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HYPOMETABOLIC HOMEOSTASIS IN OVERWINTERING AQUATIC AMPHIBIANS

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Summary

Many amphibians encounter conditions each winter when their body temperature is so low that normal activities are suspended and the animals enter into a state of torpor. In ice-covered ponds or lakes, oxygen levels may also become limiting, thereby forcing animals to endure prolonged periods of severe hypoxia or anoxia. Certain frogs (e.g. *Rana temporaria*) can dramatically suppress their metabolism in anoxia but are not as tolerant as other facultative vertebrate anaerobes (e.g. turtle, goldfish) of prolonged periods of complete O_2 lack. Many overwintering amphibians do, however, tolerate prolonged bouts of severe hypoxia, relying exclusively on cutaneous gas exchange. *Rana temporaria* overwintering for 2 months in hypoxic water (P_{O_2} approximately 25 mmHg) at 3 °C progressively reduce their blood P_{CO_2} to levels characteristic of water-breathing fish. The result is that blood pH rises and presumably facilitates transcutaneous O_2 transfer by increasing Hb O_2-affinity. Even after months of severe hypoxia, there is no substantial build-up of lactate as the animals continue to rely on cutaneous gas exchange to satisfy the requirements of a suppressed aerobic metabolism. Our recent experiments have shown that the skeletal muscle of frogs oxyconforms *in vitro* to the amount of O_2 available. The cellular basis for the oxyconformation of skeletal muscle is unknown, but the hypothesis driving our continuing experiments theorises that metabolic suppression at a cellular level is synonymous with suppressed ion leak across cellular membranes.

Key words: metabolic suppression, aquatic amphibian, overwintering, acid–base balance, ion channel suppression, cold torpor, cutaneous gas exchange, osmotic homeostasis.

Introduction

Amphibians have conquered a wide range of inhospitable habitats, extending from southernmost deserts to beyond the Arctic circle. Owing to their highly permeable skin, they are particularly susceptible to heat and dehydration. Desert-dwelling amphibians avoid the heat and dehydration of seasonal drought by aestivating over several weeks or months, whereas others burrow into the substratum each day only to emerge during the cool nocturnal hours to forage (for reviews, see Pinder *et al.* 1992; Guppy *et al.* 1994). In the most extreme arid environments, some species' foraging and reproductive activities may be condensed into a few months each year (Seymour, 1973; Loveridge and Withers, 1981; McClanahan *et al.* 1983), the rest of the time being spent underground in a state of dormancy. As amphibians extend into more northern temperate climes, a wide variety of species adopt very similar avoidance strategies. By burrowing a metre or more into the soil or by secreting themselves in natural crevices (e.g. around tree roots), the animals can reach below the frost line and avoid exposing their bodies to lethal cold temperatures (Froom, 1982). Several of the most northern-based amphibians (e.g. *Rana sylvatica*, *Hyla versicolor*, *H. crucifer*, *Pseudacris triseriata*) are indeed remarkable for their ability to undergo reversible extracellular freezing (Schmid, 1982; Storey, 1985; Layne and Lee, 1987; Storey and Storey, 1984, 1985, 1988) as they hibernate at the soil surface covered by leaf litter and snow. Overwintering under water certainly avoids the problems of freezing temperatures, but often means that animals are trapped under ice-cover for several months. During such times, small ponds or lakes can become effectively closed systems for gas exchange, particularly if additional snow cover inhibits light penetration for photosynthetic activity. Thus, although freezing temperatures are avoided, the prospect of a progressive hypoxia must be solved by adjustments in respiratory (i.e. cutaneous) gas exchange and metabolism. In addition, hypothermia may in itself, or in combination with hypoxia, lead to secondary effects on osmotic and ionic regulatory processes across the highly permeable and metabolically active skin.

Many ectothermic vertebrates respond to the rigours of periodic heat and cold by entering into quiescent states that are variously referred to as dormancy, torpor, aestivation or hibernation. These states are almost always accompanied by a lowering of basal metabolic rate, which lessens the impact of ATP demand on endogenous energy reserves (Hochachka and Guppy, 1987; Storey and Storey, 1990; Flanigan *et al.* 1991; Guppy *et al.* 1994). Hypometabolic states thereby extend survival time until favourable conditions of climate or food availability return. During prolonged aestivation in amphibians, basal metabolism can be reduced to 10–20 % of the normal resting metabolic rate (Fig. 1). On the basis of

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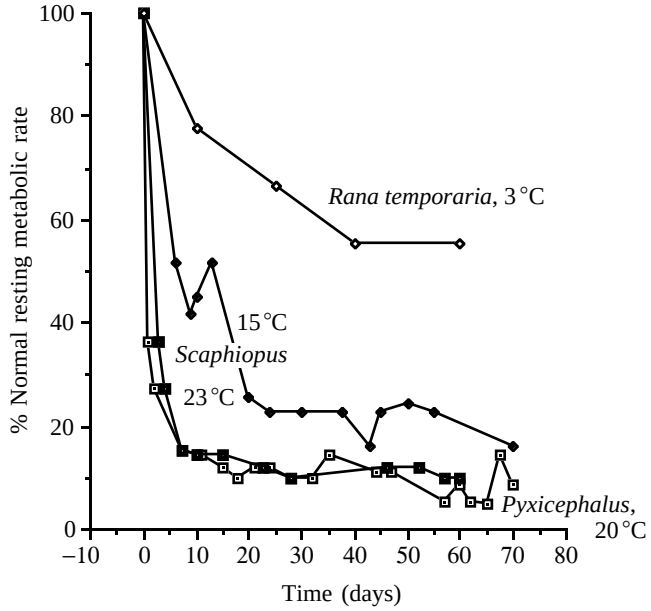

Fig. 1. Metabolic suppression during aestivation in *Scaphiopus couchii* and *Pyxicephalus adspersus* (Seymour, 1973; Loveridge and Withers, 1981) compared with the reduction in metabolic rate in *Rana temporaria* observed during prolonged submergence at 3 °C in normoxic water (P. H. Donohoe, R. G. Boutilier and T. G. West, unpublished results).

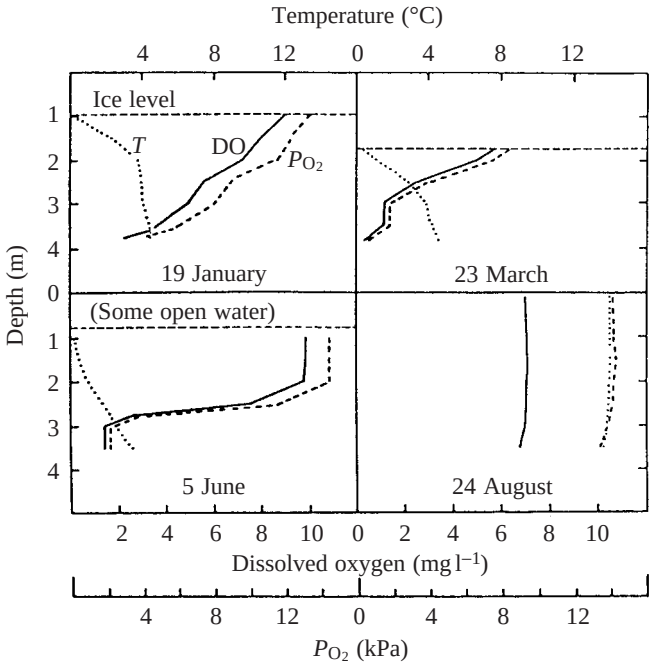

Fig. 2. Levels of dissolved O_2 (DO), oxygen partial pressures (P_{O_2}) and temperature (T) in a high-altitude lake inhabited by *Rana mucosa*. Data are shown over the course of a year. Ice-cover during the winter months is the lower margin of the ice layer (taken from Bradford, 1983, with permission). 1 mmHg=0.133 kPa.

studies showing profound metabolic depression in response to cold in reptiles and mammals (e.g. Herbert and Jackson, 1985; Geiser, 1988), one would predict that overwintering amphibians might also adopt this energy-conserving strategy. Although numerous studies have demonstrated a measurable reduction in the metabolic rate of 5 °C-acclimated frogs compared with those at higher temperatures (Dunlap, 1971, 1980; Packard, 1972; Carey, 1979; Weathers, 1976; Bradford, 1983), the energetic savings during prolonged overwintering are generally assumed to be of the order of only 5% or less (Penney, 1987); i.e. not much more than would be predicted on the basis of temperature effects alone. However, as pointed out by Pinder *et al.* (1992), virtually all of the studies examining aquatic overwintering have used 5 °C as the lowest experimental temperature, whereas in nature these animals are routinely exposed to temperature ranges of 0–5 °C (Fig. 2; Bradford, 1983). Indeed, when we exposed submerged *Rana temporaria* to temperatures of 3 °C for several months, metabolic rate became depressed by up to 50% in normoxic conditions (Fig. 1). In other words, the Q_{10} for metabolic rate between 0 and 5 °C is time-dependent, increasing as hibernation proceeds. Similar hypothermia-induced increases in Q_{10} have been observed in both turtles hibernating under water (Herbert and Jackson, 1985) and hylid frogs hibernating on land (Dunlap, 1971, 1980; Packard, 1972). Thus, despite a historical literature that has suggested otherwise (see Pinder *et al.* 1992), it appears that at least some hibernating frogs can dramatically suppress their metabolic rate in response to prolonged cold-submergence. This has important ramifications for an animal that must rely utterly on endogenous energy reserves for an entire winter season, sometimes lasting more than 6 months.

Hypometabolism and acid–base balance

Enforced diving

At experimental temperatures of 5 °C and higher, the enforced submergence of air-breathing ectotherms such as frogs and turtles is not ecologically relevant since, in the absence of ice-cover, such animals never become trapped under water; they dive and surface voluntarily according to their metabolic needs for oxygen (Shelton and Boutilier, 1982; Glass *et al.* 1983; Burggren, 1988; Jackson, 1989; Boutilier, 1991). Although perceived threats at the surface may delay voluntary air-breathing under certain conditions and lead to the recruitment of anaerobic metabolism (Gatten, 1981; Boutilier, 1989), air-breathing ectotherms would rarely, if ever, face periods of prolonged submergence at higher temperatures. The literature on enforced dives at higher temperatures has nevertheless been instrumental in defining the major physiological and metabolic responses to O_2 lack. In the early stages of such an enforced dive, the animals carry on-board reserves of oxygen in the blood and lungs, and various cardiovascular responses aid in the selective distribution of oxygen-rich blood to hypoxia-sensitive tissues (e.g. brain, kidney) and away from hypoxia-tolerant tissues such as the skeletal muscle (Jones, 1972; Burggren and Shelton, 1979;

Shelton and Boutilier, 1982; Shelton *et al.* 1986). As the internal oxygen stores run down, however, there is a progressive recruitment of anaerobic metabolism to make up for the energetic shortfall. Enforced dives of an hour or more at temperatures of 15–25 °C normally lead to a marked lactacidosis, and acid–base status can take several hours to days to recover completely to the pre-dive level (Toews and Boutilier, 1986; Jackson, 1986; Boutilier *et al.* 1992). Certainly the greatest energetic economy under these circumstances would be for the animal to reduce its metabolic demands for ATP consumption since this reduction would have the dual advantage of conserving glycolytic substrates as well as reducing self-pollution by the deleterious end-products of anaerobic metabolism. Indeed, both anuran amphibians and chelonian reptiles employ this strategy, reducing overall heat production, and therefore total metabolism, during enforced submergence (Leivestad, 1960; Jackson, 1968). Where the responses of the frog and turtle depart most dramatically is in their relative tolerance to hypoxia and anoxia. The turtle *Chrysemys picta* is perhaps the most anoxia-tolerant of all vertebrate ectotherms. It can survive periods of total oxygen lack for 3 and 10 days at temperatures of 10 and 15 °C, respectively (Herbert and Jackson, 1985). Ranid frogs, in contrast, can tolerate anoxia for only 3–8 h at similar temperatures (Hutchison and Dady, 1964; Pinder *et al.* 1992; Wegener and Krause, 1993).

Cold-submergence

The formation of an ice-cover in many northern ponds and lakes means that resident air-breathing ectotherms such as turtles and frogs must rely exclusively on cutaneous respiration for all of their gas exchange requirements. In nature, winter ice-cover can persist for several months (Fig. 2) and, while little is known of how the animals respond to the actual conditions *in situ* (Penney, 1987), several studies have simulated the conditions of overwintering in the laboratory (Ultsch and Jackson, 1982; Jackson and Heisler, 1983; Herbert and Jackson, 1985; Jackson, 1986). When the frog *Rana temporaria* is denied access to air at 2–3 °C, so as to mimic the conditions of an ice-covered pond in nature, the animals respond by progressively suppressing their aerobic metabolic rate and entering into a state of cold torpor (Fig. 1). Their metabolic rate after 2 months of submergence in aerated water is approximately 50 % of that seen in the resting air-breathing animal at 3 °C. Under these conditions, cutaneous gas exchange is evidently sufficient to maintain all of the aerobic metabolic requirements of the frog for periods of up to 4 months; i.e. blood O_2 saturation remains high, there is no significant build-up of lactate and the extracellular acid–base status is the same at 4 months as it was prior to the simulated overwintering submergence (Fig. 3; P. H. Donohoe, R. G. Boutilier and T. G. West, unpublished data). These responses are in marked contrast to those of the turtle *Chrysemys picta*, whose submergence in aerated water at 3 °C leads to a severe hypoxaemia and corresponding lactacidosis, culminating in plasma lactate levels of more than 50 mmol l^{-1} after 14 weeks

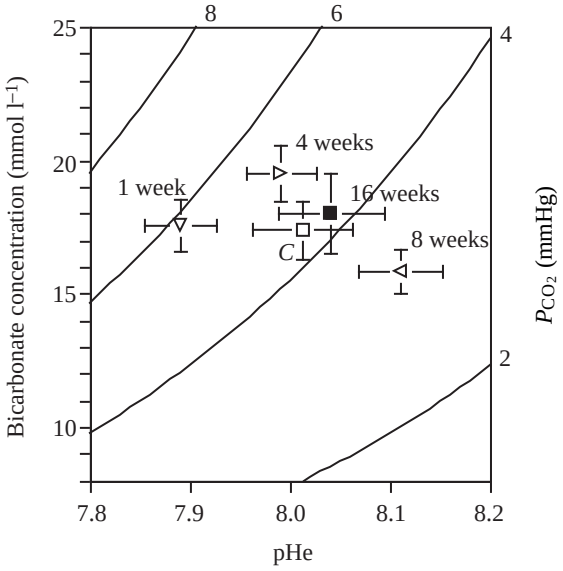

Fig. 3. pH–[bicarbonate] diagram illustrating changes in acid–base status of cold-submerged frogs (*Rana temporaria*) over 16 weeks of hibernation in normoxic conditions in the laboratory (R. G. Boutilier, P. H. Donohoe, G. J. Tattersall and T. G. West, unpublished results). Curved lines are CO_2 isopleths. Values are means ± S.E.M., N=6. 1 mmHg=0.133 kPa. *C*, control value before submergence.

of submergence (Herbert and Jackson, 1985). Thus, even though the turtle possesses some capacity for cutaneous O_2 uptake, which is particularly evident at low temperatures (Ultsch and Jackson, 1982; Herbert and Jackson, 1985), it is clearly unable to utilise the O_2 store in the aquatic environment with the same efficacy as the amphibian.

It is noteworthy that over the first 2 weeks of submergence at 3 °C, the blood P_{CO_2} of both the frog and turtle increases, leading to respiratory-induced decreases in blood pH (Herbert and Jackson, 1985; Fig. 4). As hibernation proceeds, however, the P_{CO_2} of the frog decreases, presumably as a consequence of the reduced metabolic rate and skin capillary recruitment (Burggren and Moalli, 1984; see below), and blood pH is eventually restored (Fig. 4). At the same time, the acidosis in the turtle continues to build as a result of the hypoxaemia-induced lactacidosis. Thus, under well-oxygenated conditions in the aquatic medium, the skin of the frog effectively supplants the air-breathing system, ensuring adequate protection against the development of a hypoxia-induced metabolic acidosis or a hypercapnia-induced respiratory acidosis.

Aquatic hypoxia and behavioural hypothermia

Overwintering frogs may encounter various degrees of aquatic hypoxia in ice-covered ponds and lakes. In many cases, the upper reaches of the ponds remain well-oxygenated over several months of ice-cover, with a distinct ‘oxycline’ developing at 1–3 m depth (e.g. Fig. 2). Although there have been various accounts of frogs (e.g. *Rana pipiens*) overwintering buried in or below the reducing zone at lake

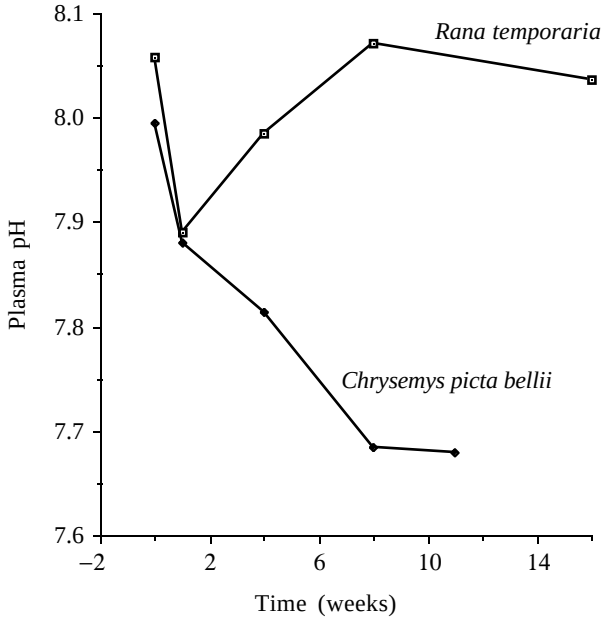

Fig. 4. Changes in blood pH during prolonged cold-submergence in normoxic water in the turtle *Chrysemys picta bellii* (data from Herbert and Jackson, 1985) and the frog *Rana temporaria* (P. H. Donohoe, R. G. Boutilier and T. G. West, unpublished results).

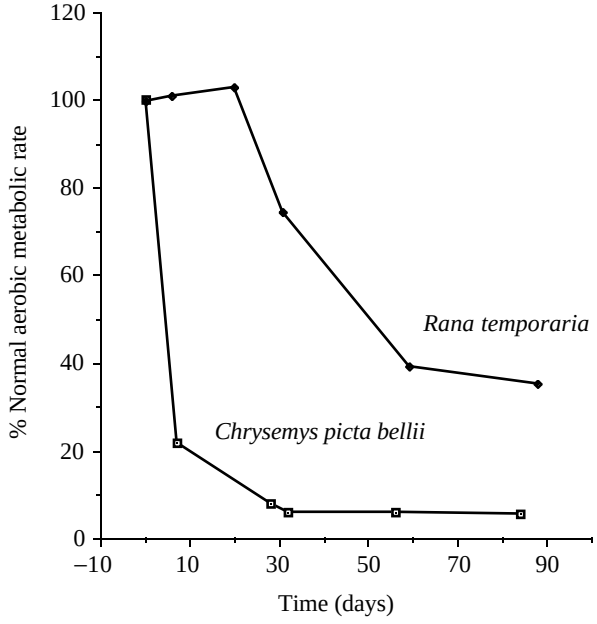

Fig. 5. Time course of metabolic depression during prolonged cold-submergence in anoxic water in the turtle *Chrysemys picta bellii* (data from Herbert and Jackson, 1985) and in hypoxic water (P_{O_2} progressively reduced to 60 mmHg over 90 days) in the frog *Rana temporaria* (P. H. Donohoe, R. G. Boutilier and T. G. West, unpublished results).

bottoms (see Hutchison and Dady, 1964), laboratory studies do not lend support to the notion that they can tolerate these conditions over an entire winter (i.e. frogs can survive anoxia for periods of only a few days to a week at 3–5 °C; Christiansen and Penney, 1973; Pinder *et al.* 1992). Nevertheless, these animals would have the capacity occasionally to 'dive' into anoxic waters to escape predation, and to periodically 'surface' to cooler oxygenated waters to restore any metabolic or respiratory imbalances.

Despite being generally inactive at low temperatures, amphibians do move about under the water and also appear to select preferred microenvironments over the course of a winter (Stinner *et al.* 1994). Indeed, overwintering frogs might offer a most ecologically relevant test of the behavioural hypothermia paradigm (Wood, 1991; Wood and Malvin, 1991, 1992), whereby environmental hypoxia evokes the selection of temperatures lower than the normoxic preferred temperature. Thermal and chemical conditions in ice-covered lakes can vary from 0 °C and normoxic at the ice–water interface to 4 °C and markedly hypoxic at depths of 2–4 m (Fig. 2; Bradford, 1983). As hypothermia and hypoxia may both have confounding effects on various rate processes and cellular transport mechanisms, it is possible that hibernating amphibians might occasionally adjust their position in the water column throughout the winter so as to select a preferred oxygen:temperature ratio. Behavioural hypothermia in amphibians has only been demonstrated at temperatures much higher than those experienced during overwintering (Wood and Malvin, 1991, 1992). Even so, the advantages of selecting lower temperatures in response to hypoxia would be the same, namely: (1) maintenance of the O_2 saturation of the blood through increases in P_{50}, (2) hypothermia-induced decreases in metabolic rate and conservation of energy reserves, and (3) reduced energetic costs of systemic oxygen delivery (Wood, 1991).

Respiratory and metabolic responses to hypoxia

The environmental extremes of cold, combined with oxygen lack, can have profound effects on the acid–base balance throughout the course of a winter. The low temperatures themselves ensure that the blood pH of normoxic ectotherms is high (approximately pH 8.0 at 3 °C) at the start of the overwintering episode (e.g. Fig. 4). However, as the metabolic demand for oxygen outpaces the ability to scavenge it from the environment, animals resort to anaerobic metabolism which inevitably leads to a lactacidosis. The prevailing theory has been that amphibians are poorly adapted to survive overwintering periods in which the water becomes severely hypoxic (Penney, 1987; Pinder *et al.* 1992). There is certainly no question that overwintering frogs cannot survive prolonged periods of *anoxia*, as certain turtles can (Fig. 5), but our recent studies on *Rana temporaria* reveal that the animals are capable of overwintering in severely hypoxic water without incurring any significant extracellular acidosis. Indeed, over the first 2 weeks of submergence in water having a P_{O_2} of 55–60 mmHg, extracellular pH (pHe) increased as a result of a combined respiratory and metabolic alkalosis (Fig. 6). During this time, the blood P_{CO_2} decreased from 4 mmHg to approximately 2.5 mmHg. Over a further 2 weeks of continued hypoxic

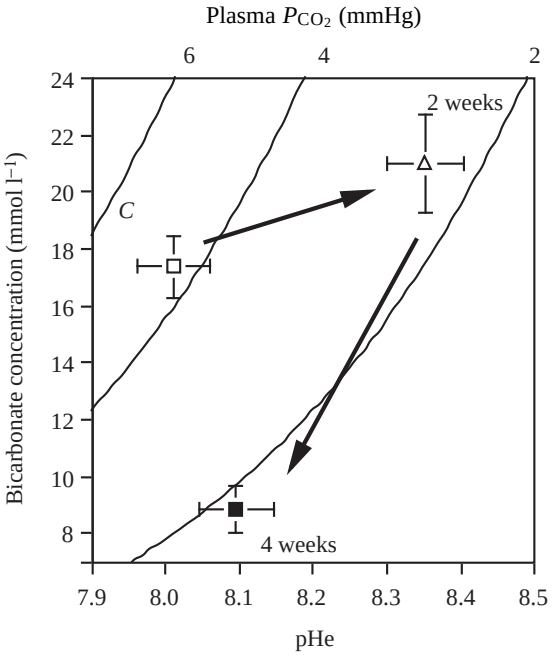

Fig. 6. pH–[bicarbonate] diagram illustrating changes in acid–base status of frogs (*Rana temporaria*) submerged in hypoxic water at 3 °C (P_{O_2} approximately 55–60 mmHg) (P. H. Donohoe, R. G. Boutilier and T. G. West, unpublished results). Curved lines are CO_2 isopleths. Values are means ± S.E.M., *N*=6. 1 mmHg=0.133 kPa. *C*, control value before submergence.

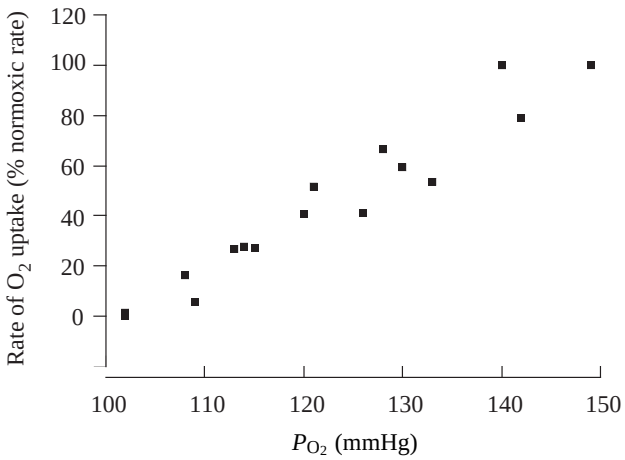

Fig. 7. Aerobic metabolic response of frog (*Rana temporaria*) sartorius muscle *in vitro* during progressive hypoxia at 5 °C (*N*=4 frogs).

submergence, a combined respiratory alkalosis and metabolic acidosis occurred, indicating that cutaneous O_2 uptake could no longer satisfy the metabolic demands of the animal. Even though the HCO_3^-/CO_2 system was adjusted to a new set-point, pHe remained elevated (Fig. 6), presumably aiding the maintenance of a high blood O_2-affinity.

Earlier studies on cutaneous gas exchange concluded that gas flux through the amphibian skin was largely passive and poorly controlled; i.e. that there was little if any capacity for modulating the rate of gas exchange in response to increased requirements for O_2 or CO_2 exchange (McKenzie and Jackson, 1978; Jackson, 1978; Boutilier *et al.* 1980; Moalli *et al.* 1981). However, most of these studies were conducted on animals at high temperatures (i.e. 15–30 °C) and therefore high metabolic rates. At hibernation temperatures, however, the rate of oxygen consumption ($\dot{M}_{O_2}$) of the entire animal is well below the maximum cutaneous $\dot{M}_{O_2}$ possible at such temperatures; i.e. cold-submerged bullfrogs are capable of maintaining their normoxic $\dot{M}_{O_2}$ as aquatic P_{O_2} levels decrease from 140 to 80 mmHg (Pinder, 1987). They accomplish this through a decrease in arterial P_{O_2}, which effectively maintains the P_{O_2} gradient across the skin, and through an increase in cutaneous diffusing capacity (Pinder, 1987). The increased diffusing capacity is probably the consequence of skin capillary recruitment (increasing the functional surface area for gas exchange; Burggren and Moalli, 1984) and a hypoxia-induced redistribution of blood to the cutaneous circulation (Boutilier *et al.* 1986).

Metabolite homeostasis during hypoxia

Metabolic suppression as part of an overall overwintering survival strategy

To summarise our discussion so far, we have emphasised that behavioural and whole-animal metabolic events in overwintering frogs are governed largely by ambient environmental temperature and oxygen level. Cold-induced reductions in activity level and metabolic rate processes, as well as avoidance of freezing and of environmental anoxia, are undoubtedly key features of the overwintering survival strategy in these organisms. It would also be advantageous for the organism to have some flexibility in its physiological and metabolic state. This would allow it to cope with instability in environmental variables independently of behavioural actions, which tend to be energetically costly. For example, cardiovascular adjustments during periods of environmental hypoxia may produce a blood circuit that becomes increasingly restricted to the gas-exchange surface (the skin) and the oxygen-sensitive core organs. By hyperperfusing the skin and redistributing tissue blood flow, the animal would avoid inadequate oxygenation in organs such as the heart and brain, despite the reduced gradient of P_{O_2} across the respiratory surface. In this situation, metabolic flexibility in the system would probably be most evident in the tissues that experience reduced perfusion. For example, the inactive and principally oxyconforming skeletal muscle mass (Fig. 7) is likely to display reduced oxygen consumption during extended periods of blood flow (and oxygen) limitation (Hogan *et al.* 1992; Stainsby *et al.* 1993). It is possible that the total (aerobic + anaerobic) metabolism of skeletal muscle could be defended at a constant rate if the reduction in oxidative ATP production were counterbalanced with parallel increases in anaerobic ATP generation *via* the glycolytic pathway. Although this might be a reasonable response in the short term, sustained glycolytic activation would rapidly deplete muscle energy reserves during periods of prolonged oxygen limitation. A more expedient

response would be to suppress the total metabolism (i.e. total ATP turnover) of the skeletal muscle mass in order to extend the utilisation of glycolytic reserves throughout the ensuing months of overwintering. Indeed, the whole-animal response to progressive overwintering hypoxia appears to be an even further suppression of metabolism (compare Fig. 5 with Fig. 1), given that the oxyconforming skeletal muscle represents such a large proportion of total body mass. In the section that follows, we will examine some aspects of metabolic plasticity that seem relevant to hypoxia-tolerance across vertebrate species and consider the relative ability of frogs to become hypometabolic in order to endure periods of low oxygen availability.

Comparative aspects of metabolic suppression: where do frogs fit in?

One goal of comparative studies of animal hypometabolism is to establish general principles by identifying responses that may be shared across species. When dealing with the specifics of cellular energetics, a certain complexity arises owing to the variety of different regulatory processes that may be involved. In fact, recent reviews concede that it is probably not possible to point to any one downregulated ATP-utilizing process as being singularly important to the long-term energetic economy and survival of hypoxia-tolerant organisms – with ion and acid–base homeostasis, transport processes, protein and urea synthesis and muscle contraction events all being contributing factors (Guppy *et al.* 1994; Storey and Storey, 1990). Similarly, several mechanisms may be invoked on the energy supply side. Suppression of glycolytic ATP generation involves modification of enzymes by phosphorylation/dephosphorylation reactions, masking of enzyme catalysis, possibly through protein binding to subcellular structures, and allosteric regulation of glycolytic enzymes (Harrison *et al.* 1991; Hochachka and Matheson, 1992; Storey, 1993; Guppy *et al.* 1994). Nevertheless, it may be that a general cellular hypometabolic response involves only a few primary signals that affect a few key processes. For example, activation of protein kinases, which in turn affect cellular protein phosphorylation state, can have a potentially broad-reaching and cascade-like inhibitory effect on a number of cellular functions, and for this reason is appealing as being universally relevant across hypoxia- and anoxia-tolerant species (Storey, 1993; Guppy *et al.* 1994). Furthermore, comparisons of tissue metabolic correlates of anoxic exposure have identified other, more basic, generalisations regarding the capacity for metabolic suppression in animals. Two fundamental characteristics are: (1) that a high, tissue-glycolytic capacity is important to long-term anoxic survival and recovery and (2) that it is critical to balance cellular ATP supply and demand throughout anoxic episodes. *R. temporaria* has only a moderate capability to withstand complete environmental anoxia. It displays characteristics of anoxia-tolerance capacity that are intermediate between those of ‘good vertebrate anaerobes’, such as turtles (*Pseudemys scripta* and *Chrysemys picta*) and carp (*Carassius carassius* and *C. auratus*), and more aerobically adapted species, such as rainbow trout (*Oncorhynchus mykiss*). This intermediate capacity is indicated from comparisons of whole-animal abilities to recover from anoxia at high ambient temperatures, from observations of differential organ responses to anoxia in *R. temporaria* itself, and from indicators of tissue energy balance and of tissue glycolytic capacity across species.

The work of G. Wegener and colleagues (reviewed by Wegener and Krause, 1993), on the behavioural and metabolic responses to complete anoxia in *Rana temporaria*, provides us with clues about the relative sensitivity of this animal to oxygen deprivation. These authors note that reflex movements in the frog were lost gradually (over 100 min) during exposure to pure nitrogen gas (at 20 °C) and that recovery in air was delayed if anoxia was continued beyond the point of paralysis. Recovery was usually inhibited if air-exposure was not initiated within 50 % of the anoxic period before paralysis was first noted. After 3 h of continued anoxia, brain concentrations of ATP, creatine phosphate (CrP) and glycogen had declined precipitously, while the concentration of ADP had doubled. Skeletal muscle glycogen and CrP levels were reduced by 40–50 %, but in contrast to brain tissue, ATP and ADP levels in skeletal muscle were maintained at the control concentrations throughout the anoxic insult (Wegener and Krause, 1993). Adenylate energy charge declined to a value of less than 0.4 in hypoxic brain, while muscle and heart maintained their energy charge at the control level, i.e. >0.9. At the same time, whole-body metabolic rate (measured as heat dissipation) in anoxia-exposed animals was depressed to approximately 30 % of the normoxic rate. These observations raise three points regarding the relative anoxia-tolerance of *R. temporaria*. First, they indicate that central nervous system (CNS) function in *R. temporaria* is most vulnerable to oxygen deprivation, in agreement with previous conclusions that amphibian (*R. catesbeiana*) brain tissue responded to anoxia in a manner similar to that seen in anoxia-intolerant mammalian brains (Sick and Kreisman, 1981). The different sensitivities of the frog brain and the turtle brain (Lutz *et al.* 1985) to oxygen deprivation would seem to account in large part for the reduced ability of frogs compared with the turtle to recover from prolonged anoxia. Second, they draw attention to the importance of maintaining cellular energy balance during anoxia. In brains of the more anoxia-tolerant turtles and fish (*Chrysemys picta* and *Carassius carassius*), cellular ATP and adenylate energy charge were defended to a far greater extent during anoxia than in *R. temporaria* (Lutz *et al.* 1985; Doll *et al.* 1994; Johansson *et al.* 1995; Buck and Bickler, 1995). Third, differential responsiveness of central (brain) and peripheral (skeletal muscle) tissues to complete anoxia, in conjunction with the short-term capacity of *R. temporaria* to suppress reversibly whole-body metabolism at 20 °C, indirectly addresses the possibility that this species is better equipped to deal with prolonged hypoxia than with anoxia. On the one hand, the defence of the energy balance in muscle

suggests that this tissue may be relatively tolerant to reduced oxygen supply and, given its large relative mass (approximately 60 % of body mass in frogs), it is likely that most of the whole-body suppression of metabolism can be attributed to inactivity and metabolic downregulation in muscle during anoxia/hypoxia. On the other hand, loss of energy balance and function in the oxygen-deprived CNS of frogs identifies this species as being sensitive to anoxia.

One consistent feature of organ homeostasis in hypoxia-tolerant species is stability in the concentrations of adenine nucleotides (ATP, ADP, AMP). Stable [ATP], in particular, is often considered central to the viability of tissues during oxygen deprivation. In the anoxic turtle brain, the decline in [ATP] in the initial stages of exposure is eventually compensated for as steady-state adenylate levels are re-established at, or above, the control normoxic level (Lutz *et al.* 1985; Kelly and Storey, 1988). A similar constancy in [ATP] is evident from studies with turtle hepatocytes (Land *et al.* 1993). Sick *et al.* (1993) point out that the turtle brain is not likely to be any more tolerant of energy failure than are oxygen-sensitive brains, in the sense that glycolytic blockade in anoxic turtle brains induces the characteristic mammalian responses of irreversible ATP loss and suppression of electrical activity. Instead, aerobic-to-anaerobic transitions in the turtle brain are highly dependent on the maintenance of a tight balance between energy-consuming processes and pathways of energy production. In a model of metabolic homeostasis in turtles and rats, it was indicated that the anoxic rat brain lost ATP very rapidly (to below 10 % of normal values within 5 min of anoxia) because glycolysis, CrP hydrolysis and downregulation of energy-consuming processes were ineffective in making up for the anoxic inhibition of oxidative metabolism (Sick *et al.* 1993). In turtles, however, these mechanisms appear to combine to buffer cellular [ATP] near the normoxic level. Interestingly, turtles do show a transient reduction in brain [ATP] and [ADP] early in an anoxic episode, the timing of which coincides with an increased level of adenosine in the extracellular space. Rising [adenosine] appears to influence energy balance in tissues by modulating brain glycogenolysis, through effects on enzymes and on substrate supply, and by depressing cellular functions that influence maintenance rates of ATP hydrolysis (e.g. neurotransmitter release, ion channel density, synaptic activity) (reviewed by Lutz and Nilsson, 1993). Adenine nucleotide levels recover rapidly, and the level of adenosine decreases after only 100 min of anoxia, leaving the possibility that secondary modulators of brain energetics come into play after the adenosine signal has diminished. Alternatively, or additionally, observations of cycling of extracellular adenosine, correlated with changes in intracellular [ATP], over 24 h of anoxia may mean that any hypometabolic effects of adenosine in turtle brain can remain influential over long periods (Lutz, 1995). Monitoring [ATP] has more than just diagnostic value in assessing tissue energy coupling and overall tissue viability during anoxia. [ATP] has been strongly implicated as a regulator of cellular K^+ homeostasis (Spruce *et al.* 1985; Krnjevic, 1993) and, in an indirect regulatory capacity, as the source of tissue interstitial adenosine, which is a suspected modulator of membrane/energy coupling in animal tissues (Magistretti *et al.* 1986; Nilsson, 1991; Nilsson and Lutz, 1992). The possible roles for [ATP] and adenosine in anoxic or ischaemic responses of vertebrate tissues have been reviewed elsewhere (Krnjevic, 1993; Lutz and Nilsson, 1993; Sick *et al.* 1993).

The anoxic frog brain responds like the anoxic mammalian brain in terms of K^+ balance (Sick and Kreisman, 1981) and in terms of changes in adenine nucleotide levels (Wegener and Krause, 1993), whereas protection of adenine nucleotide levels in frog skeletal muscle (Wegener and Krause, 1993) is a response characteristic of anoxia-tolerance. *R. temporaria* do show some tolerance to complete anoxia but, given that brain energy state becomes unstable immediately on exposure to anoxia, it seems likely that these animals are better adapted to confront periods of hypoxia rather than prolonged anoxia. More study is needed to examine this possibility and to elucidate the integrative mechanisms which might lead to hypoxic suppression of metabolism and to maintenance of aerobic energetics in central tissues.

Fuels for frogs

It is known that energy reserves fluctuate seasonally in amphibians (Smith, 1950; Harri, 1975). Observations by Smith (1950) indicate sequential deposition of lipid and carbohydrate stores over the summer and autumn (with fat being deposited earlier than liver glycogen) and the dramatic reduction of these stores by winter's end (liver glycogen is almost completely depleted from a peak store of approximately 1000 μmol g^{-1} and fat-body mass is reduced to approximately 1 % of peak autumn values), suggesting that the animal relies on both fuel types for overwintering metabolism. Observations of a doubling of fat-body triglyceride lipase activity in cold-acclimated *R. temporaria* (Harri, 1975) agree with an increased capacity for fat mobilisation in overwintering animals. In addition, low levels of insulin binding to liver membranes in the winter frogs (Scapin and Incerpi, 1992) are expected to remove a stimulus for glycogen synthetase activation and therefore to favour mobilisation of liver glycogen. If insulin effectiveness in frogs is generally diminished in the winter months, then this could also promote fat availability since part of the normal insulin response is to reduce plasma free fatty acid levels (Frayn, 1992). Hence, it would seem that the animal is metabolically poised to utilise both lipid and carbohydrate during the winter months.

At present, however, there are far too few metabolic correlates (hormone and enzyme quantifications) or direct *in vivo* measurements (respiratory quotients and plasma turnover rates) of substrate utilisation available to determine which substrate types and storage sites are preferred at different stages in an overwintering period. The variability in the rate of decline of fat and liver glycogen stores over the winter months makes it difficult to identify any temporal pattern in their depletion (Smith, 1950). However, using rates of oxygen

Table 1. *Estimated capacity for fat-body and liver glycogen stores to maintain overwintering metabolic rate in* Rana temporaria

	Rate of oxygen uptake ($\mu mol\ h^{-1}$)	Time to oxidize fat-body lipids, 225 µmol per animal (days)	Time to oxidize liver glycogen, 625 µmol per animal (days)	Total time of fuel oxidation (days)
Normoxic (day zero)	9.6	76	16	92
Normoxic (submerged 90 days)	5.7	129	28	157
Hypoxic (to 50 mmHg over 88 days)	2.9	254	54	308

Fat metabolism based on the assumption that fat body mass in *R. temporaria* (0.25 g in a 25 g animal; Smith, 1950) is 80 % triglyceride and that the typical triglyceride, $C_{57}H_{107}O_6$, requires 78 mol O_2 per mol triglyceride oxidized (Flatt, 1992).

Glycogen metabolism based on liver glycogen of 1000 $\mu mol\ g^{-1}$ (Smith, 1950) and 6 mol O_2 used per mol glucosyl unit oxidized.

Table 2. *Normoxic glycogen content in liver, heart and skeletal muscle of vertebrates that display varying capacities for hypoxia/anoxia-tolerance*

	Liver	Heart	Skeletal muscle
Turtle	1000	250	300–400
Goldfish	1000–2000	150	100–400
Frog	750–1000	50–150	50–100
Trout	200–300	20–50	20–50

Glycogen content is measured in $\mu mol\ g^{-1}$.

Data are compiled from Smith (1950), Merrick (1954), Daw *et al.* (1967), Hyvarinen *et al.* (1985), Penney (1987), Schulte *et al.* (1992) and T. G. West (unpublished observations).

consumption for *R. temporaria* held at 3 °C (P. H. Donohoe, unpublished observations) and peak storage levels of fuels as recorded by Smith (1950), it is possible to estimate the time it takes to deplete each fuel store in overwintering animals (Table 1). The calculations presented are intended only as rough estimates and to provide a means of comparing the relative energetic value of each fuel type. Two points emerge from the analysis. The first is that fat-body triglyceride has far greater potential for sustaining overwintering metabolic rate than does liver glycogen. Even if the muscle glycogen store (see Table 2) is taken into account (assuming total muscle mass is approximately 60 % of body mass), triglyceride in the fat-body alone has at least twice the potential of glycogen for supporting aerobic metabolism in the whole animal. The second point is that suppression of oxygen consumption as a result of the duration of submergence and as a result of hypoxia can significantly delay the oxidative depletion of body substrate stores and therefore extend the period that *R. temporaria* can withstand overwintering dormancy. In fact, suppression of metabolism is probably essential since combined fat-body and liver glycogen stores would provide only a few months of oxidizable fuels at the rate of oxygen uptake observed in the initial stages of normoxic water submergence. The aerobic metabolic rate suppression (by about one-third to one-half) noted after prolonged submergence at 3 °C delays complete depletion of these stores by an extra 2 months. Furthermore, if the animal remains aerobic during the progressive reduction of ambient P_{O_2} (down to approximately 50 mmHg; Table 1), then the further suppression of metabolism would allow for a 3.3-fold increase in the number of days that liver glycogen and fat-body lipids can support oxidative processes in overwintering animals. The observation that liver glycogen and fat-body mass are depleted over a period of 6–8 months before new stores start to be laid down (Smith, 1950) is consistent with some interval of metabolic rate reduction being important to the survival of overwintering *R. temporaria*. Conclusions about whether shifts in preference for different fuel types or storage sites might be associated with shifts in whole-animal metabolic rate await further investigation.

A final point to discuss about fuels in frogs relates to glycolytic capacity. A high glycolytic capacity is a characteristic feature of anoxia-tolerant systems (Hochachka, 1980). This capacity is manifest in good vertebrate anaerobes as relatively large stores of glycogen in different tissues (Table 2) and as relatively high activities of glycolytic enzymes (Christensen *et al.* 1994). Glycolytic capacity alone cannot be used as a criterion for judging organismal tolerance to low oxygen levels since high glycogen concentrations and glycolytic enzyme activities are also characteristic of aerobically adapted animals that possess high power output muscles (e.g. tuna white muscle; see Arthur *et al.* 1993). Nevertheless, it is instructive to examine glycolytic potential across species that display a certain capacity for aerobic metabolic rate suppression (as discussed above for *R. temporaria*) and different capacities for tolerance to environmental anoxia. A relatively straightforward analysis of tissue glycogen content (Table 2) suggests that frogs have moderate glycolytic capacities, in keeping with points discussed above that identify amphibians as having modest tolerance to anoxia; i.e. intermediate between that of turtles and that of mammals. Frog muscle glycogen stores are intermediate compared with those of the anoxia-tolerant turtles and goldfish and the more aerobically adapted rainbow trout. Similarly, cardiac enzyme profiles indicate that *Rana temporaria* occupies an intermediate position in glycolytic potential among these species (Christensen *et al.* 1994). The hepatic glycogen content of frogs, however, is within the same range as seen in anoxia-tolerant species, and whole-brain glycogen levels are approximately 20 $\mu mol\ g^{-1}$ in frogs (*R. pipiens*), turtles (*P. scripta*) and goldfish (*C. carassius*) (McDougal *et*

al. 1968). Both frogs (discussed earlier) and trout (see Boutilier *et al.* 1988) display the ability to reduce aerobic metabolic rate in response to progressive hypoxia, but the trout has limited capacity for survival in low-oxygen conditions (Boutilier *et al.* 1988). The superior survival capacity of frogs over trout in environments with fluctuating ambient oxygen levels may reflect their higher hepatic glycogen stores, essential for supplementing fermentable substrate in the oxygen-sensitive brain.

Long-term tolerance of anoxia is undoubtedly the result of sufficient glycolytic activation in combination with suppression of metabolism so that the organism will not exceed its glycolytic potential. This combination is probably best illustrated through comparative observations of cardiac performance *in vitro*. The interesting point from these studies is that hearts from both anoxia-tolerant and anoxia-intolerant species remain functional when deprived of oxygen as long as power output is reduced to a very low level. In fact maximum steady-state power output of anoxic turtle hearts at 15 °C is similar to that observed in severely hypoxic rainbow trout hearts (Arthur *et al.* 1992; Farrell *et al.* 1994). While this similarity represents subphysiological performance in the case of the trout, the significance of the results is that for a system to show long-term reversible tolerance to anoxia there must be considerable reductions in the energy-consuming reactions in order to attenuate glycolysis and allow for extended maintenance of energy balance.

Osmotic and ionic homeostasis

The cell membrane

Little is known about the cellular basis for metabolic rate depression. One hypothesis is that *metabolic suppression* at a cellular level is synonymous with *ion channel suppression* where hypoxia evokes adjustments in ion channel densities so as to lower membrane permeabilities and, therefore, the energetic costs associated with maintaining electrochemical gradients (Hochachka, 1986). In most mammalian organs or tissues, O_2 lack leads to just the opposite result; i.e. hypoxia-induced membrane failure, as ions drift towards their thermodynamic equilibrium. In facultative vertebrate anaerobes, however, hypoxia-induced membrane destabilization of the kind seen in mammals is either slow to develop or does not occur at all (Lutz, 1988). The catastrophic K^+ efflux seen in anoxic and ischaemic mammalian heart muscle and brain is thought to be caused by the opening of ATP-sensitive K^+ (K_{ATP}) channels (Noma, 1983; Spruce *et al.* 1985; Hansen, 1985) as concentrations of ATP fall below some critical level during hypoxia (Weiss and Lamp, 1987). If the K_{ATP} channels of facultative vertebrate anaerobes were similarly ATP-sensitive, the observed maintenance of ionic gradients in these animals (Lutz, 1988) could only be explained by accelerated ion pumping rates or by compensatory ionic changes in other passive leak pathways. The former seems unlikely, however, since the reduced ATP synthesis rates during anaerobiosis would presumably be insufficient to pace any accelerated Na^+/K^+-ATPase activity. Alternatively, preserved ionic gradients during hypoxia or anoxia could be explained by a relative decrease in membrane permeability (i.e. ion channel suppression), so that the energetic costs of maintaining cellular ion balance (e.g. through Na^+/K^+ exchange) could be drastically reduced. In this regard, glycolysis appears to be the preferred source of ATP for K_{ATP} channels in normoxic mammalian heart (Weiss and Lamp, 1987) and, if glycolysis were even more effective in preventing K_{ATP} channel opening in facultative vertebrate anaerobes, this could form the basis for a greater capacity for ion channel arrest in anoxia.

The mammalian response to anoxia includes hyperpolarization in neuronal cells, marked by a small shift of K^+ into the extracellular space. Hyperpolarization is deemed protective in that it suppresses energy-dependent electrical activity and may spare ATP for cellular homeostatic mechanisms, one of which is the direct inhibition of further K^+ leakage through ATP-dependent K^+ channels. However, the effect is transient, and eventually the energetic imbalance and decline in [ATP] described above lead to irreversible loss of membrane ion gradients. Part of the evidence that changes in [ATP] have a direct influence on K^+ conductance in mammals comes from observations that brain slices or cells pre-loaded with CrP (improving the cellular buffering of ATP) display smaller anoxia-induced [K^+] changes (for a review, see Krnjevic, 1993). In turtles, extracellular [K^+] reaches a new steady-state level that is 2–3 mmol l^{-1} above that seen in normoxic turtles, and this coincides with a fall in evoked potential activity (Sick *et al.* 1993). The loss of ion homeostasis evident in rats never develops in turtles, and this appears to be due largely to continued energy coupling, effective defence of adenine nucleotide concentrations and the maintenance of a low K^+ conductance through ATP-dependent K^+ channels.

Ionic exchange mechanisms and cellular energy metabolism are also intimately linked in intracellular pH regulation. Owing to the well-documented depressant effects that decreases in pH can have on the force output and contraction characteristics of cardiac and skeletal muscle of ectotherms (Langerstrand and Poupa, 1980; Renaud and Stevens, 1984; Wasser *et al.* 1990), it is important that there be effective mechanisms for regulating intracellular pH (pHi) during anoxia. For example, hypoxia-induced decreases in pHi of mammalian heart muscle lead to increased Na^+/H^+ exchange and a rise in intracellular Na^+ activity (Bountra and Vaughan-Jones, 1989) as protons are eliminated from the cell. pH regulation *via* Na^+/H^+ exchange can, however, be energetically costly. For example, in nucleated erythrocyte models of pHi regulation (Boutilier and Ferguson, 1989), activation of membrane Na^+/H^+ exchange elevates aerobic metabolic rate in direct proportion to the rise in cell [Na^+] as a result of increased Na^+/K^+-ATPase activity. The latter normally accounts for 20 % of the total aerobic metabolic rate but rises to 40–50 % of the cellular energy budget in pH-regulating erythrocytes (Ferguson and Boutilier, 1988, 1989; Tufts and Boutilier, 1991). Thus, pHi regulation

by a combination of accelerated Na^+/H^+ exchange and suppression of energy metabolism appears paradoxical. In addition to Na^+/H^+ exchange, recent experiments on normoxic frog muscle indicate that decreases in pHi enhance the opening of ATP-sensitive K^+ channels (Standen *et al.* 1992). Thus, the apparent maintenance of ionic gradients during anoxia in certain vertebrate anaerobes is made all the more surprising since falling ATP levels as well as pHi should conspire to increase rather than to decrease membrane permeability to K^+ *via* K_{ATP} channels. Thus, the strategies for pHi regulation during anoxia in facultative vertebrate anaerobes may be fundamentally different from those of their hypoxia-sensitive counterparts.

Even less is known about the signalling pathways involved in the transition to hypometabolic states during anoxia. There has been speculation that decreased pHi or increased intracellular P_{CO_2} may trigger metabolic rate depression (Lutz, 1988; Warburton *et al.* 1989; Wasser *et al.* 1990, 1993), but this has yet to be confirmed. Plasma catecholamine concentrations increase in proportion to the magnitude of the hypoxic acidosis in amphibians (Boutilier and Lantz, 1989), and titres can remain elevated above normoxic levels for several days in some ectotherms (Boutilier *et al.* 1988). Although catecholamine release is generally viewed as an emergency response to stress, such release could be an important early response to chronic anoxia if, through desensitisation of tissues (Sibley and Lefkowitz, 1985), it led to a downregulation of metabolic responsiveness. While there are many other possible systemic co-mediators of metabolic rate (e.g. corticosteroids, insulin, glucagon, thyroid hormones), there has been intense interest of late in more local chemical triggers. As an end-product of ATP degradation, adenosine offers a direct measure of cell metabolic status, and the identification of A_1 and A_2 receptors indicates that adenosine can serve as a negative feedback signal to correct imbalances between energy supply and demand (Belardinelli and Shryock, 1992). For example, concentrations of adenosine are inversely proportional to energy charge in the isolated heart of the frog *Rana ridibunda* (Lazou and Beis, 1986), and adenosine is therefore an obvious candidate for signalling when the rates of ATP synthesis fall below the rates of ATP usage. While the fluid and electrolyte composition of the intracellular fluids are governed by cell membrane transport processes, the cutaneous epithelium offers the first line of defence against perturbations in osmotic and ionic homeostasis.

The cutaneous epithelium

The skin of amphibians is a major osmoregulatory organ which, along with the kidney, serves to maintain ionic and osmotic homeostasis. However, exposure to cold temperatures (4–5 °C) disrupts this homeostasis: even though the rate of osmotic water influx declines, a cessation of urine production for some 12–18 h leads to a marked retention of water, a decrease in extracellular osmolality and a corresponding increase in body mass by as much as 10 % (Schmidt-Nielsen and Forster, 1954; Parsons and Lau, 1976; Bradford, 1984). During subsequent acclimation to the cold for several months, urine production rate reaches a new steady state at approximately 25 % of its former warm-acclimated value (DeHaan and Bakker, 1924; Parsons and Lau, 1976), body water content stabilises and the initial decrease in extracellular osmolality is reversed as electrolyte concentrations are slowly restored (Jørgensen *et al.* 1978; Bradford, 1984). Although cold-submergence of frogs in hypoxic or anoxic water can lead to even greater water retention than in their normoxic counterparts (Christiansen and Penney, 1973), little is known about the regulation of fluid and ion balance under these conditions. It is conceivable that an altered set-point in osmotic balance during hypoxia could place the animal in a more energetically favourable condition; i.e. a more dilute extracellular fluid would have the combined advantage of reducing the osmotic influx of water as well as lowering the energetic costs of transcutaneous ion transport.

As amphibian skin plays a dual role in gas and ion exchange, conditions that maximise the uptake of oxygen (e.g. vasodilation, increased surface area, decreased diffusion distances) could have the effect of enhancing the passive loss of ions (e.g. Na^+, Cl^-) from the animal to the environment (Stiffler, 1988; Boutilier, 1988; Boutilier *et al.* 1992). Active transport processes located in the apical membrane of mitochondria-rich cells (H^+-ATPase) and in the basolateral membrane of principal cells (Na^+/K^+-ATPase) are thought to be responsible for energising the uptake of Na^+ and Cl^- from the dilute medium surrounding the skin (Harvey, 1992). Ionic and osmotic homeostasis are therefore inextricably linked to the ready supply of ATP to fuel the ATPases. In well-mixed and normoxic waters, this linkage normally presents no problems to the animals since the skin is presented with high partial pressures of oxygen. In hypoxic conditions, however, ionic and osmotic homeostasis can only be achieved if the rates of ATP production are sufficient to supply the demands of the active transport systems at the skin. Presumably an ‘osmoregulatory compromise’ must be struck between the permeability requirements for effective gas exchange *versus* those operating to maintain water and electrolyte balance. For example, a reduction in the passive leak of ions from animal to environment (i.e. epithelial ion channel suppression) would effectively decrease the demands for active ion pumping and therefore ATP consumption. Although this hypothesis has not been examined directly as a function of hypoxia, the skin of frogs acclimated to 5 °C exhibits increased electrical resistance compared with that of 16 °C-acclimated animals (Koefoed-Johnsen and Ussing, 1974). The pathway accounting for this higher resistance is thought to be a cellular Cl^- shunt, suggesting some degree of ion channel suppression in response to low temperatures. Other studies have shown that the osmotic water permeability of frog skin decreases when animals are transferred from 15 to 5 °C (Parsons and Lau, 1976) and that such effects may be mediated through seasonal changes in winter responsiveness to hormones such as antidiuretic hormone (Parsons *et al.* 1978).

Epilogue

The survival strategies available to overwintering frogs are diverse, with temperature (through simple Q_{10} effects) influencing activity and metabolic rate processes, with instinct and behaviour guiding the animal away from intolerable environmental extremes (freezing and prolonged anoxia), and with physiological and metabolic plasticity accounting for further economies in energy expenditure. The combined effect of these adaptations must be to sustain whole-animal metabolic rate at a minimum homeostatic level, so that on-board fuel reserves do not become limiting to survival.

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