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Carlos Arturo Navas
José Eduardo Carvalho
Editors

Aestivation

Molecular and Physiological Aspects

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Aestivation

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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.

Carlos Arturo Navas
José Eduardo Carvalho

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Chapter 1

Metabolic Depression: A Historical Perspective

Philip C. Withers and Christine E. Cooper

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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).

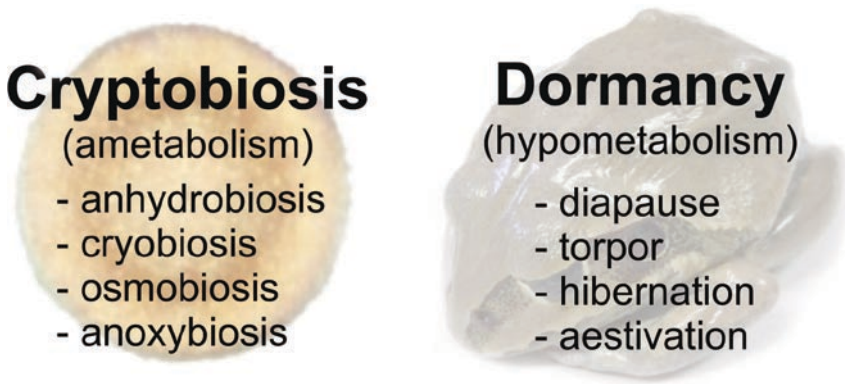


Fig. 1.1 Schematic summary of types of cryptobiosis, which is an ametabolic state (e.g., an anhydrobiotic *Artemia* cyst), and dormancy, which is a hypometabolic state (e.g., an aestivating *Cyclorana* frog). Terminology adapted from Keilin (1959)

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 polyhyphus*; 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 O₂ h⁻¹ is reduced to approximately 0.007 μl O₂ h⁻¹, then is further reduced for dormant encysted embryos to 0.0001 μl O₂ 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 Q₁₀ 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 woolly 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.