to absorb water from subsaturated air is the Australian walking stick, *E. tiaratum*, which aestivates in the egg stage but quite likely many more examples will be found as the study of insect aestivation expands.

10.5 Mechanisms to Reduce Water Stress

10.5.1 Protein and Molecular Changes

Protein and molecular responses common during dehydration are likely essential to insects during aestivation, but few insect studies have focused on this topic. In terrestrial snails, several stress-related proteins (HSPs, etc.) change in abundance as a result of

aestivation and appear to prevent dehydration-induced stress and mortality (Reuner et al. 2008). Insects may have similar responses during aestivation, but little is known. It is likely more efficient to upregulate proteins to prevent damage rather than repair damage after it has already occur (França et al. 2007; Lopez-Martinez et al. 2008, 2009).

Hsps are among the most studied proteins in relation to water balance (Tammariello et al. 1999; Bayley et al. 2001; Hayward et al. 2004; Sinclair et al. 2007; Lopez-Martinez et al. 2008, 2009). Three types of Hsps (smHsp, Hsp70, and Hsp90) have been linked to dehydration and diapause in insects, but no studies have been conducted on aestivating insects thus far. Many insects produce Hsps continually during diapause (Rinehart et al. 2007), but whether this is a component of aestivation is unknown. Nondiapausing insects also express one suite of Hsps during dehydration and a different suite during rehydration (Hayward et al. 2004; Lopez-Martinez et al. 2009). The increase of Hsps either prevents stress damage by acting to prevent unwanted biochemical interactions, by repairing protein damage or prompts the disassembly of proteins damaged by dehydration (Parsell and Lindquist 1993; Feder and Hofmann 1999; Rinehart et al. 2007). Recently, we have shown that Hsps are essential for insects to reach their normal dehydration tolerance by blocking their expression with RNA interference (Benoit et al. 2009b). Late embryogenesis abundant proteins (LEAs) seem to act by stabilizing the structure of proteins as the water content within the insect declines (Kikawada et al. 2008). Antioxidant enzymes, such as catalase and superoxide dismutase (SOD), are elevated during dehydration, presumably to reduce damage from oxygen radicals formed from desiccation-induced stress (França et al. 2007; Lopez-Martinez et al. 2008, 2009). Changes in proteins involved with the membrane and cytoskeleton may be fairly common in relation to dehydration (Li et al. 2009). One of the main functions of proteins involved with the cell membrane is to restructure the membrane to reduce water movement out of the cells as the hemolymph osmolality changes. Cytoskeletal protein changes serve to stabilize the cells during the pressure and size changes caused by the osmotic stress of dehydration (Li et al. 2009).

10.5.2 Osmolality, Solutes and Regulation of Size and Volume

Insect hemolymph osmolality, measured in mOsm kg^{-1}, ranges between 100 to 1,400 mOsm kg^{-1} and for most insects is around 400–500 mOsm kg^{-1} (Hadley 1994). It is important to note that increasing osmolality, even 2–3×, reduces water loss only slightly, and the net water flux out of the insect persists unless the individuals are in an environment near saturation or above their CEH (Willmer 1980; Chown and Nicolson, 2004). The alimentary canal efficiently regulates ion content, and usually maintains osmolality within 200–300 mOsm kg^{-1} range for most insects that reside in mesic and xeric regions, without causing internal damage. In some cases, insects sequester ions as insoluble forms, usually in the fat body, during dehydration and these ions can be release when the hemolymph volume increases during rehydration, effectively buffering the osmolality changes within the insect (Chown and Nicolson 2004). Additionally, combining free fatty acids and amino acids into large insoluble molecules can be used to regulate osmolality.

Poor osmoregulators usually have osmolalities that vary nearly 1000 mOsm kg^{-1} (Hadley 1994; Elnitsky et al. 2008), and such insects usually reside in moist microhabitats. Osmolality fluctuations are influenced by a variety of molecules, including, but not limited to, salts (NaCl, KCl), polyols (glycerol), sugars (trehalose), free amino acids (proline, etc.), and free fatty acids.

Some of these molecules that increase during dehydration have protective qualities (Goyal et al. 2005; Benoit et al. 2009a). Trehalose and glycerol are two of the most common molecules that can suppress water loss and reduce stress (Yoder et al. 2006; Watanabe 2006). Trehalose is especially important during severe dehydration by preventing unwanted protein interactions, decreasing metabolism by altering fluid dynamics and protecting proteins and cellular membranes (Crowe et al. 1992; Suemoto et al. 2004; Goyal et al. 2005; Yoder et al. 2006; Benoit et al. 2009a). Additionally, it is important to note that glycerol and trehalose act to prevent heat and cold stress, resulting in cross-tolerance between temperature and dehydration stress (Yoder et al. 2006; Benoit et al. 2009a). Proline, as a free amino acid, may have similar effects (Yancey 2005; Ignatova and Gierasch 2006). This amino acid has been documented to increase during stress in a few insects (Michaud and Denlinger 2007; Michaud et al. 2008), but future studies are needed to determine the exact function of proline within insects during dehydration. Dehydration-induced changes have also been documented for glucose and sorbital (Hadley 1994; Elnitsky et al. 2008). Currently, only two studies have looked for changes in protective osmolytes during aestivation. In the case of the blossom weevil, *Anthonomus pomorum*, trehalose was increased (Koštál and Šimek 1996), but no changes in osmolytes were noted in Mediterranean tiger moth, *Cymbalophora pudica* (Koštál et al. 1997).

Another factor that contributes to regulation of osmolality and water content is volume regulation and/or compartmentalization (Zachariassen and Einarson 1993; Hadley 1994; Zachariassen and Pedersen 2002). For example, a significant portion of water may be lost from one water pool (i.e., the hemolymph), but water in another region of the body (e.g., salivary glands and midgut) may remain relatively constant. Typically, water in the tissues is conserved at the expense of the hemolymph. This is particularly intriguing since the osmolality of the hemolymph is usually higher than within the cells during dehydration. Even though the exact mechanisms for retaining water in a tissue at the expense of the hemolymph are not known it is extremely important in order to retain the integrity of biologically active tissue in the body. Overall, much of the stress induced by dehydration can be reduced by regulating osmolality and water pools of the insects.

Body size change represents a mechanism that can increase dehydration tolerance of insects. A key example is provided by the experiments of Gibbs and colleagues on adaptations of *Drosophila* for dehydration resistance. In these studies, dehydration was used as a selective mechanism for *D. melanogaster*, and it was determined that increased water content (over 30%), along with respiratory changes, were the factors that increased dehydration resistance (Gibbs et al. 1997; Folk et al. 2001). Of interest, is the fact that size changes are not present in cactophilic, desert-adapted *Drosophila* when compared to mesic species, thus raising the question whether size changes are ecologically relevant or can only be used for interspecies comparisons

(Gibbs and Matzkin 2001). Size changes have also been noted during dormancy in insects. The size of the northern house mosquito, *C. pipiens*, increases substantially in preparation for the winter (Benoit and Denlinger 2007). Interestingly, the water pool does not increase; instead this represents an increase in the dry mass, but even so, the decrease in the surface area to volume ratio reduces water loss (Benoit and Denlinger 2007). Increasing body size would be a fairly simple mechanism for increasing dehydration resistance in aestivating insects.

10.5.3 Membrane Restructuring

As mentioned in Sect. 5.1, membrane changes can result in response to dehydration or in preparation for dormancy (Holmstrup et al. 2002; Tomčala et al. 2006). Only a few such studies have been conducted in general and even fewer on insects. Of interest, dehydration-induced changes are minor in comparison to membrane restructing that results from the induction of dormancy (Michaud and Denlinger 2006; Tomčala et al. 2006). Nearly all of these studies have focused on cold tolerance or the diapause syndrome (Koštál et al. 2003; Michaud and Denlinger 2006; Tomčala et al. 2006), but I expect that such changes also occur during aestivation. Most responses during diapause involved changes in glycerophospholipids that improve membrane fluidity and improve cold tolerance.

10.6 Extreme Dehydration Tolerance (Anhydrobiosis)

Anhydrobiosis as a mechanism to survive dry seasons deserves special attention. A loss 30–40% of body water content causes death in most insect (Hadley 1994). However, a few species can survive even after losing over 70% of their water content, and anhydrobiotic species can lose over 95% of their water content and still survive (Crowe et al. 1992; Danks 2000; Watanabe 2006). Most organisms capable of anhydrobiosis are relatively small, such as tardigrades, springtails and chironomid larvae (Watanabe 2006). Nearly all insects capable of losing over 70% of their water content are larvae within the midge family, Chirnomidae (Suemoto et al. 2004; Watanabe 2006; Benoit et al. 2007a). Thus, the remaining discussion in this section focuses predominately on the mechanisms used by chironomid larvae to tolerate extreme dehydration.

For midge larvae to tolerate high levels of dehydration, a particular sequence of changes has been noted. First, water loss has to occur at a relatively controlled rate, which is done so by decreasing cuticular permeability, aggregating, or building structures to reduce water loss (Watanabe 2006). After dehydration reaches a certain point, high concentrations of certain ions trigger the synthesis of trehalose and other protective molecules such as glycerol and free amino acids (Watanabe 2006). Slow dehydration yields more osmolytes than rapid dehydration, which is the result of more time available to respond to dehydration stress (Kikawada et al. 2005;

Benoit et al. 2007a, 2009a). Within the insects, aquaporins regulate water movement between compartments (Kikawada et al. 2008). These channel proteins seem to be particularly important in tandem with cells accumulating trehalose, ensuring that vitrification and water-replacement occurs properly. Additionally, LEA and Hsp genes are upregulated during this time to avoid protein denaturation and aggregation (Watanabe 2006; Kikawada et al. 2008). Throughout this entire process, metabolism is suppressed to prevent oxidative stress and respiratory water loss (Benoit et al. 2007a, Lopez-Martinez et al. 2008). Oxidative stress proteins such as catalase and SOD may be increased to reduce dehydration-induced oxidative stress. Normal biological activity is resumed once larvae become rehydrated due to the presence of liquid water.

Overall, this ability to tolerate complete dehydration allows larvae to reside in seasonal water pools. When the dry season begins, these habitats quickly desiccate and the larvae respond accordingly. These responses by the larvae to dehydration prevent subsequent damage from heat and other stresses (Watanabe 2006; Benoit et al. 2009a). For *P. vanderplanki*, this cross tolerance is extremely important with temperatures in the African dry season pools reaching 60–70°C (Watanabe 2006). Cross tolerance is also evident in *B. antarctica*: dehydrated larvae are more tolerant to high Antarctic temperatures (30°C; Benoit et al. 2009a). Thus, high levels of dehydration allow chironomid larvae to survive a range of environmental stresses.

10.7 Water as a Development Cue

Moisture serves as a cue for breaking aestivation in a number of tropical insects after a long period of water scarcity (Masaki 1980; Denlinger 1986; Pires et al. 2000). Although water may be commonly used to break aestivation, it is used less commonly to initiate aestivation. Waiting until the dry season arrives may be too late to program the entrance into dormancy. Thus, other triggers such as temperature or photoperiod serve more commonly as the cues for the onset of aestivation. One key aspect is that a single, short exposure to water or high humidity does not usually cause termination of dormancy; termination more frequently requires a sustained exposure to wet conditions (Denlinger 1986; Watanabe 2006). Brief rains, which are not infrequent during the dry season, can replenish water stores, but using a brief rain to terminate aestivation could be disastrous if the favorable conditions prompted by the onset of rainy season have not been initiated. One way to prevent this accidental break is by suppressing moisture-triggered development until a fixed period of latency has elapsed (Denlinger 1986; Pires et al. 2000).

10.8 Conclusions and Future Directions

Since maintaining water balance is such a high priority for insects, it is not surprising that a variety of methods are used to accomplish this task, as shown in Table 1.

Table 10.1 Factors that influence water balance characteristics of insects during dormant periods. + indicates it has been documented, (+) indicates it has not been demonstrated, but it will likely be established based on related insect physiology studies

	Aestivation	Diapause
Suppression of water loss		
Reduced respiration	+	+
Spiracle control	+	+
Increased cuticular lipids	(+)	+
Cuticle modifications	(+)	+
Presence of 2nd barrier	+	+
Size increases	(+)	+
Dry waste	+	+
Water intake		
Water vapor absorption	(+)	+
Metabolic water production	+	+
Ingestion of free water	+	+
Stress-reduction		
Proteins		
Heat shock proteins	(+)	+
Late embryo genes is abundant	(+)	(+)
Antioxidant	(+)	+
Cytoskeletal	(+)	+
Protective or colligative solutes		
Glucose	+	+
Glycerol	+	+
Proline	(+)	+
Sorbital	(+)	+
Trehalose	+	+
Membrane alterations	(+)	+
Osmolality regulation	+	+

As Table 1 indicates many of the same methods are likely used during aestivation and winter diapause, and I suspect that responses during aestivation and winter diapause will be seen as even more similar when more experiments have been completed. Why are the responses of diapausing and aestivating insects so similar? During winter (diapause) and summer (aestivation), the stresses in respect to moisture requirements are similar: a lack of free water and food resources and low ambient relative humidity. The only major difference is temperature. While temperatures are low during the winter resulting in lower water loss rates, aestivating insects are exposed to high temperatures, which further reduce their ability to retain water. Additionally, only certain biological factors can be altered without expending considerable energy, thus, overlap between the responses of aestivation and diapause is even more likely. Due to higher temperatures, aestivation, particularly during the dry seasons in tropical regions, is likely a more challenging period to maintain water balance than winter diapause even though both types of dormancy use similar methods to increase dehydration resistance.

The paucity of research on insect aestivation indicates a clear need for more focus on this topic if we are to understand the mechanisms involved in water balance of dormant insects. Particularly, studies that focus on environmental cues that trigger aestivation-based changes in behavior and physiology are required. This is not an easy task. To use insect diapause as an example, many groups have focused on triggers, changes and termination of diapause, but considerably more research is still needed to elucidate the fine points of these mechanisms (Denlinger 2002). With the considerable overlap noted in the water requirements of winter diapausing and aestivating insects, it is likely that other physiological similarities exist. Based on this observation, it is reasonable to use research on winter diapause as a baseline for expanding research on aestivation. Previous papers by Denlinger (2002) and Koštál (2006) discuss changes in diapause that could be utilized for comparative work on aestivation.

Responses of other aestivating organisms, such as snails, anurans, and fish (Storey 2002, 2004) have been studied more thoroughly, and offer potential insights that may also be applicable to insect aestivation. To reduce water stress, mechanisms such as reduced respiration and metabolism, increased cuticle barriers, changes in osmolality, and the ability to tolerate losing a high proportion of water content are used by organisms such as snails and anurans (Storey 2002). Many proteins have been studied for noninsect aestivating organisms (Storey 2002), and comparative studies could be beneficial for elucidating protein expression themes for insects.

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Chapter 11
Diapause and Estivation in Sponges

Stephen H. Loomis

Contents

Abstract Sponges can be found in fresh or saltwater habitats. As part of their lifecycle, many sponges produce gemmules as a means of surviving environmental challenge. In most sponges, the gemmules contain cells that are initially in a state of metabolic arrest that is controlled by endogenous factors. This state is known as diapause. Following a period of exposure to unfavorable conditions, the cells in the gemmule transit from diapause into a state known as quiescence in which metabolic depression is controlled by environmental factors. When favorable conditions return, the gemmules germinate and produce a new sponge. Production of gemmules is triggered by environmental factors such as decreased temperature or desiccation and involves cell aggregation of thesocytes and the laying down of the gemmule coat. Thesocytes contain yolk platelets as an energy store and high concentrations of polyols that maintain high osmotic concentration in the cells of the gemmules. The high osmotic concentration maintains metabolic depression and turns off cell division. It is the inability to reduce the osmotic concentration that maintains the gemmules in diapause. Transition to quiescence requires the ability of the cells in the gemmules to convert the polyols to glycogen, and thus reduce the osmotic concentration. At this stage, the cells are able to reduce osmotic concentration but do not until favorable conditions return. Early in the germination process, the polyols are

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converted to glycogen, reducing the osmotic pressure and releasing the inhibition of cell division and metabolic rate. Both cell division and metabolic rate increase eventually leading to germination of the gemmules and production of a new sponge.

11.1 Introduction

Sponges are diploblastic, benthic, filter-feeding organisms that are an important ecological component of a wide variety of habitats world-wide. They can be found in fresh water, estuarine, and marine habitats, and can be present in intertidal, shallow, and deep-sea environments (Worheide et al. 2005; Hooper and van Soest 2002). Marine sponges are common in Antarctica (Dayton et al. 1974; Worheide et al. 2005), temperate regions (Freeman et al. 2007; McDougal 1943; Thakur and Müller 2004), the tropics (Wiedenmayer 1977; Schmahl 1990; Sterrer 1986), and even the Arctic (Plotkin and Boury-Esnault 2004). Likewise, freshwater sponges can be found in ponds, streams, and bogs from the Arctic through the tropics (Frost 1991; Ricciardi and Reiswig 1993; Droscher and Waringer 2007; Manconi et al. 2008). Sponges can represent the predominant benthic component of marine (Freeman et al. 2007) and freshwater ecoystems (Frost 1978). Sponges are important competitors, can provide a food source for other organisms, are known to be involved with a variety of symbiotic relationships (Wulff 2006), play an important role in nutrient cycling and primary production (Frost 1978; Frost and Williamson 1980), and are increasingly known for the presence of bioactive compounds that may be useful in medicine (Thakur and Müller 2004). Recently, sponges have been used to help decipher evolutionary relationships among the metazoans (Maldonado 2004; Worheide et al. 2005).

Despite their biological importance, relatively little is known about the physiology and biochemistry of sponges, especially, as it relates to their ability to enter the states of diapause and quiescence (Casceres 1997; Guppy and Withers 1999; Hand and Podrabsky 2000). Sponge life cycles can vary from species to species; however, a "generalized life cycle" (Fig. 11.1) helps to introduce the concepts of quiescence and diapause and how they relate to the life history of sponges. Adult sponges quickly grow during favorable environmental times when they lay down most of their biomass and undergo sexual reproduction. When the environment becomes less favorable (during times of lake draw-down or as the temperature drops in the fall in temperate regions, for example), many sponges produce asexual reproductive structures called gemmules.

Gemmules contain one type of cell (thesocytes) packaged in collagenous and glass (spicule) capsules that are left behind when the adult sponge dies. In many species, when the gemmules are produced, the cells are in a state of metabolic depression that is endogenously controlled. In this diapause state, gemmules will not germinate and continue development even if conditions become favorable again (Hand and Podrabsky 2000). Instead, the gemmules must go through a period of exposure to unfavorable conditions during which diapause is broken and gemmules

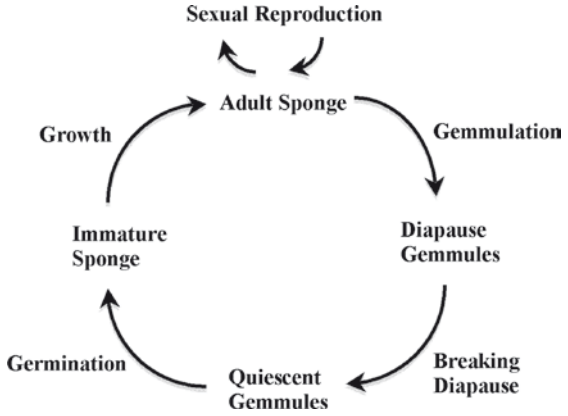

Fig. 11.1 Generalized life cycle of a sponge

enter a state known as quiescence. In quiescence, metabolic depression is maintained by environmental factors (such as low temperature or desiccation). If favorable conditions return, metabolism and cell division are turned up, the cells will begin to differentiate, and will eventually emerge from the gemmules to develop into an adult sponge (Hand and Podrabsky 2000). Therefore, diapause can be defined as an endogenously controlled state of metabolic depression that is an obligate step in the life cycle of the sponge, whereas, quiescence can be defined as an environmentally controlled state of metabolic depression (See Hand and Podrabsky 2000 and Guppy and Withers 1999 for reviews on diapause and quiescence in animals). It is not necessary for gemmules to go through diapause to reach quiescence and there is a great deal of variability among sponge species and within individual species as to the details of the life cycle.

In this chapter, I will review our current understanding of the cell biology and biochemical mechanisms of each of these steps in the life cycle focusing in particular on (1) gemmulation, (2) diapause, (3) breaking of diapause, (4) quiescence, and (5) germination. Most of what we know about these stages has come from work on freshwater sponges; however, I will mention marine sponges whenever it is appropriate. Opportunities for new directions of study are also highlighted.

11.2 Gemmulation

Gemmule are comprised of thesocytes surrounded by a collagen and spicule coat (Fig. 11.2). The thesocytes from freshwater sponges are usually binucleate and are packed with yolk platelets and lipid inclusions along with numerous ribosomes, endoplasmic reticulum, mitochondria, and golgi (Simpson 1984). The yolk platelets are thought to provide energy reserves in the form of basic proteins and glycoproteins (De Vos 1971; Harrison and Cowden 1975; Simons and Muller 1966),

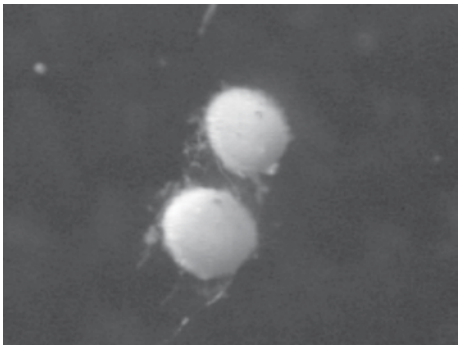

Fig. 11.2 Gemmules of *Anheteromeyenia ryderi*

polysaccharides (Harrison and Cowden 1975) and lipids (Kauffold and Spannhof 1963) during diapause, quiescence, and, especially, during germination.

Gemmule formation is thought to occur in response to a shift from favorable to unfavorable conditions that may include an increase or decrease in temperature and/or desiccation (Fell 1998). The time of year that gemmules form in natural populations can be extremely variable even for the same species. Pronzato et al. (1993) examined the timing of gemmule production by populations of *Ephydatia fluviatilis* along a climatic gradient in Italy. In northern populations, gemmules form early and the sponge spends less time as active adults in the summer. In the southern populations, gemmules are formed during the summer and the adults are active during the winter. The same is true when comparing populations in the United States and Belgium (Harsha et al. 1983; Poirrier 1974; van de Vyver and Willenz 1975). The timing can also be variable for two species in the same body of water. For example, *E. fluviatilis* exists as gemmules from May through August in Lake Pontchartrain, Louisiana while *Spongilla alba* exists as gemmules from late September through early May (Poirrier 1974, 1976).

The extreme variability in the timing of gemmule production leads to the question of what environmental cues are responsible for initiating gemmulation. Rasmont (1963) found that the size and nutritional condition of the sponge influence gemmule formation. Larger sponges that were fed more bacteria formed more gemmules. He suggested that large healthy sponges produced an inducer that initiated gemmule formation. He also proposed that there was an endogenous component controlling gemmulation because gemmules stored at 3°C for different lengths of time form different numbers of gemmules. Pronzato et al. (1993) also argue that there may be an endogenous rhythm controlling the life cycle of *E. fluviatilis* because they have identified a population that undergoes a brief period of gemmule formation in a very stable habitat. Simpson and Gilbert (1973) argue that exogenous and endogenous factors such as prior gametogenesis and larva production may be important in determining when a sponge gemmulates. Water temperature may play a major role in determining the seasonality of the life cycle of freshwater sponges (Harsha et al. 1983; Simpson and Gilbert 1973). Gemmules from *S. alba* are produced in mid fall when the water temperature falls below 22°C (Poirrier 1976) and gemmules of *E. fluviatilis* are

produced when water temperatures rise above 30°C (Poirrier 1974). Light appears to inhibit gemmule formation and may interact with temperature (Simpson 1984). Low levels of silicon dioxide in the incubating medium results in smaller gemmules, with some even lacking spicules (Petermans-Pe et al. 1975; Simpson 1984).

Cyclic nucleotides may be important in initiating gemmule formation in some freshwater sponges. Incubating freshwater sponges in theophylline (an inhibitor of cyclic nucleotide phosphodiesterases) stimulated gemmule formation (Rasmont 1974). Presumably, the increase in cyclic nucleotide concentration resulting from the inhibition of its breakdown increased the production of cell aggregates, the first stage in gemmule formation (see below). Adding cyclic nucleotides along with aminophylline (also an inhibitor of cyclic nucleotide phosphodiesterase) directly to the sponges stimulated gemmule formation in *Spongilla lacustris* but not in *E. fluviatilis.* (Simpson 1984; Simpson and Rodan 1976), so this effect may not be universal.

Supposedly, the cyclic nucleotides would be produced as a result of the presence of an environmental factor stimulating its production in the sponge cells through signal transduction. Very little is known of the possible signal transduction pathways that might link environmental cues to gemmule production. Genes for elements of two signaling pathways have been identified from sponges. The phospholipase C gene, part of the inositol phosphate signaling pathway, was identified in *E. fluviatilis* (Koyanagi et al. 1998) and mitogen-activated protein kinase gene was identified in *Suberites domuncula* (Bohm et al. 2000), but neither has been linked to gemmule production.

Simpson and Gilbert (1973) have divided gemmule formation into three stages: early, mid, and late. During early gemmulation, the first visible sign of gemmule production is the formation of aggregates of two or three types of ameboid cells in the mesenchyme of the sponge. In most freshwater sponges, trophocytes provide the nutrients for development of yolk platelets through cytolysis or phagocytosis by the thesocytes (Simpson and Fell 1974). Harrison and Cowden (1975) identified a third cell type (granular cells) in the aggregates of *Eunapius fragilis* that is involved in gemmule coat formation. Following aggregation, mid gemmulation is marked by the production of a layer of columnar cells around the aggregate. These cells will form the gemmule coat during the late stage of gemmulation (Simpson and Gilbert 1973). The last step in gemmule formation is an increase in the osmotic pressure of the thesocytes (Simpson and Fell 1974). In most freshwater sponges, newly formed gemmules have a relatively high osmotic pressure. Zeuthen (1939) found that gemmules of *S. lacustris* contain thesocytes that are in osmotic equilibrium with a 220 mOsm solution of sodium chloride. Likewise, Schmidt (1970) found that thesocytes from the gemmules of *E. fluviatilis* lysed when placed in distilled water but when released into a 115 mOsm solution of sodium chloride they remained stable. The development of high osmotic pressure in these cells is most likely due to the synthesis of polyols from glycogen. Loomis et al. (1996b) identified sorbitol as the major component of the ethanol-soluble extract of gemmules of *E. fragilis* and Loomis et al. (2009) found that myo-inositol is the major ethanol-soluble component in gemmules of *Anheteromeyania ryderi*. The amount of polyol present in the gemmules can account for the increased osmotic pressure observed in the thesocytes of these sponges (Loomis et al. 2009)

11.3 Diapause

Newly formed gemmules can be in the states of diapause or quiescence, and the distinction between these states is not always clear (Fell 1998). Fell and others have measured the depth of diapause by determining the time of exposure to low temperature and/or desiccation that will break diapause (Bazer and Fell 1986; Fell 1995; Fell 1994). Fell (1995) defines gemmules undergoing shallow diapause as those gemmules that will hatch after only a few days of exposure to low temperature (5°C) and gemmules undergoing deep diapause as those gemmules that must be exposed to low temperatures for a month or more before they will hatch. The depth of diapause varies greatly both within and among species. For example, *S. lacustris* produces three types of gemmules: thick-capsuled gemmules, green thin-capsuled gemmules and yellow thin-capsuled gemmules (Fell 1995). When produced, the geen thin-capsuled gemmules are in quiescence, the thick-capsuled gemmules are in shallow diapause and the yellow thin-capsuled gemmules are in deep diapause (Fell 1995). *Eunapius fragilis* (Fell 1995) and *Anheteromeyenia ryderi* (Bazer and Fell 1986) undergo deep diapause, whereas, in *Heteromeyenia tubisperma* diapause is much more shallow (Bazer and Fell 1986).

Depth of diapause has also been determined in a few species of sponges by measuring the metabolic rate of diapausing gemmules and comparing that with the metabolic rate of quiescent gemmules and the metabolic rate at the time of emergence of the first cells from germinating gemmules. Rasmont (1962) measured the rate of oxygen consumption of both diapausing and postdiapausing gemmules of *Ephydatia mulleri* and found that the metabolic rate of diapause gemmules was depressed by a factor of 3 when compared with quiescent gemmules. Brondsted and Lovtrup (1953) measured oxygen consumption of gemmules of *S. lacustris* during germination. *S. lacustris* produces two types of gemmules, green and brown. The quiescent green gemmules had an initial rate of oxygen consumption five times greater than that of postdiapause, brown gemmules. Further, the respiration rate of brown gemmules increased by a factor of 7 during germination whereas the respiration rate of green gemmules only increased 1.5-fold. Loomis et al. (1996a) measured both the oxygen consumption rate and heat production of gemmules of *E. fragilis* during diapause, quiescence, and germination. The metabolic rate of quiescent gemmules was twice that of diapause gemmules and the rate of oxygen consumption and heat production steadily increased to six times that of quiescent gemmules. Clearly, the metabolic rate of diapause gemmules is significantly depressed and stays depressed until diapause is broken at which time it increases a small amount.

Very little is known of the endogenous control mechanisms that maintain the gemmules in diapause. Loomis et al. (2009), Rozenfeld (1971), Schmidt (1970), Simpson et al. (1973), and Zeuthen (1939), have all demonstrated that the intragemmular osmotic pressure of diapause or quiescent gemmules is high (between 100 and 220 mOsM) and that the osmotic pressure is reduced below 40 mOsM during the early stages of germinationton. Further, germination is inhibited as long as the osmotic pressure is more than 50 mOsM. Rozenfeld (1974) examined the effects of an

unidentified inhibitor (gemmulostasin) of germination on DNA, RNA, and protein synthesis during germination and found that DNA synthesis was completely inhibited and that RNA and protein synthesis were delayed in the presence of the inhibitor. Since gemmulostasin was never identified, Simpson et al. (1973) and Simpson and Fell (1974) have proposed that gemmulostasin actually represents an increased osmotic pressure. If this is true, cell division, translation, and protein synthesis would be inhibited by high osmotic pressure. Indeed, Loomis et al. (2009) have recently shown that germination, metabolic rate, and cell division of quiescent gemmules of *E. fragilis* and *A. ryderi* are controlled by increased osmotic pressure in the cells. High levels of polyols (sorbitol in *Eunapius* and myo-inositol in *Anheteromeyenia*) maintain the osmotic pressure in the thesocytes above 100 mOsM. These compounds are most likely produced from glycogen during the formation of gemmules (see above) and are present in diapause gemmules.

Cyclic neucleotides such as cAMP may also play a role in inhibition of germination. Simpson and Rodan (1976) found that cAMP levels in *S. lacustris* were high in quiescent gemmules and declined rapidly during the early stages of germination. When cAMP levels were maintained by inhibition of cAMP phosphodiesterase, the thesocytes did not begin cell division.

11.4 Breaking of Diapause

The breaking of diapause usually requires exposure of gemmules to adverse environmental condition for a period of time (Fell 1998) For example, diapause can be broken by exposing gemmules to low temperature (5°C) for a few days, (in the case of shallow diapause) or months (in the case of deep diapause). Newly formed gemmules of *E. fragilis* exposed to 3°C for 3 weeks followed by exposure to 20°C for 27 weeks did not germinate. The same gemmules later hatched following further cold treatment of up to 18 weeks (Fell 1995, 1998). Diapause may also be broken by desiccation in the gemmules of some sponges (Fell 1987a, 1987b). Desiccation increased germination of gemmules of *E. fragilis* kept at 20°C and exposure to cold for a few weeks, prior to desiccation greatly reduced the time of exposure to low temperature required for breaking of diapause (Fell 1987b).

One of the first steps in the germination of *E. fragilis* is the reduction of sorbitol levels and concomitant decrease in intracellular osmotic pressure (Loomis et al. 1996b, 2009). Sorbitol appears to be converted to glycogen through the activation or *de novo* synthesis of sorbitol dehydrogenase (SDH) and an unknown mechanism of activation of glycogen synthase (Loomis et al. 1996b). These steps appear to set off the cascade of events that result in emergence of the cells from the gemmules. The molecular switch for breaking of diapause may involve the change in the cell's ability to synthesize or activate the enzymes that convert polyols to glycogen. For example, in *E. fragilis*, the gene coding for SDH may not be capable of being switched on during diapause. During the breaking of diapause, the ability to switch on the SDH gene could occur transitioning the gemmules from diapause into quiescence.

11.5 Quiescence

Once diapause is broken, gemmules are kept in depressed metabolic state by continued exposure to adverse conditions. Low temperature, desiccation, oxygen deprivation, osmotic stress, low or high pH, and short day photoperiod have all been shown to inhibit germination in postdiapausing gemmules (Benfey and Reiswig 2005; Loomis et al. 2009; Loomis et al. 1996b; Rasmont 1954, 1963; Reiswig and Miller 1998). Various ions such as Na^+, Cl^-, and SO_4^{2-} have a long-term effect on inhibition of germination of *E. fragilis* (Fell 1992). Divalent cations (except calcium) also inhibit germination of postdiapausing gemmules from *S. lacustris*; however, calcium appears to overcome this inhibition (Ostrom and Simpson 1979). The effects of ions on germination may be important in those sponges that gemmulate as a result of desiccation.

In *E. fragilis* and *A. ryderi*, low temperature is the major factor inhibiting germination in postdiapausing gemmules (Loomis et al. 2009). The primary effect of low temperature is to maintain the high osmotic pressure in the gemmules, inhibiting the early stages of germination.

11.6 Germination

When conditions become favorable, the quiescent gemmules begin germination (Fig. 11.3). For example, gemmules from *E. fragilis* remain metabolically depressed as long as the temperature is maintained below 5°C (Fell 1995; Loomis et al. 1996b). When the temperature is raised to 20°C, germination is initiated and cells emerge from the gemmules within 48–72 h (Fell 1995, 1998).

The process of germination begins with the division of thesocytes, gradual breakdown of yolk platelets, and differentiation of thesocytes into other cell types (Simpson and Fell 1974; Fell 1998; Simpson 1984). The foramen or micropyle (the structure through which the cells move to the outside) eventually opens and cells migrate out of the gemmule to differentiate into a small sponge.

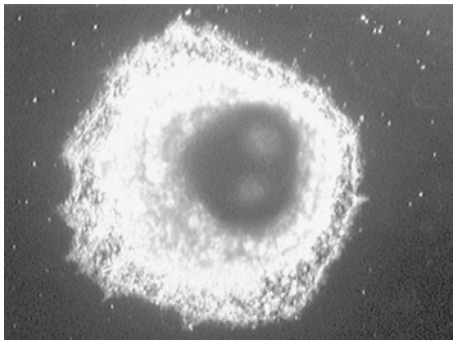

Fig. 11.3 Newly germinated sponge of *Eunapius fragilis*

During germination, the metabolic rate increases slowly and reaches a level six to eight times that of quiescent gemmules at the time of cell emergence (Bronsted and Lovtrup 1953; Loomis et al 1996b). Polyol levels decline dramatically during the early stages of germination and appear to be converted to glycogen (Loomis et al. 1996a; Loomis et al. 2009). In *E. fragilis*, loss of sorbitol is due to the increase in activity of SDH (Loomis et al. 1996b). In *E. fragilis* and *A. ryderi*, initiation of cell division is correlated with the reduction of the osmotic pressure of the thesocytes to below 50 mOsM (Loomis et al. 2009).

At least in some sponges, germination is initiated when the temperature rises and activates the metabolic pathways responsible for conversion of polyols to glycogen (Loomis et al. 2009). Very little is known of the control mechanisms of this pathway during early stages of germination, but in *E. fragilis* it most likely involves upregulation of the gene encoding SDH. The total activity of glycogen synthase does not change during germination of gemmules of *E. fragilis,* but the activity of glucose-6-phosphate dependent glycogen synthase is much higher than that of glucose-6-phosphate independent glycogen synthase (Loomis et al. 1996b). Since glucose-6-phosphate is an intermediate in the pathway from sorbitol to glycogen, it may be that a buildup of glucose-6-phosphate as a result of sorbitol catalysis activates glycogen synthase. Total glycogen phosphorylase activity increases more than double during germination, but the proportion of glycogen phosphorylase declines, attenuating the apparent increase in activity (Loomis et al. 1996b). Since cAMP levels do not change during germination, it is most likely not involved in control of conversion of sorbitol to glycogen (Loomis et al. 1996b).

11.7 Summary

Because there is a great deal of variability among sponge species in factors involved in control of gemmulation, diapause, quiescence, and germination, it is difficult to identify the commonalities. Even though our understanding of control mechanisms is rudimentary in most sponges, a story is starting to immerge from studies of *E. fragilis*. Fig. 11.4 summarizes our current state of knowledge along with some "leaps of faith" that still need investigation.

Gemmulation is initiated by environmental cues that include decreasing temperature in northern populations and desiccation in southern populations. The result is aggregation of the cells that will become thesocytes and formation of the gemmule coat. During thesocytes formation, platelets are produced and glycogen is converted to sorbitol, increasing the osmotic pressure in the cells. These thesocytes are in a state of diapause because they do not have the ability to convert glycogen to sorbitol most likely because the SDH gene is downregulated. Osmotic pressure above 100 mOsM inhibits cell division and maintains metabolic depression. Following a long exposure to cold or a period of desiccation, the cells gain the ability to convert sorbitol to glycogen and enter quiescence. Cell division and metabolic depression are maintained by high osmotic pressure. An increase in temperature

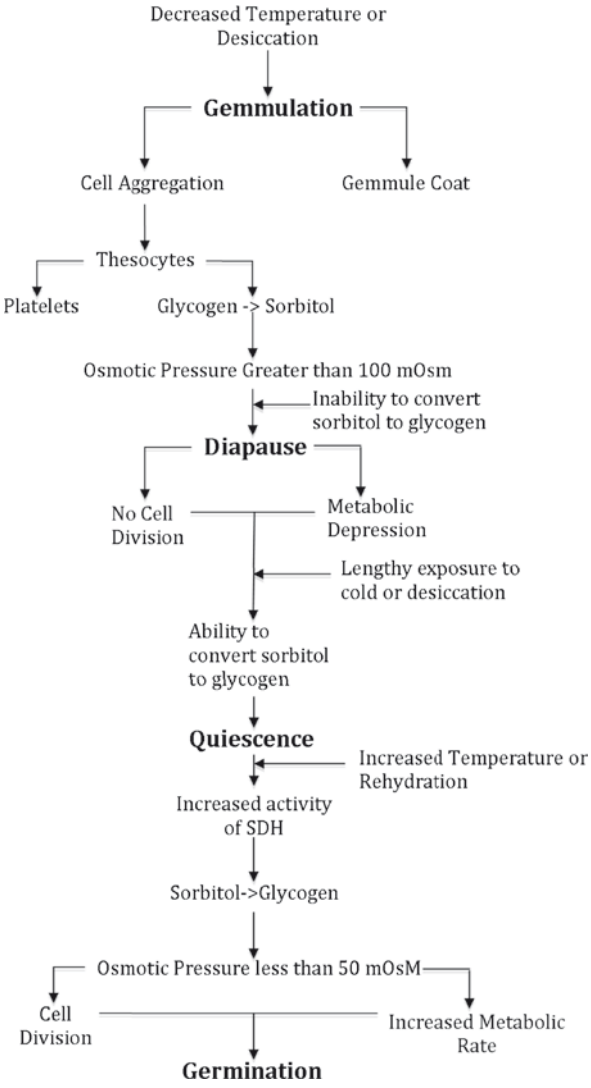

Fig. 11.4 Proposed mechanisms of control of the life cycle of *Eunapius fragilis*

triggers activation of the SDH gene and *de novo* synthesis of SDH. Sorbitol is then converted to glycogen, lowering the osmotic pressure below 50 mOsM. Cell division is initiated and the metabolic rate begins to increase triggering germination.

Obviously, there is a great deal that we still need to understand about the mechanisms of control of the life cycle of *E. fragilis*. We need to confirm that there is inhibition of the SDH gene during diapause and that the gene is activated during germination. We have not identified transcription factors acting as switches between diapause and quiescence. The clock that determines the amount of time a gemmule

must be exposed to adverse conditions before switching to quiescence needs to be identified, and control of the rest of the metabolic pathway leading from sorbitol to glycogen needs to be elucidated. One approach to deciphering these factors may be through the use of microarrays to identify differences in gene activity in diapause, quiescent, and germinating gemmules. Likewise, a proteomics approach my be fruitful in understanding some of these mechanisms. A number of other sponges must be studied to determine commonalities in mechanisms in light of the unusual diversity of responses that different species of sponges or even individuals within a species have to changes in their environment. Only then, will we begin to unravel the complexity of diapause and quiescence in sponges.

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Chapter 12
Aestivation in the Fossil Record: Evidence from Ichnology

Daniel I. Hembree

Contents

Abstract Aestivation is a physiological and behavioral response to high temperature or low moisture conditions. Therefore, it is typically not considered to be capable of being preserved in the fossil record. However, most aestivating organisms produce a burrow to protect themselves from the harmful environmental conditions that trigger aestivation. These structures can be preserved in the rock record as trace fossils. While trace fossils are abundant in the continental fossil record, few are definitively associated with aestivation. Interpreting aestivation behavior from fossil burrows requires a detailed examination and interpretation of the surrounding sedimentary rocks and comparisons with taxonomically and ecologically similar extant organisms. Currently, only four types of aestivation structures are recognized in the fossil record: Pleistocene earthworm chambers, Devonian to Cretaceous lungfish burrows, Permian lysorophid burrows, and Permian to Triassic dicynodont burrows. The trace fossil evidence suggests that aestivation evolved independently among continental organisms in several clades during the middle to late Paleozoic.

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12.1 Introduction

Aestivation is a state of inactivity and metabolic reduction in response to a lack of water or high temperature. It is a common part of the life cycle of animals that occupy periodically dry habitats. Decreasing moisture levels and increasing temperature are considered as the signal for entering and leaving a state of aestivation. Extremes of either environmental variable can lead to extensive periods of aestivation. Typically, the aestivating animal burrows into moist soil or mud, forms a cocoon to slow water loss, and becomes inactive to conserve energy (Pinder et al. 1992). Aestivation burrows are used by extant annelids, mollusks, arthropods, fish, amphibians, reptiles, and mammals as temporary shelters from extreme, short-term climatic or environmental conditions.

Simple to complex burrows are constructed to create a soil-buffered microenvironment more suitable to the animal's requirements for survival (Hasiotis et al. 2007). Daily temperature variation, for example, decreases with depth in the soil. At 40 cm below the soil–air interface, there is little to no daily temperature fluctuation (Lavelle and Spain 2005). Aestivation behaviors, therefore, allow many animal groups to occupy a wider variety of habitats and climate settings that would otherwise be possible (Mayhew 1968; Lee 1985; Greenwood 1986; Heatwole and Taylor 1987; Pinder et al. 1992; DiMichele et al. 2006). These burrows may be occupied from a few months to several years and are generally only used once (Pinder et al. 1992). Successions of cross-cutting burrows may result from several years of seasonality, producing many generations of burrows originating from the same surface.

The evolutionary history of aestivation is a subject of some debate. Because aestivation is a physiological and behavioral process with no consistent expression in organism morphology, it is not generally considered to be capable of being studied in the fossil record. Given the common behavior of burrowing among many aestivators and the rapidly developing field of ichnology, however, this assumption is changing. This chapter will review the best documented occurrences of aestivating continental organisms in the fossil record and discuss the significance of the geologic history of aestivation to biologists and geologists.

12.2 Evidence of Aestivation in the Fossil Record

Aestivation is a physiological response to environmental conditions. Such activities are generally not considered capable of being preserved in the fossil record. However, most aestivating organisms produce a physical structure, typically a burrow, to protect themselves from the harmful environmental conditions that trigger aestivation. These structures can be preserved in the rock record as trace fossils. Trace fossils are the preserved remains of burrows, tracks, trails, nests, borings, or any other record of the interaction between an organism and a substrate (Bromley 1996). In most cases, the substrate is a layer of loose sediment or soil, but it can also include wood, leaves, shells, rock, or even individual mineral grains depending on

the size of the trace-making organism. Trace fossils are invaluable to paleontologists because they provide direct evidence of organism behavior in the fossil record.

Although the occurrence of fossil burrows within a sedimentary rock unit provides evidence that a group of organisms burrowed, they may not necessarily have been produced for the purpose of aestivation. Organisms burrow into the substrate for a number of reasons including the construction of permanent dwellings or temporary concealment, locomotion below the surface, and deposit feeding or active hunting (Bromley 1996; Hasiotis 2007; Hasiotis et al. 2007). Interpreting aestivation behavior from fossil organisms and their burrows, therefore, requires a detailed examination and interpretation of the surrounding sedimentary rocks as well as comparisons with taxonomically and ecologically similar extant organisms.

Sedimentary rocks are the product of specific depositional environments. Each environment imparts a unique suite of properties on sediments and soils. These features can provide indirect evidence of aestivation through lithologies and sedimentary structures that are suggestive of arid or seasonally arid climates. Environments with arid climates typically consist of large volumes of wind-blown sediment including silt and sand (Kocurek 1996; Parrish 1998). Preserved eolian deposits such as dunes composed of large scale cross bedded sandstone and siltstone are highly suggestive of arid environments. Evaporites, layers of water-soluble minerals such as halite and gypsum, form when seawater or lake water evaporate faster than it is replenished (Kendall and Harwood 1996; Parrish 1998). Evaporites, therefore, are indicative of environments where rates of evaporation exceed precipitation and runoff. Many types of soils, preserved in the geologic record as paleosols, also form in regions that are permanently or seasonally arid. Paleosols that form in permanently arid regions are often poorly developed contain high concentrations of evaporite minerals such as calcite, halite, and gypsum often concentrated in dense layers close to the soil surface (Retallack 2001; Buol et al. 2003). Paleosols that formed in seasonally wet and dry regions may also contain some evaporate mineral horizons of calcite as well as shrink-swell structures (slickensides and pseudoanticlines) that develop as clay minerals expand and contract as soil moisture levels fluctuate over time (Retallack 2001; Buol et al. 2003).

Sedimentary structures are physical features in sedimentary rock that produced by abiotic or biotic depositional and postdepositional processes. Two types of sedimentary structures that are useful in interpreting the occurrence of aestivation are mud cracks and rhizoliths. Mud cracks form when originally water-saturated clay minerals dry and contract. When the water filling ponds, lakes, and streams evaporate, plants may colonize the newly exposed ground given enough time. The plant roots penetrate the sediment deposited within the formerly aqueous environment. The roots may be preserved as rhizoliths which are indicative of subaerial exposure surfaces (Retallack 2001). These structures, when associated with fossils and burrows, add support to the interpretation of aestivation behavior in various fossil groups.

The investigation of modern aestivating organisms in the field and laboratory also aids in the interpretation of fossil aestivators. Through direct observation, paleontologists can determine the types of sediments in which aestivation shelters

are constructed as well as the processes of construction and associated behaviors. Through study of the organism within the shelter, paleontologists can observe the morphological and physiological responses to aestivation including coiling the body to reduce water loss or the production of a waterproof skin or mucus cocoon. Finally, by casting the physical structures produced by aestivating animals, the architectural and surficial morphology of burrows constructed by different taxonomic groups in different environments can be assessed.

Aestivation is generally associated with terrestrial and freshwater organisms. Animals first colonized these environments approximately 440 million years ago during the Silurian (Behrensmeyer et al. 1992; Shear and Seldon 2001). Since the Mississippian, most modern groups of organisms that include species capable of aestivation were present in continental environments including myriapods, arachnids, insects, and amphibians (Behrensmeyer et al. 1992; Shear and Seldon 2001). The evolution of aestivation among continental organisms likely occurred independently in several clades during the middle to late Paleozoic. For example, there is evidence of aestivation among lungfish since the Devonian of eastern North America and amphibians since the Permian of central and western North America (Hasiotis 2002; Hembree et al. 2004; Small and Evans 2006).

The ideal periods in which aestivation should be common are those that experienced above average temperatures and low average precipitation. The Permian is often associated with this type of global climate system (Crowley 1994; Worsley et al. 1994). Burrowing by continental animals was very common and continental trace fossils have been reported from Permian paleosols of North America, South America, Africa, Antarctica, and Asia (Romer and Olson 1954; Olson and Bolles 1975; Berman 1976; Smith 1987; McAllister 1990; Miller et al. 2001; Hasiotis et al 2002; Hembree et al. 2004;). It is likely that many of these structures were related to aestivation-related behaviors.

12.3 Examples of Aestivation in the Fossil Record

The following is a review of four of the best documented examples of aestivation by continental organisms in the fossil record, including Pleistocene earthworms, Paleozoic, and Mesozoic lungfish, Permian lysorophid amphibians and Permian dicynodonts. In all of the examples, aestivation is interpreted from the morphology of the burrow, comparison to modern organisms, and sedimentological evidence of periods of drought.

12.3.1 Annelids

Earthworms are a major part of modern soil ecosystems around the world. While they are part of the permanent soil fauna, many species do respond to changes in the surface environment. The majority of earthworm species are capable of entering

a state of aestivation or hibernation within burrow chambers. Lee (1985) described three types of earthworm burrows: (1) vertical burrows made by anecic species that feed on surface debris, (2) horizontal burrows made by endogeic species that feed on organic material in the soil, and (3) vertical burrows connected with spherical chambers where earthworms aestivate during dry or cold seasons.

Burrow types 1 and 2 are produced throughout the year and result in the homogenization of the soil. Type 3 burrows, however, are produced only in response to poor environmental conditions and are used both as refuges for aestivation and hibernation. Aestivation chambers are produced by the extant earthworms *Hormogaster elisae, Martiodrilus heterostichon,* and *Aporrectodea caliginosa* in soils where seasonal variation is extreme (Lee 1985). *A. caliginosa* is capable of burrowing up to 40 cm into the soil to construct a chamber in which the earthworm rolls up to avoid desiccation during the summer. The earthworm excavates a spherical, mucus-lined chamber in which an individual earthworm coils into a tight ball. When the period of temperature or water-related stress is over, the earthworm exits the chamber leaving coiled mass of fecal pellets (Lee 1985).

12.3.1.1 Trace Fossil Evidence: Earthworms

Aestivation burrows of earthworms were recently reported by Verde et al. (2007) from the Upper Pleistocene Sopas Formation of northern Uruguay. The new ichnospecies, *Castrichnus incolumis*, consists of a 30 mm diameter ovoid chamber surrounded by a multilayered wall of ball-shaped, 2 mm diameter, pellets (Verde et al. 2007). On the inside of the chamber, the pellets are flattened giving the interior a smooth surface. The chamber is filled with a string of 5 mm diameter, rounded pellets that are coiled into a ball. The ovoid chambers are preserved in full relief and weather out of the outcrop as distinct structures.

These structures were interpreted as aestivation burrows by Verde et al. (2007) based on comparisons with modern earthworm aestivation burrows. The morphological feature of these trace fossils that indicates they were used as aestivation shelters is the multilayered wall of packed fecal pellets (Verde et al. 2007). The extant earthworm *M. heterostichon* produces lined, subsurface chambers before entering an aestivation state (Jiménez et al. 2000). The pellets composing the walls are rounded and smaller than those that typically fill tunnels associated with daily locomotion and feeding behaviors. The earthworms produce the chamber lining by applying fecal material directly to the chamber wall. The lining provides a moisture retaining seal that prevents the desiccation of the earthworm during the period of dormancy (Lee 1985). When excavated from modern soils, the aestivation chambers of *M. heterostichon* occur as discrete structures covered with clay and the pelleted lining (Jiménez et al. 2000). When emerging from the chamber after the period of dormancy ends, the earthworm defecates within the chamber and emergence burrow while moving upward through the soil. This produces a coiled string of fecal pellets that fills the aestivation chamber (Jiménez et al. 2000).

12.3.2 Fish

Modern fish inhabiting lakes and floodplain ponds in regions subject to drought use burrows to provide protection from desiccation when these water bodies evaporate during the dry season. Various species of gobies, catfish, mudskippers, and African and South American lungfish use burrows as aestivation chambers (Atkinson and Taylor 1991). The African lungfish *Protopterus annectens* is the classic example of an extant aestivating fish. *Protopterus* lives in floodplain swamps of the Gambia River (Greenwood 1986). The climate of this region is characterized by alternating wet and dry seasons in which rainy seasons may last 3–5 months and flood large areas of the alluvial plain (Greenwood 1986). During this wet period, lungfish and other species of fish are active in streams as well as ephemeral ponds and swamps. At the end of the wet season, *P. annectens* burrows into the floor of the drying swamps and enters an aestivation state whereas other fish species reenter the rivers.

Protopterus constructs aestivation burrows by biting into the moist sediment of the pond floor and forcing its body downward using lateral motion of the body and tail (Greenwood 1986). The diameter of the aestivation burrow is, therefore, close to that of the fish's body and between 30–250 mm in depth. When the burrow is completely excavated, the lungfish turns 180° so that its snout is pointed upward. By doing so, the lungfish compresses the soft mud at the base of the burrow and widens it into a flask-shaped chamber, approximately twice the diameter of the fish. While the mud of the pond floor is still wet, the lungfish may secrete a large volume of mucus. When the mud finally dries down to the depth of the aestivation chamber, the mucus sheath also dries to form a close-fitting, water-tight cocoon that protects the fish from water loss through diffusion and evaporation. While in an aestivation state, *Protopterus* can survive for several months to a year without food or water (Fishman et al. 1986; Greenwood 1986; Sturla et al. 2002). Once the wet season returns, surviving individuals bite through the cocoon and encasing mud to return to the pond.

12.3.2.1 Trace Fossil Evidence: Lungfish

Lungfish burrows are the oldest recognized vertebrate burrows, and have been used to infer the presence of seasonal droughts in the geologic past since their description from the Permian of Texas (Romer and Olson 1954). The oldest known lungfish burrows occur in Devonian fluvial deposits of the Catskill Formation in central Pennsylvania (Hasiotis 2002). Lungfish burrows have also been recently described from Mississippian deposits of northwestern Kentucky (Garcia et al 2006). However, lungfish burrows do not become common until the Permian in continental deposits of the North American mid-continent including Texas, Kansas, and New Mexico (Romer and Olson 1954; Carlson 1968; Berman 1976; MacAllister 1990; Hasiotis et al. 2002). Lungfish burrows have also bee reported from the Upper Triassic Chinle Formation of the Colorado Plateau (Dubiel et al.

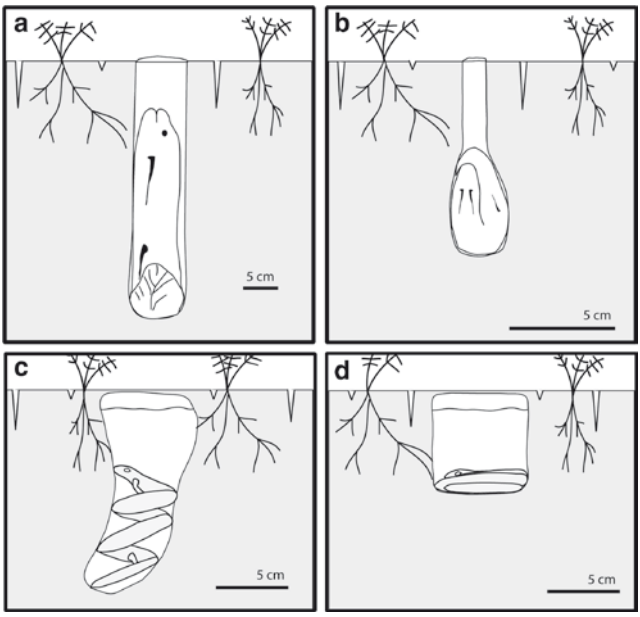

Fig. 12.1 Diagrammatical vertical cross sections of fossil aestivation burrows of Permian lungfish (**a**, **b**) and lysorophid amphibians (**c**, **d**). Lungfish aestivation burrows include elongate, cylindrical burrows (**a**) and flask-shaped burrows (**b**). Lysorophid aestivation burrows include downward tapering burrows (**c**) and short, cylindrical burrows (**d**)

1987; Hasiotis and Mitchell 1989; Dubiel et al. 1989; Gobetz et al. 2006), and potentially the Cretaceous of Denmark (Surlyk et al. 2008).

The burrows of the Permian lungfish *Gnathorhiza* are the most completely described in the literature and are typically used as the type example of lungfish aestivation burrows by paleontologists (Romer and Olson 1954; Carlson 1968; Berman 1976; Hasiotis et al. 1993; Miller et al. 2001). *Gnathorhiza* aestivation burrows possess two basic morphologies (Fig. 12.1a,b). The first type consists of a 1–10 cm diameter, cylindrical shaft with a circular-to-elliptical cross section. These burrows have a relatively consistent diameter from the burrow opening to the termination. The second type consists of a flask-shaped burrow with a 1–10 cm diameter, cylindrical upper shaft that leads to an expanded, bulbous chamber. In both morphological types, the burrows are 10–50 cm long and primarily vertical with a deviation of no more than 5–8 degrees. When preserved in place, fossil lungfish burrows have distinct sides and bases but indistinct tops that grade into the host rock. The disrupted upper surface of the burrow cast is attributed to biological and physical reworking of the sediment surface after the burrow was vacated. The well-defined walls define the edges of the excavated burrow. These walls are generally smooth, although some possess small bumps or horizontal to vertical striations (Hasiotis et al. 1993). These surficial features are interpreted as snout indentations and scale or fin scratches, respectively, and are a result of the physical

excavation of the burrow by the lungfish (Hasiotis et al. 1993). The burrows may possess an outer shell of packed mudstone containing scales and small bones and an inner core of mudstone, siltstone, bone fragments, and, occasionally, a complete lungfish (Carlson 1968). There is a less than 10% occurrence of complete lungfish skeletons within burrows at a single locality (Carlson 1968). This is often used to estimate an average mortality rate among aestivators under normal conditions. Fossil beds in which more than 10% of the burrows contain complete lungfish skeletons are considered to have been produced by periods of severe or long-term drought.

The interpretation of fossil lungfish burrows as aestivation shelters is supported by three different lines of evidence. The occurrence of fossil skeletons of *Gnathorhiza* within the fossil burrow casts as well as the fin scratch marks on the burrow walls supports the interpretation that the burrows were produced by lungfish. The fossil burrows are identical to those produced by modern lungfish entering an aestivation state (Hasiotis et al. 1993). In addition, when lungfish burrows are found in the fossil record, they occur in dense clusters along a single stratigraphic horizon. This indicates that all of the lungfish present in the lake or pond burrowed at one time. The cooccurrence of sedimentary structures that suggest the evaporation of a water body such as mud cracks, casts of evaporite minerals, and rhizoliths, with the burrowed layers support the interpretation that these burrowing events were in response to drought conditions. Finally, in many thick Permian deposits, lungfish burrows may occur in several layers within a singe sedimentary unit; each successive burrow layer crosscutting the layer below (Romer and Olson 1954; McAllister 1990; Hasiotis et al. 2002). These multiple layers of lungfish burrows suggest that the burrowing behavior was intermittent and potentially seasonal. In the modern, aestivation behavior produces very similar cross cutting layers of burrows (Greenwood 1986; Pinder et al. 1992).

12.3.3 Amphibians

Aestivation is common among extant amphibians. Many aestivating amphibian species may be active for as few as 2 months of the year; their lives condensed into brief active periods during favorable conditions (Pinder et al. 1992). Aestivating frogs and toads are mainly terrestrial and inhabit regions with arid to semiarid climates subject to variable and seasonal rainfall (Mayhew 1968; Pinder et al. 1992). The spadefoot toads of North America, *Scaphiopus* (Pelobatidae), the Australian *Cyclorana* (Hylidae), and the African bullfrog *Pyxicephalus* (Ranidae) are some of the best-documented terrestrial aestivators (Zug et al. 2001). For example, the anuran *Scaphiopus couchii* inhabits California deserts with air temperatures that reach 50°C, mean annual precipitation of less than 6 cm, and no permanent water bodies (Pinder et al. 1992). These anurans aestivate for 7–10 months per year, and are active for short periods of time after seasonal rains create ephemeral ponds in which they breed (Pinder et al. 1992). The larvae develop quickly and metamorphose into

adults before the waters evaporate. Other amphibians capable of aestivation are aquatic and inhabit regions with a more temperate climate including the anurans *Xenopus* and *Ceratophrys* and the North American salamanders *Ambystoma, Siren,* and *Amphiuma* (Pinder et al. 1992; Zug et al. 2001). These amphibians burrow into the wet mud of drying ponds in a manner similar to aestivating lungfish.

Amphibian aestivation burrows vary in depth depending on the size of the animal, the season, average temperature, and the density of the soil. Spadefoot toad burrows range from 20–70 cm deep, whereas African bullfrog burrows are 80–150 cm deep (Emerson 1976; Pinder et al. 1992). Aestivation burrows are typically nonrandomly distributed and occur in small patches with a high burrow density surrounded by large areas with a few or no burrows. Over 39 spadefoot toad burrows have been reported from one 6 m^2 area (Pinder et al. 1992). Like lungfish, many aestivating amphibian produce cocoons composed of one to several layers of shed skin or a layer of secreted mucus in order to reduce evaporative water loss (Pinder et al. 1992). Cocoons of shed epithelial cells surrounding aestivating aquatic anurans are capable of reducing evaporative water loss by 90% and enable survival up to 150–250 days in dry soil (Pinder et al. 1992). The salamander *Siren intermedia* produces a similar dry, inelastic cocoon that prevents dehydration for up to 250 days (Reno et al. 1972; Etheridge 1990).

12.3.3.1 Trace Fossil Evidence: Lysorophids

Because aestivation is common among modern amphibians, it is reasonable to expect this behavior in extinct lineages of amphibians. Amphibian fossils are well-documented in Paleozoic deposits (Carroll 1988; Holman 2003, 2006). Many of these fossil occur in small, lens-shaped mudstone units interpreted as the deposits of small ponds or lakes. While many of these fossils occur as articulated skeletons, few occur within distinct burrows. This may be due to the use of "primitive" burrowing techniques of amphibians such as simple compression through wet mud (Wake 1993). If the animal died while aestivating, such burrowing processes would result in an articulated skeleton encased in mudstone. Despite the prevalence of aestivation among extant anurans, no anuran aestivation burrows have been reported from the fossil record.

There is at least one well-documented example of a Paleozoic aestivating group of amphibians, the lysorophids. Lysorophids are elongate amphibians with highly reduced limbs. The lysorophid amphibians occur from the Mississippian to the Permian in deposits of the North American midcontinent (Wellstead 1991). Mississippian and Pennsylvanian fossils of lysorophids occur within laminated, carbonaceous mudstones interpreted as lakes or small ponds. These species are not associated with burrows. The Permian lysorophid *Brachydectes elongatus* (Lysorophidae), however, is found in burrows within thin freshwater units and paleosols interpreted as ephemeral pond deposits (Olson and Bolles 1975; Hembree et al. 2004, 2005) (Fig. 12.1a,b). The lysorophid burrows tend to occur in large clusters along a single stratigraphic horizon with concentrations up to 20 burrows

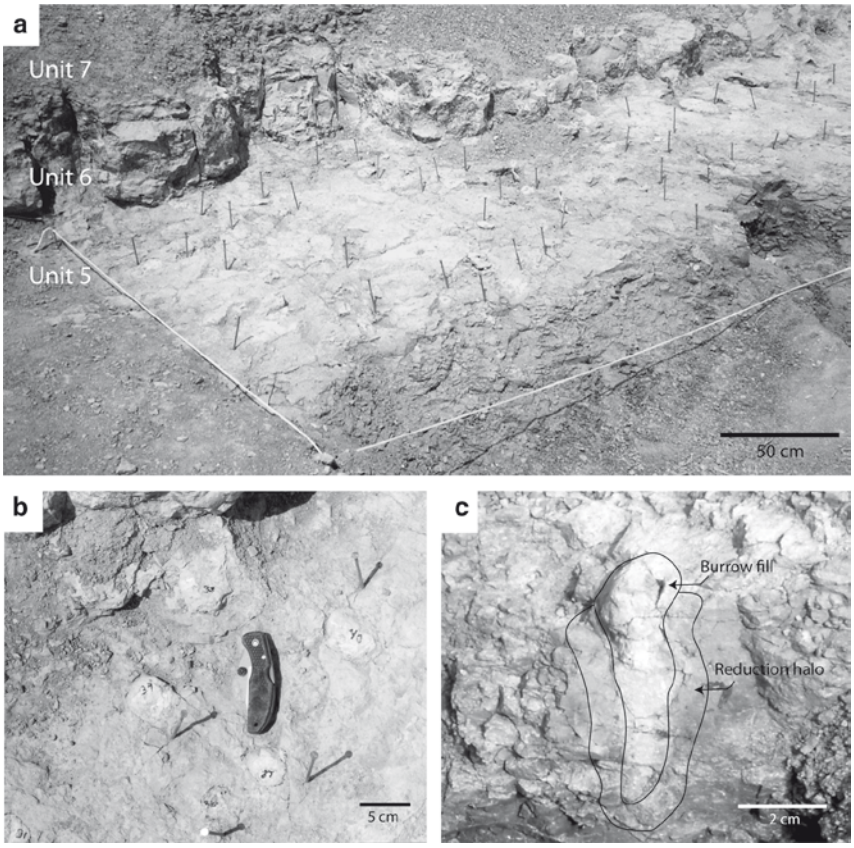

Fig. 12.2 Lysorophid burrows preserved *in situ*. (**a**) A resistant, ledge-forming green mudstone (Unit 6) interpreted as the deposits of an ephemeral pond. The mudstone contains large clusters of burrows of lysorophid amphibians. Each nail indicates the position of a preserved burrow. (**b**) Plan view of *in situ* aestivation burrows. (**c**) Cross sectional view of the cast of a lysorophid burrow extending into the underlying paleosol (Unit 5). The burrow is surrounded by a reduction halo of green mudstone

per square meter (Fig. 12.2). These burrow clusters extend downward from subaerial exposure surfaces characterized by the presence of desiccation cracks, dense concentrations of fragmented freshwater fossils, rhizoliths, and other pedogenic features (Hembree et al. 2004).

Lysorophid burrows are downward tapering shafts that are elliptical in cross section, 1–8 cm in diameter, and 10–15 cm long (Hembree et al. 2005) (Fig. 12.3a–c). The axis of the burrow tends to be subvertical, with some specimens deviating up to 40 degree (Fig. 12.3d). The burrows lack a lower chamber typical of lungfish burrows and instead become narrower at the termination. Fossil material of lysorophids occurs in 20–50% of burrows. This fossil material may consist of articulated skeletons coiled in loose whorls within the burrow cast or disarticulated bones concentrated near the base of the burrow (Hembree et al. 2004, 2005) (Fig. 12.4).

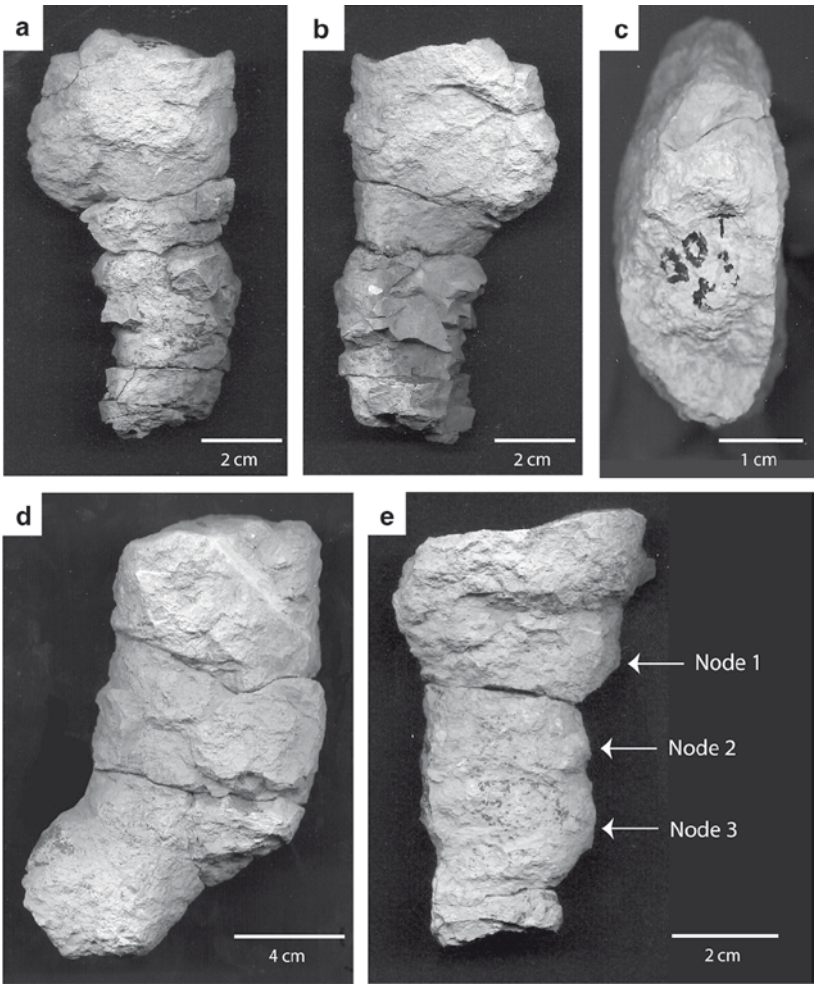

Fig. 12.3 Front, rear, and top (**a**–**c**) view of a typical lysorophid aestivation burrow. The burrow is primarily vertical and displays a downward tapering morphology. (**d**) Large lysorophid burrow with a 40° deviation from the vertical. (**e**) Surface nodes on the outer surface of a downward tapering lysorophid burrow

Lysorophid burrows have distinct sides and bases but lack a lining. The outer surface of lysorophid burrows is characterized by large, irregularly spaced nodes likely created by the animal's snout as it wedged its way into the pond floor (Hembree et al. 2005) (Fig. 12.3e).

The interpretation of fossil lysorophid burrows as aestivation shelters is supported by their occurrence in dense clusters along a single stratigraphic plane in association with subaerial exposure features such as mud cracks, fragmented fossil lags, rhizoliths, and pedogenesis. This indicates that the lysorophid population burrowed at one

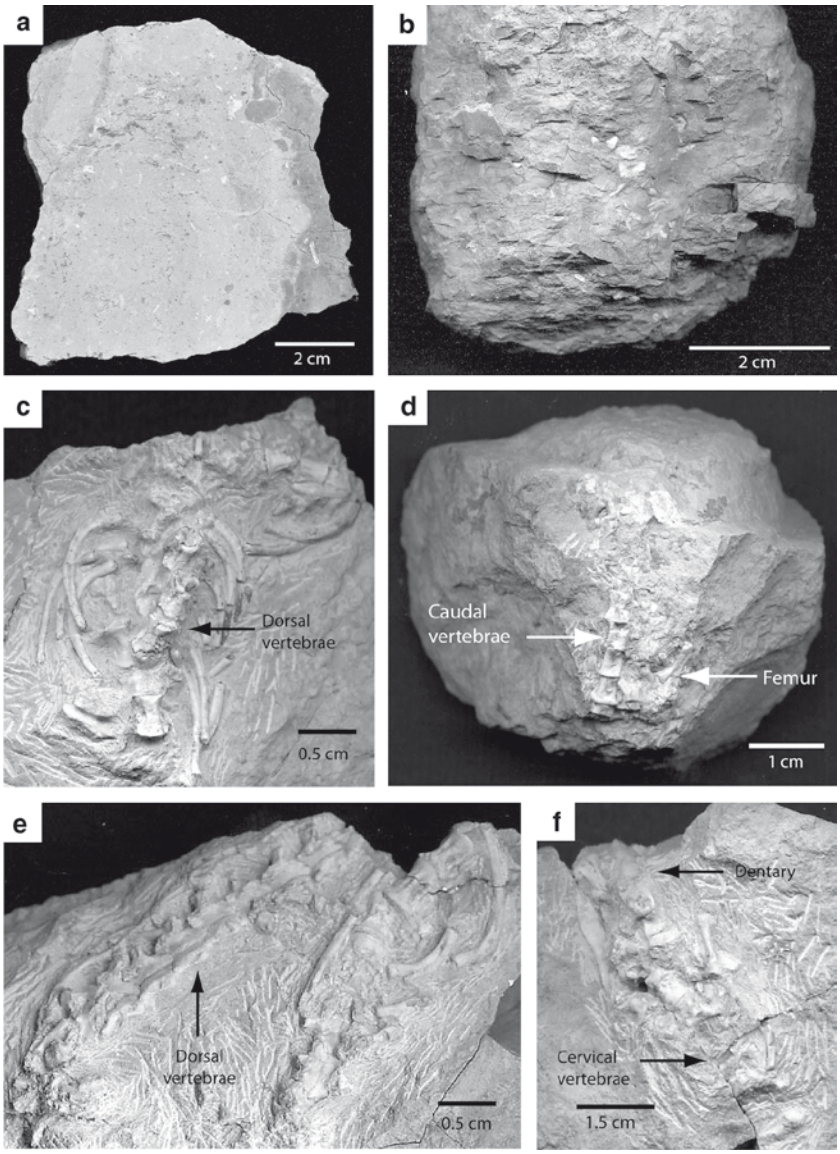

Fig. 12.4 Lysorophid fossils found within aestivation burrows. (**a**) A cut and polished burrow cast showing the sharp boundary between the sediment fill and surround red paleosol matrix. (**b**) Lower portion of a fossil burrow with disarticulated specimens of a lysorophid. (**c**) Horizontally oriented, partially articulated dorsal vertebrae and ribs of a lysorophid at the base of a burrow. (**d**–**f**) A vertically oriented, nearly complete skeleton of a lysorophid within an aestivation burrow. Articulated caudal vertebrae and hindlimbs are preserved in the borrow termination (**d**) Below the coiled, articulated dorsal vertebrate in the center of the burrow (**e**) Which are below the articulated cervical vertebrate and cranium (**f**)

time in response to the evaporation of the small pond. These clusters also occur in multiple, stacked layers that cross-cut one another, suggesting that these burrowing events occurred several times over the life of the pond ecosystem and may have been a seasonal event. Finally, the lysorophids have a similar morphology and inhabit a similar environment to such modern aestivating amphibians as the North American salamander *Amphiuma means*. These salamanders also inhabit small floodplain ponds and burrow en masse in dense clusters during dry seasons (Pinder et al. 1992).

12.3.4 Amniotes

While not as common as among amphibians, aestivation is found among most major reptile groups (lizards, snakes, turtles, and crocodilians) that inhabit arid regions (Mayhew 1968; Zug et al. 2001; Chapple 2003). Aestivation behavior in reptiles seems to be triggered in response to extreme temperatures and seasonal drought. The most commonly cited example of the North American gopher tortoise, *Gopherus agassizii*, uses underground burrows as a general shelter, nest, and refuge from daily to seasonal temperature fluctuations (McGinnis and Voigt 1971; Zimmerman et al. 1994; Duda et al. 1999). Monitor lizards also construct simple burrows as refuges from daily to seasonal temperature variations (Traeholt 1995).

12.3.4.1 Trace Fossil Evidence: Dicynodonts

Large, helical burrow casts in the Late Permian Beaufort Group of the Karoo Basin, South Africa have terminal chambers that contain coiled skeletons of the dicynodont *Diictodon* (Therapsida), a small herbivorous mammal-like reptile (Smith 1987). Burrows with similar morphology but without fossils in the terminal chamber are also known from the Lower Triassic Fremouw Formation of Antarctica (Miller et al. 2001). The burrow casts are 50–75 cm deep, vertically oriented, spiraling shafts composed of fine-grained sandstone and siltstone. The angle of the upper entrance ramp is 10–32 degree. The diameter of the shaft increases from 6 cm at the entrance ramp and gradually increases through two dextral coils, to about 16 cm at the base of the spiral. At the base of the burrow, the tunnel straightens again and widens into a horizontal, terminal chamber (Smith 1987). Linear ridges interpreted as scratch marks from claws are preserved on the outer walls and floor (Smith 1987).

Data collected from these fossil burrows suggest that they were not simple dwelling chambers or nests, but were used as refuges from extreme environmental conditions. The first piece of evidence is the relative rareness of these burrows in comparison to the large number of *Diictodon* body fossils (Smith 1987). A similar helical burrow, *Daimonelix*, produced by Miocene beaver *Palaeocastor* is very common in Miocene paleosols of the North American mid-continent and occurs in high densities of up to 190 individual burrows (Martin and Bennett 1977). The absence of large numbers of *Diictodon* burrows in the Beaufort Group and Fremouw Formation suggests that the

burrows were not constructed often or maintained over long periods of time. In addition, these regions were subjected to extreme variations in temperature and precipitation during the Late Permian and Early Triassic (Crowley 1994; Worsely et al. 1994). These environmental conditions would have been similar to modern arid regions where extant reptiles make use of burrows as seasonal shelters. Finally, the helical morphology of the *Diictodon* burrows is suggestive of a refuge from high temperature and low moisture conditions. The helical structure helps to limit air circulation between the surface environment and the terminal chamber (Meyer 1999). This would allow the humidity inside the terminal chamber to rise above that of the surface. These lines of evidence do suggest that the helical *Diictodon* burrows could have been used as aestivation shelters. However, since these were terrestrial animals, the presence of a subaerial exposure surface cannot be used as support, and the assessment of the burrows as aestivation structures is tentative.

12.4 Discussion

Given the trace fossil evidence, aestivation likely evolved independently among continental organisms in several clades during the middle to late Paleozoic. By the Permian, aestivation was present within groups of continental fish and tetrapods including lungfish, amphibians, and mammal-like reptiles. The relatively common occurrence of aestivation structures within Permian fossil deposits is likely related to the extremes in temperature and low precipitation in the interior of Pangea (e.g., Crowley 1994; Worsely et al. 1994). Given the presence of aestivation burrows before the Permian, however, aestivation was not the result of this environmental condition. Instead, it may have provided those organisms occupying central Pangea a selective advantage over those incapable of surviving long-term drought.

There are a number of modern groups of aestivating organisms for which aestivation structures are not currently recognized in the fossil record. These missing organisms, such as insects, are well-represented in the fossil record by both body and trace fossils. A large number of burrows, cocoons, chambers, and nests from Devonian to Pleistocene paleosols have been attributed to a variety of insects including beetles, wasps, bees, ants, and termites (Hasiotis 2002). Some of these trace fossils may have been produced for the purposes of aestivation; however, there is no compelling evidence to support this interpretation. Continued research with modern aestivating insects may reveal distinct trace morphologies that will allow paleontologists and ichnologists to confidently attribute specific trace fossils to aestivation behavior.

The presence of aestivation burrows within a sedimentary unit is suggestive of seasonal variations in temperature and precipitation. Horizons of aestivation burrows, therefore, provide paleontologists with invaluable information in reconstructing the history of a fossil ecosystem. Assuming that aestivation is an annual event, and is induced by regular, seasonal droughts, these burrow layers may be used as timelines. For example, the occurrence of multiple layers of lungfish or lysorophid aestivation burrow clusters within a single lake deposit may be used to estimate the

number of times the lake dried up and refilled. If the time between each aestivation event was 1 year, then the burrow layers may also be used to estimate average sedimentation rates, aiding in the determination of the depth of the water body, local drainage patterns, and erosion rates.

While aestivation is difficult to ascertain in the fossil record, the production of subsurface shelters by organisms does allow this behavioral and physiological response to extreme environmental conditions to be preserved. By studying the behavior of modern aestivating organisms and applying this data to the fossil record, more fossil examples may be found and increase our knowledge of the evolutionary history of aestivation.

12.5 Summary

The study of aestivation in the fossil record requires the application of ichnology. Because aestivation is a physiological and behavioral response and has no consistent morphological expression, the recognition of subsurface shelters produced by aestivating organisms is necessary. While trace fossils are abundant in the continental fossil record, the number definitively associated with aestivation is low. There are currently only four types of aestivation structures recognized in the fossil record: Pleistocene earthworm chambers, Devonian to Cretaceous lungfish burrows, Permian lysorophid burrows, and Permian to Triassic dicynodont burrows.

The interpretation of fossil burrows as aestivation shelters requires the recognition of sedimentological features that indicate periods of desiccation or drought. Interpreting aestivation behavior from fossil organisms and their burrows, therefore, requires a detailed examination and interpretation of the surrounding sedimentary rocks. In addition, comparisons of fossil taxa with taxonomically and ecologically similar extant organisms provide support for the interpretation of aestivation. Given the prevalence of aestivation among extant reptiles, amphibians, and arthropods, it is likely that many more fossil aestivation burrows remain to be found. While the earliest recognized aestivation structures are associated with Devonian lungfish, the fossil record of this physiological and behavioral process may be even older.

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