Physiological Ecology of Aquatic Overwintering in Ranid Frogs

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ABSTRACT

In cold-temperate climates, overwintering aquatic ranid frogs must survive prolonged periods of low temperature, often accompanied by low levels of dissolved oxygen. They must do so with the energy stores acquired prior to the onset of winter. Overwintering mortality is a significant factor in their life history, occasionally reaching 100% due to freezing and/or anoxia. Many species of northern ranid frogs overwinter in the tadpole stage, which increases survival during hypoxic episodes relative to adults, as well as allowing for larger sizes at metamorphosis. At temperatures below 5 °C, submerged ranid frogs are capable of acquiring adequate oxygen via cutaneous gas exchange over a wide range of ambient oxygen partial pressures ($P_{O_2}$), and possess numerous physiological and behavioural mechanisms that allow them to maintain normal rates of oxygen uptake across the skin at a relatively low $P_{O_2}$. At levels of oxygen near and below the critical $P_{O_2}$ that allows for aerobic metabolism, frogs must adopt biochemical mechanisms that act to minimise oxygen utilisation and assist in maintaining an aerobic state to survive overwintering. These mechanisms include alterations in mitochondrial metabolism and affinity, changes in membrane permeability, alterations in water balance, and reduction in cellular electrochemical gradients, all of which lead to an overall reduction in whole-animal metabolism. Winter energetic requirements are fueled by the energy stores in liver, muscle, and fat depots, which are likely to be sufficient when the water is cold and well oxygenated. However, under hypoxic conditions fat stores cannot be utilised efficiently and glycogen stores are used up rapidly due to recruitment of anaerobiosis. Since ranid frogs have minimal tolerance to anoxia, it is untenable to suggest that they spend a significant portion of the winter buried in anoxic mud, but instead utilise a suite of behavioural and physiological mechanisms geared to optimal survival in cold, hypoxic conditions.

Key words: hibernation, overwintering, north-temperate amphibian, frog, submergence, metabolic suppression, hypoxia tolerance, critical oxygen tension.

CONTENTS

I. Introduction ................................................................................................................. 120
II. The overwintering environment ...................................................................................... 120
III. The ecology of overwintering among ranid frogs: field studies ....................................... 121
    (1) Bullfrog (\textit{Rana catesbeiana}) ...................................................................................... 121
    (2) Green frog (\textit{Rana clamitans}) ..................................................................................... 121
    (3) Northern leopard frog (\textit{Rana pipiens}) ......................................................................... 122
    (4) Mountain yellow-legged frog (\textit{Rana muscosa}) ............................................................. 122
    (5) Columbia spotted frog (\textit{Rana luteovitris}) and Oregon spotted frog (\textit{Rana pretiosa}) .............. 122
    (6) European water frogs (\textit{Rana temporaria}, \textit{R. esculenta}, \textit{R. ridibunda}, \textit{R. lessonae}) ............................... 123
    (7) Overwintering mortality ................................................................................................... 123
    (8) Hibernaculum location and overwintering survival ......................................................... 123
    (9) The ecological importance of overwintering tadpoles .................................................. 123

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I. INTRODUCTION

Due to seasonal low temperatures, amphibians in north temperate climates are generally forced to hibernate [or overwinter; see Ultsch (1989) for a discussion of terminology] on land or underwater. If they hibernate on land, challenges include desiccation and low temperature. In North America, one approach to terrestrial hibernation is to overwinter below the frost line in a moist microenvironment, and this strategy is utilised by toads, terrestrial salamanders, and some frogs. Another strategy is to overwinter at lesser depths and tolerate freezing, which occurs in several terrestrial frog species, but apparently not in salamanders (Storey & Storey, 1986). The wood frog (Rana sylvatica), which occurs as far north as Alaska and hibernates on land, has received much attention in this regard (reviews include Layne & Lee, 1995; Storey & Storey, 2004).

Amphibians that hibernate underwater are either aquatic or semi-aquatic. Neotenic salamanders (e.g. Necturus maculosus; Petranka, 1998) and newts (e.g. Notophthalmus viridescens; Jiang & Claussen, 1992) may remain relatively active during winter, and even feed, but there are almost no data on their wintertime physiology. Until recently, the same was true for frogs, but in the past few decades there has been a spate of studies on frogs submerged at low temperature, particularly with regard to hypoxia tolerance (Donohoe & Boutilier, 1998, 1999; Donohoe, West & Boutilier, 1998, 2000; Tattersall & Boutilier, 1997, 1999a, b, c; West & Boutilier, 1998). Virtually all these studies have been with frogs in the family Ranidae. Ranid frogs in northern climates that hibernate underwater avoid desiccation and (normally) freezing as challenges. However, once the surface ice forms and persists, the frogs must endure extended periods of apnoea, becoming dependent upon the surrounding water for exchanges of respiratory gases, ions, and water. It is this under-ice environment and adaptations of frogs to it that is the subject of our review.

II. THE OVERWINTERING ENVIRONMENT

It is not possible to make a universal statement about the physico-chemical environment surrounding an overwintering frog. Two factors, however, will be of paramount importance: temperature and dissolved oxygen. If the water temperature at the surface remains above freezing, then the dissolved oxygen tension will typically remain high, due to wind-mixing, low biochemical O₂ demand, and photosynthesis. Furthermore, frogs are not torpid at low temperatures, so it is possible that if a free water surface is available, they could breathe air. Whether this occurs in nature at low temperatures is unknown, but in a laboratory setting, the presence of gas in the lungs after long-term maintenance at 3 °C in normoxic water (G.J. Tattersall, unpublished observations) indicated that air-breathing had occurred; had it not, the lungs would have collapsed (Ultsch, Brainerd & Jackson, 2004a). However, if the air temperature becomes cold enough that ice forms, then the overwintering frogs become apnoeic and must depend upon extrapolmonary (largely cutaneous) gas exchange. At temperatures low enough to cause ice formation, aquatic gas exchange is sufficient if the PwO₂ is relatively high, specifically above the critical O₂ tension (Pc), here defined as the PcO₂ below which the animal is no longer able to maintain its standard rate of oxygen consumption. The Pc is not thought to exceed 50 mmHg (1 mmHg = 133.3 kPa) for ranid frogs (Bradford, 1983; Tattersall & Boutilier, 1999a; Ultsch, Reese & Stewart, 2004b). Whether the PwO₂ falls below the Pc in a hibernaculum depends upon a number of variables, including the type of water body, whether there is a current, the depth, the time of winter and its duration and severity, and the substratum. Frogs hibernating in flowing water, even though the surface may be frozen, are unlikely to experience hypoxia severe enough to present a respiratory challenge (Bradford, 1983; Crocker et al., 2000; Ultsch, Graham & Crocker, 2000). However, in lentic habitats, where many ranid frogs overwinter, PwO₂ can fall below Pc, even to the point of anoxia, if the water is shallow, the environment eutrophic, and the ice is covered with snow for long periods. In the case of anoxia, frogs are relatively physiologically defenseless, being capable of surviving only a few days at best (Bradford, 1983; Stewart, Reese & Ultsch, 2004). Of most interest are hibernaculum environments that become hypoxic, but not anoxic, where there may be stratification for both oxygen and temperature (Fig. 1), with warmer, hypoxic water on the bottom (4 °C, <20 mmHg) and colder, partially oxygenated water just below the ice.
Since feeding does not occur during overwintering (data from *Rana temporaria*, G. J. Tattersall, personal observations; Pasanen & Koskela, 1974) and gut motility is severely reduced (data from *Rana pipiens*; Gossling, Loesche & Ottoni, 1980), energy intake is nil. Thus an overwintering frog may not only have to cope with hypoxia, but also with a finite supply of on-board energy stores. The crucial question in terms of understanding the biology of overwintering then becomes one of how a frog stays alive by balancing its energy supply and demand with a potential oxygen decrement; in some cases many do succumb and massive die-offs (winterkill) are well documented (see below).

### III. THE ECOLOGY OF OVERWINTERING AMONG RANID FROGS: FIELD STUDIES

Except for wood frogs (*Rana sylvatica*), North American ranid frogs normally hibernate underwater, and a number of species have tadpoles that overwinter (Collins & Lewis, 1979). In Europe, terrestrial overwintering among ranid species is more common, ranging from occasionally to commonly (see below). However, even among North American species that typically hibernate aquatically, overwintering on land is not precluded if a suitable hibernaculum can be located, and does occur under some circumstances (see below). In North America and Europe, typical poolside and streamside frogs are ranids, and we will deal exclusively with the genus *Rana* for both ecological and physiological discussions. Following is a summary of the ecology of overwintering of several ranid species.

(1) **Bullfrog (*Rana catesbeiana*)**

This species occurs as far north as Nova Scotia and southern Ontario. Hibernation is typically aquatic, although terrestrial hibernation has been reported (Bohnsack, 1952). Friet (1993) tracked bullfrogs during the winter in Nova Scotia, where ice covered his study site for 14-16 weeks. Bottom water was hypoxic (about 37 mmHg) for about four months. The $P_{wO_2}$ was higher closer to shore, so there were horizontal $PO_2$ gradients. As in other studies with hibernating frogs in lake and pond habitats (see below and other species accounts), most of the frogs stayed near the shore. What frogs he saw were not buried in the mud. This observation does not preclude mud burial, but it seems unlikely due to the anoxia that prevails in unstirred mud beyond the first few millimeters. Stinner, Zarlinga & Orcutt (1994) also found hibernating bullfrogs (in Ohio) to remain unburied; five out of six were located in shallow water near a stream inlet, and one was in 0.5 m of water. Movements occurred throughout the winter, although activity was minimal during the coldest portion. In Missouri, bullfrogs in two farm ponds had disappeared from view by 24 October when water temperature fell to 14°C and air temperature was 6°C; seining captured frogs, which suggests that they were not buried (Willis, Moyle & Baskett, 1956). Bullfrogs have also been found overwintering in seeps, a hibernaculum that may be used more frequently for overwintering by amphibians than is commonly appreciated (Sweet, 1960).

(2) **Green frog (*Rana clamitans*)**

Bohnsack (1951) described terrestrial hibernation of a single green frog in Michigan. The nearest pond (30 m distant) had dried, but there was another about 115 m away. Possibly the frog was caught between ponds by the cold weather, as green frogs typically hibernate underwater. Grant *et al.* (2004) found six juveniles submerged under a rock at the edge of a small pond in Maryland, along with three adult pickerel frogs (*Rana palustris*) and seven juvenile bullfrogs. In New York, Lamoureux & Madison (1999) radio-tracked 11 green frogs to their overwintering sites.

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**Fig. 1.** Time profiles of temperatures of a shallow pond in Canada (45°N) throughout the winter of 2004-2005. Temperature loggers placed at fixed depths reveal the rapid changes occurring near the surface and slower changes at the bottom. Top trace shows six months of temperature data; the middle and bottom traces show only the period of time during which ice was present at the surface. A considerable range (arrows in middle trace) of temperatures (0-6°C) is available to inhabitants of the frozen pond. The lower trace shows the thermal gradient (-1×slope) of the temperature-depth relationship, based on temperature loggers placed at 4 cm intervals. The thermal gradient is steepest early in the winter when the ground temperature warms the deeper waters, gradually becoming shallower as the winter progresses as ground temperature cools and the pond becomes stratified based on the temperature-dependent density of water (G.J. Tattersall, unpublished observations).
The frogs moved 80-560 m from breeding ponds to streams (five; three more untracked frogs were found under rocks in streams), seeps (four; two additional unmarked frogs were also found in the seeps), inside a beaver dam (one), and in a ditch (one). All sites provided flowing water during the winter and remained unfrozen. The tracked frogs were adults, but 80 metamorphs (and no adults) were collected on the pond side of a drift fence in the spring, suggesting they overwintered in the breeding pond. In Michigan, stream-dwelling frogs did not migrate to hibernate, but did so to breed, returning to their stream home ranges after breeding (Martof, 1953).

(3) Northern leopard frog (Rana pipiens)
This is the most northern ranid frog in North America that hibernates underwater, ranging well into central Canada where its hibernaculum can be covered by ice for more than half of the year. Emery, Berst & Kodaira (1972) studied leopard frogs under the ice in an Ontario pond. During early March, at a bottom water temperature of 2.5 °C, they found 15 frogs as deep as 3.1 m below the ice, all resting in small pits that they apparently excavated. The frogs were mostly lightly covered with silt. Two made slow voluntary movements and others lifted from pits were capable of slow swimming movements. The \( P_eO_2 \) was about 80 mmHg. Resident trout (Salmo gairdneri, Salvelinus fontinalis × S. namaycush hybrids) ate frogs during winter, but not during summer. Nevertheless, the frogs did not burrow into the substratum for concealment, probably because of the requirement for cutaneous \( O_2 \) uptake that could not occur if the frogs were buried.

Other studies report that overwintering leopard frogs occupy aquatic microenvironments that facilitate cutaneous \( O_2 \) uptake, that they often migrate to such sites from breeding and/or feeding grounds, that they often hide among rubble, under boulders, and in similar microhabitats that provide concealment but still permit water flow over the skin, and that the frogs retain mobility at temperatures as low as 0.5 °C (Blair, 1951; Cunjak, 1986; Merrell, 1977; Ultsch et al., 2000). Thus, access to dissolved oxygen appears to take precedence over concealment, if both cannot be provided in available hibernacula.

Leopard frogs have also been found to overwinter in numbers terrestrially. Usually caves or deep rock crevices are used (Rand, 1950); as is the case for the closely related R. palustris, which uses caves more frequently for overwintering than other frogs, although the frogs are typically in or near water within the caves (Fenolio, Graening & Stout, 2005; Johnson, 1987; Resetarits, 1986). Winter temperatures in such hibernacula, however, must remain above freezing for survival to occur, so the further north a frog occurs, the less likely it is to overwinter successfully on land.

(4) Mountain yellow-legged frog (Rana muscosa)
This is a high-altitude frog, currently found mainly in California. Bradford (1983) reported that in some winters in the Sierra Nevada mountains almost all adults died in shallow (<4 m) lakes, while tadpoles often survived. The tadpoles cannot survive anoxia; those kept at less than 5 mmHg at 4 °C died within several hours. However, the lower \( P_eO_2 \) at 4 °C for tadpoles (15 mmHg) than for frogs (30 mmHg) indicated that the tadpoles were better adapted to severe hypoxia. Thus tadpoles can serve as a population reservoir, allowing a population to persist without the necessity of immigration after a winterkill, so long as a lake does not become anoxic. As overwintering under ice can last up to nine months, the presence of overwintering tadpoles is an important aspect of the life history of this species, and probably of other northern ranid species. In a river, adult frogs survived, as would be expected from the higher \( P_eO_2 \) found in flowing water, regardless of ice cover (Bull & Hayes, 2002; Crocker et al., 2000; Ultsch et al., 2000). Thus there remained a source of adults for repopulation of ponds that did become anoxic, resulting in death of both adults and tadpoles.

While there are vertical gradients in \( P_eO_2 \) during winter in these mountain lakes, as in most lakes, these are irrelevant to both adult and larval ranids, as they are bottom dwellers and are unable to take advantage of the higher \( P_eO_2 \) near the surface, particularly since their lungs are collapsed during the winter (Ultsch et al., 2004a) and their negative buoyancy would lead to energetically costly attempts at accessing air. However, the frogs do avoid the deepest sections of the lakes, which would have the lowest \( P_eO_2 \), favouring near-shore hibernacula, often under rock ledges or within rock crevices where they may aggregate and to which at least some may migrate overland (Matthews & Pope, 1999).

(5) Columbia spotted frog (Rana luteiventris) and Oregon spotted frog (Rana pretiosa)
These are closely related western frog species that range from Nevada to Alaska. Bull & Hayes (2002) followed adult Rana luteiventris by radiotelemetry to wintering sites in northeastern Oregon over three years. Regardless of the type of habitat (lotic or lentic), the frogs remained in shallow water less than 1 m from shore, and remained mobile throughout the winter. Where hollow chambers under banks occurred, with unfrozen water and access to air, the frogs preferentially used them as hibernacula. Such sites provide protection from predators and scouring by high water flow rates (Martof, 1953), as well as an aerial \( O_2 \) source. Some frogs (16 of 66) moved from their capture and release site to other sites for hibernation (mean distance 275 m), but most did not. Water temperatures in ice-covered ponds (44% of hibernating frogs) and lotic habitats (23% of hibernating frogs) were less than 1 °C from December through February, but \( P_eO_2 \) did not fall below about 65 mmHg in the former, or about 123 mmHg in the latter. In partially frozen ponds (29% of hibernating frogs), water temperatures were somewhat higher for the same period (up to a mean of 3.7 °C) and \( P_eO_2 \) somewhat lower (50 mmHg), but no hibernaculum became anoxic; moreover, air-breathing was a possibility in ice-free areas. When higher water temperatures were available, as in partially frozen ponds with springs that created some open water...
surrounded by ice, the frogs favoured the warmer areas and stayed in the ice-free water. *Rana pretiosa* in Washington also hibernated in shallow (mean 17.4 cm) water, under ice and amid vegetation (Watson, McAllister & Pierce, 2003).

(6) European water frogs (*Rana temporaria, R. esculenta, R. ridibunda, R. lessonae*)

We have lumped these “species” together partly because of their relationships. *Rana esculenta* is a hybridogenetic water frog, being a hybrid of *R. lessonae* and *R. ridibunda*. Matings of *R. esculenta* produce fertilised eggs, but the larvae typically fail to metamorphose (Anholt et al., 2003). Terrestrial hibernation appears to be more common among the European ranids than among northern North American ranids (other than wood frogs). Berger (1982) states that in Poland, *R. lessonae* is a terrestrial hibernator, *R. ridibunda* hibernates underwater and occasionally on land, and *R. esculenta* hibernates on land when they occur with *R. lessonae* and in water when they occur with *R. ridibunda*. This mixed behaviour is difficult to explain, and it should be noted that the studies were not done under natural conditions. In studies of a fenced natural habitat in Switzerland, Holenweg & Reyer (2000) found that both *R. lessonae* and *R. esculenta* hibernated terrestrial, although they were sometimes flooded, and often changed hibernacula during the winter; pond bottom searches produced only two *R. esculenta*. However, these authors note that other studies have reported both of these species hibernating on land and in water, so the ability to do both seems to be present in the L-E (*lessonae-esculenta*) complex. R. Semlitsch (personal communication), states that *R. lessonae* hibernates on land, *R. ridibunda* in water, and *R. esculenta* in water and on land, in general agreement with published work.

*Rana temporaria* is a wide-ranging European frog that occurs as far north as above the Arctic Circle; it may hibernate for 8–9 months at high latitude or altitude. Hibernation is typically aquatic, with frogs often migrating in large numbers to suitable sites (Elmberg, 1990; Koskela & Pasanen, 1974; Pasanen & Sorjonen, 1994). Reflecting the wide distribution of this species, hibernation sites include virtually any permanent water body with either standing or flowing water. Occasional terrestrial overwintering has been reported, even in more northern localities (Coganimeau, 1991; see Koskela & Pasanen, 1974; Pasanen & Sorjonen, 1994; Vitanen, 1965). Pasanen & Sorjonen (1994) concluded that in some winters, approximately 33% of *Rana temporaria* overwinter on land. It is not clear whether these individuals endured sub-zero winter temperatures on land, or whether they were able to avoid freezing. Possibly some populations of *Rana temporaria* may have some degree of freeze tolerance; Pasanen and Karhapää (1997) demonstrated that *R. temporaria* do possess a limited capacity for freeze tolerance, being capable of surviving frozen for 24 h.

(7) Overwintering mortality

Winter is often associated with significant mortality in ranids. Anholt et al. (2003) studied *R. lessonae* and *R. esculenta* in Switzerland over six years, and found highly variable mortality rates, from 2 to 94% (mean of 38% for *R. esculenta* and 60% for *R. lessonae*); the high mortality rates were associated with winter severity. They did not state if the frogs were hibernating on land or in water; terrestrial hibernation was found for these species in Switzerland by Holenweg & Reyer (2000). Elmberg (1990) did not find springtime return rate of *R. temporaria* to be correlated with the harshness of winter in Sweden, but there the frogs hibernated aquatically; so a harsh winter might only prolong hibernation, not necessarily make it more stressful, provided a severe hypoxia did not occur. He did find that survival rate increased with age, but mortality (estimated from recapture rates) was high, ranging from 49 to 84% (seven-year mean of 69%) in males and 67 to 95% (seven-year mean of 84%) in females. These frogs hibernated in a river and moved to a breeding pond in the spring; since mortality was estimated from return rates, the estimated mortality values are likely to be higher than the actual rates (frogs could have been carried downstream out of the study area; such non-returns were considered as deaths), but the author argues that his results are comparable with other studies of the species.

(8) Hibernaculum location and overwintering survival

The overwintering location will influence mortality. Hibernating in streams and rivers assures the presence of water throughout the overwintering period, as well as adequate oxygenation of the water, although there is a threat from scouring during winter or early spring floods. Hibernating in lentic environments such as ponds and marshes increases the probability of falling water levels and/or O2 reduction, resulting in freezing and/or severe hypoxia or anoxia. If a pond freezes to the bottom, the frogs may move into the mud to avoid freezing or being crushed by the expanding ice, but the oxygen lack in anoxic mud will kill them in a matter of days. Water overlain by ice can become anoxic, due to heavy snow cover inhibiting light penetration and reducing or eliminating photosynthesis, which can lead to winterkills of frogs (Lacht, 1991; Manion & Cory, 1952; Merrell, 1977; Merrell & Rodell, 1968). Mortality can reach 100% in such lentic environments, while frogs hibernating in nearby lotic environments can survive (Bradford, 1983). In some cases frogs can migrate to more suitable overwintering sites during autumn (Elmberg, 1990), but in other cases the nearest alternative site is too distant and the frogs must hibernate in their summer habitats. Several consecutive harsh winters can effect a serious reduction of such populations. For example, Pasanen & Sorjonen (1994) found that the number of *R. temporaria* overwintering in a gravel pit in northern Finland decreased from 2876 in 1988 to 136 in 1992, following high mortality due to falling water levels (presumably leading either to freezing or anoxia) in the 1988–89 and 1991–92 winters.

(9) The ecological importance of overwintering tadpoles

Tadpoles are better suited physiologically to overwintering in hypoxic conditions than are adults (see discussion of
tures as low as 0.6-4 °C (Tattersall & Boutilier, 1999c). Times to full exhaustion from forced activity range from 15 to 18 min at temperatures of 7 °C and below (Tattersall & Boutilier, 1999c). Immediately following these bouts of activity, oxygen consumption rates are elevated 3.5-5.5 times above resting values and take up to 12 h to return to normal. Although exhaustive exercise is unlikely to be an ecologically relevant activity during overwintering, the fact that cold, submerged frogs are capable of prolonged activity and can significantly increase their rates of O2 consumption implies that overwintering frogs are neither incapable of movement, nor are they oxygen-starved while submerged in aerated water.

IV. THE PHYSIOLOGY AND BEHAVIOUR OF OVERWINtering: LABORATORY STUDIES

The literature on the physiology of aquatic overwintering of frogs is almost exclusively limited to ranids, and to a relatively few species within that genus, including the North American *Rana catesbeiana* and *R. pipiens*, and the European *R. temporaria*. Frogs hibernating underwater can be surrounded by water that is normoxic (≈ 160 mmHg at 0-4 °C) (typical of moving water), anoxic water (mud burial), or hypoxic water (prevailing in many still-water hibernacula). By definition, hypoxic water is that with any PO2 less than air-saturation (normoxic), but typically the term is used to denote a degree of reduction in PwO2 that is likely to be stressful to an animal, which makes it a rather relative term. For the PwO2 to be hypoxic enough to present a physiological challenge for a submerged frog, it has to be near or lower than the Pc, that level of PO2 below which the animal cannot maintain normal aerobic metabolic functioning for an extended period. Given an adequate O2 supply, then other factors such as body size, ionic regulation, water balance, temperature effects, energetics, etc., come into play in determining the limits of hibernation. Since gas exchange is probably the proximate limiting factor determining survival during long-term submergence, our focus in this section will be on describing how overwintering frogs match oxygen supply and oxygen demand. Both variables can be adjusted to ensure some degree of oxygen homoeostasis. Firstly, however, we will address the issue of what level of aerobic activity (an adjustable demand-side variable) submerged frogs can sustain, prior to discussing the importance of cutaneous gas exchange to metabolic homoeostasis.

(1) Activity capacity of overwintering frogs

It has long been recognised that overwintering frogs are not strictly torpid. Previous authors have remarked that frogs are capable of reacting to stimuli and moving at temperatures as low as 0.6-4 °C (Cunjak, 1986; Emery et al., 1972; Pasanen & Koskela, 1974; Ueltsch et al., 2000). Rather than lacking mobility, frogs submerged in cold normoxic water exhibit a remarkable capacity for induced activity (Ueltsch et al., 2000), with Q10 values for locomotory parameters ranging from 1.3 to 2.5 (Tattersall & Boutilier, 1999c). (2) Aerial respiration in the cold

The influence of temperature on aerial respiration has been examined in anurans (Kruhoff et al., 1987; Wang, Abe & Glass, 1998) and shows, predictably, that low temperature decreases the drive to breathe air. These studies, however, have not examined whether air breathing occurs at temperatures consistent with overwintering nor whether frogs in their natural environments breathe air at low temperatures. Prior to ice formation, aquatic frogs still have the option of breathing air. Since cutaneous gas exchange in the cold is more than adequate to fuel their aerobic requirements (Donohoe et al., 1998; Tattersall & Boutilier, 1997; also see next section), coming to the surface when the water temperatures are below 5 °C would be unnecessary to obtain adequate oxygen. If, however, the prevention of lung collapse (Ueltsch et al., 2004a) is a sufficient drive to breathe air, the respiratory behaviour of frogs should illustrate a trade-off between the costs (predation risk and energy used for surfacing (Kramer, 1988; Kramer & McClure, 1981) and the benefits of air-breathing. As expected from Q10 effects, decreasing the temperature increases the apnoeic periods between surface breaths in *R. catesbeiana* (Fig. 2, G. Tattersall and T. Coplen, unpublished observations). Increasing the depth required to reach the surface to breathe air increases the breath holds even further, and results in virtually indefinite periods of voluntary submergence (i.e., breath-holds lasted as long as the observation period of 12 h) at the lowest temperature tested (1 °C), suggesting that the energetic investment required to reach the surface outweighs any benefit of breathing air at these low temperatures (Shannon & Kramer, 1988). It is unlikely that this lack of air-breathing results from an inability to move since frogs have been shown to have a substantial capacity for spontaneous and directed movement at temperatures as low as 0-1.5 °C (Tattersall & Boutilier, 1999c; Ueltsch et al., 2000). In the field, it is possible that predation risks play a much more important role in determining whether frogs will come to the surface. Conceivably, as the winter progresses and the temperatures decline, the trade-off between the cost of surfacing and
benefits of air breathing is what drives the frogs underwater. Eventually this voluntary submergence promotes a gradual decline in metabolism (Donohoe et al., 1990) proposed to be associated with prolonged submergence. Certainly, it would be unlikely that a metabolic suppression could take effect if the frog were required to surface on a regular basis.

One reason why air breathing behaviour in the cold might be an untenable option for frogs is that it may, paradoxically, incur slight anaerobic costs. Frogs (Rana temporaria) housed for one week at temperatures ranging from 0 to 7 °C, either denied air access or with easy access to air (housed in 10 cm of water), have remarkably different physiological parameters considering they were all in normoxic water. Cold frogs that have ready access to air (housed in 10 cm of water), have remarkably different physiological parameters compared to those that are denied air access or submerged. The resulting diffusion boundary layer poses a limitation to cutaneous gas exchange (Booth & Feder, 1991; Vogel, 1994). These diffusion boundary layers result from the viscosity of the exchange medium. The decreased fluid velocity prevents bulk flow and mixing from occurring close to the solid surface, resulting in a stagnant layer of fluid through which diffusion is the only means of gas movement. The resulting diffusion boundary layer poses a limitation to gas exchange primarily because the partial pressure of O₂ at the skin-fluid interface is much lower than the value of PO₂ in the free stream, and thus presents a relatively small partial-pressure-driving gradient across the skin (Booth & Feder, 1991). Since it is the partial pressure gradient that ultimately determines the rate of diffusion of a gas across any surface, low values at the skin-water interface will result in decreased rates of uptake, and subsequent limitations to cutaneous gas exchange.

(3) Cutaneous gas exchange in skin-breathing amphibians

As in any respiratory organ, cutaneous gas exchange is a diffusive process whereby gases move across a layer of tissue and into the nearby bloodstream. Across large distances, the molecular movement of respiratory gases, governed by Fick’s Law of Diffusion (where concentration gradient is replaced by partial pressure gradient), is not a very efficient process. The thicker the barrier between environmental respiratory gases and the blood, the longer it takes for diffusion to occur, setting a limit on metabolic turnover of aerobic tissues. The site of cutaneous gas exchange in frogs is mainly across the skin. This thickness of the epidermal barrier ranges from 18 to 60 μm in amphibians (Czopak, 1965, 1983), which exceeds that of the lung epithelium by 1-2 orders of magnitude, making cutaneous diffusive transfer of gases a relatively slow process relative to pulmonary gas exchange. As a result, cutaneous gas exchange, particularly for oxygen, is usually only adequate at low temperatures, where metabolic demand is reduced. Since temperature has little influence on the physical process of diffusion (Q₁₀ ≈ 1), but strongly influences metabolic rate, particularly at low temperatures, cold, submerged frogs can meet their metabolic demands through cutaneous diffusion of oxygen and carbon dioxide.

Cutaneous gas exchange of amphibians has been described as occurring between the skin and an “infinite pool” of gases in the surrounding environment (Feder & Pinder, 1980). Empirical evidence demonstrates that an external boundary layer of poorly mixed gases can limit cutaneous gas exchange (Booth & Feder, 1991; Vogel, 1994). These diffusion boundary layers result from the decrease in fluid velocity close to solid surfaces, due to the viscosity of the exchange medium. The decreased fluid velocity prevents bulk flow and mixing from occurring close to the solid surface, resulting in a stagnant layer of fluid through which diffusion is the only means of gas movement. The resulting diffusion boundary layer poses a limitation to gas exchange (primarily O₂) exchange primarily because the partial pressure of O₂ at the skin-fluid interface is much lower than the value of PO₂ in the free stream, and thus presents a relatively small partial-pressure-driving gradient across the skin (Booth & Feder, 1991). Since it is the partial pressure gradient that ultimately determines the rate of diffusion of a gas across any surface, low values at the skin-water interface will result in decreased rates of uptake, and subsequent limitations to cutaneous gas exchange.

Amphibians have had to develop methods of coping with these diffusion boundary layers (hypoxic boundary layers). Increased water velocity acts by disrupting the boundary layer; thus increased activity or movement would be one method of reducing boundary layers (Burghgren & Feder,
Rana pipiens

In the field, water movement can be effected by locating to between 12 and 18 mmHg (occasionally in natural situations (Emery et al., 1972), removing accumulated silt and renewing water next to the skin in the process. The fact that frogs are not torpid at low temperatures, and can move about even in water that is near 0 °C (Emery et al., 1972; Tattersall & Boutilier, 1999; Ultsch et al., 2000) may well be pertinent to considerations of hypoxic boundary layers and gas exchange, even under normoxic conditions.

(4) Physiological responses to submergence in normoxic water

All ranid species that have been studied during submergence in cold normoxic, or near-normoxic water, have sufficient cutaneous exchange capabilities for both O2 and CO2. For example, in R. pipiens submerged at a PwO2 of 159 mmHg at 3 °C, blood P02 falls to about 9 mmHg, but there is no increase in lactate or decrease in pH (Stewart et al., 2004), attesting to a lack of anaerobiosis characteristic of other frogs submersed in cold normoxic water (Donohoe et al., 1998; Tattersall & Boutilier, 1999). Submergence induces a significant decrease in PCO2 (Donohoe et al., 1998; Tattersall, 1998) which would result in a decrease in the P50 of the blood, as would the low temperature. Maginniss, Song & Reeves (1980) found the P50 of R. catesbeiana blood at 5 °C to be 13.5 mmHg, and Pinder (1983) reported P50 values from R. pipiens at 5 °C ranging between 12 and 18 mmHg (PCO2 not stated). With the lower temperature used by Stewart et al. (2004), an estimate of a P50 no higher than 10 mmHg seems reasonable, which means that the blood would be close to 50% saturated with O2 at 9 mmHg. When P50 is reduced to about half air saturation (80 mmHg), the blood P02 falls to about 3 mmHg (Fig 3), but without a significant reduction in air uptake (Ultsch et al., 2004b), suggesting that a blood P02 of 9 mmHg when the frog is submersed in normoxic water at 3 °C is in excess of that needed to support the aerobic metabolic rate. With the reduction in metabolic rate induced by both prolonged submergence and the low temperature, the hibernating frog can remain essentially a cutaneous water breather throughout months of overwintering.

Despite the low values reported for cardiac blood P02 of submersed frogs at overwintering temperatures (Fig 3; Ultsch et al., 2004b), it is evidently sufficient for oxygen delivery to the tissues, since oxygen uptake is maintained without recruitment of anaerobiosis (see above). Blood isolated from cold, submersed frogs (Rana temporaria) has a similarly high affinity for oxygen (Fig 4; Tattersall, 1998). Temperature, however, still has a significant effect on blood P50 (8.1 and 12.3 mmHg at 1.5 and 7 °C, respectively, at 3.9 mmHg CO2). Increasing the PCO2 to 7.8 mmHg increases the P50 to 13.0 and 15.3 mmHg at 1.5 and 7 °C, respectively (Tattersall, 1998). These affinities should be reasonable estimates of blood oxygen affinity in vivo, since similar ranges of PCO2 are found in cold-submersed frogs (Stewart et al., 2004; Tattersall & Boutilier, 1997). A high affinity for oxygen at low temperatures would permit more than adequate loading of oxygen at the skin over a broad range of environmental P02. Although a large P02 gradient is required to drive oxygen exchange across the skin (Pinder, 1987), the reduced metabolic rate during submergence (Donohoe et al., 1998), in conjunction with the low blood P02 and high O2 affinity associated with low temperatures, appears to be sufficient to meet tissue oxygen demands. Furthermore, the Bohr coefficient of frog blood at 1.5 °C is much higher than that at 7°C (-0.71 compared to -0.29; see Fig 4), demonstrating that at the lowest temperatures, the effect of tissue [H+] on haemoglobin binding in overwintering frogs may be strong enough to act favourably for off-loading oxygen at the tissues in spite of the extremely high affinity for O2 (Tattersall, 1998).

(5) Physiological responses to submergence in anoxic water

Only CO2 elimination is relevant in anoxic water, and frogs have no difficulty eliminating CO2 to the water while hibernating so long as the PCO2 of the water is not elevated. The greatly reduced metabolic rate, coupled with the ease with which CO2 is lost through the integument in amphibians due to the high solubility of CO2 in water and a well-perfused cutaneous vasculature can effect a respiratory alkalosis during prolonged cold submergence that may be of ecological consequence in combating the metabolic lactic acidosis that occurs during short bouts of anoxia (Stewart et al., 2004). Although frogs do not tolerate anoxia for long periods, that does not necessarily mean that they have no adaptations to anoxia, nor that the ability to survive anoxia for a matter of days is irrelevant. Ponds, in particular, have a heterogeneous distribution of O2 during winter, both in space and time, ranging from anoxia in the mud to potential supersaturation in the water column during periods of intensive photosynthesis. Vertical P02 profiles are of little import to a hibernating frog, which is a bottom dweller, but horizontal (shore to depth) P02 gradients do occur, and can be exploited by frogs, which remain mobile even at very cold temperatures. Moreover, anoxia may be transient, due to excessive snow cover on a pond that may melt or be swept away. Therefore the ability to survive anoxia for relatively short periods (days), could be crucial. Since the leopard frog (R. pipiens) has been frequently reported to be subject to winterkills due to anoxia, and is one of the species for which data on the physiological responses to anoxia are available, we use this species as a model.

At 3-5 °C, anoxic R. pipiens (and R. temporaria and R. catesbeiana) can survive no more than 4-7 days (Christiansen & Penney, 1973; Donohoe & Boutilier, 1999; Stewart et al., 2004). After four days of anoxia, R. pipiens exhibits many of
the same physiological responses in the plasma shown by anoxia-tolerant turtles over much longer periods: decreases in $\text{PCO}_2$, pH, $\text{[HCO}_3^-]$, $\text{[Na}^+]$ (often not seen in turtles), and $\text{[Cl}^-]$, and increases in total $\text{[Ca]}$, total $\text{[Mg]}$, $\text{[glucose]}$, $\text{[lactate]}$, and haematocrit (Stewart et al., 2004). Most of these changes, when they occur in turtles, can be considered compensatory to the primary challenge of a continual production of lactic acid (reflected by the steady increase in lactate levels), but in the leopard frog (and bullfrog) the rate of lactate accumulation is so rapid that the fall in pH (Fig. 5) quickly overwhelms any compensatory responses. Primarily because of their shells, even relatively anoxia-intolerant species of turtles have a greater whole-body buffering capacity than frogs (Table 1). In Table 1, the reference decrease in pH is to 7.4 because the frogs die at that pH. Although frogs and turtles have a similar initial pH, the turtles can survive to as low as pH 7.0, and are therefore not only better able to buffer a fall in pH, but are also capable of surviving a greater pH decrement.

While 4-7 days of survival in anoxic water in the cold is not impressive relative to ectotherms such as turtles or crucian carp ($\text{Carassius carassius}$), survival is still a matter of
days, not minutes or hours. Thus, it is fair to state that frogs have limited tolerance to anoxia compared to many turtles. They can, however, still tolerate short periods of anoxic challenge, even at room temperature (Knickerbocker & Lutz, 2001; Wegener & Krause, 1993). That said, understanding their potential short-term mechanisms for anoxia tolerance is important from a comparative perspective as well as from an ecological one. For example, properties of the frog’s mitochondria may help bestow it with limited anoxia tolerance. Normally, under oxygenated conditions, the mitochondrial F₁F₀-ATPase protein is responsible for producing ATP by harnessing the potential energy stored in the proton motive force (i.e., the mitochondrial membrane potential) generated from the electron transport chain and aerobic glycolysis. During anoxia, this protein can become a net ATP consumer, in a form of ‘cellular treason’ (St-Pierre, Brand & Boutilier, 2000a). When oxygen is in short supply, the electron transport chain is not functional; thus the protons normally transferred across the inner mitochondrial membrane to maintain the proton motive force are not available. By operating in reverse, the F₁F₀-ATPase can maintain mitochondrial membrane potential and prevent cell death, providing a paradoxical role for the same enzyme.

![Graph](image)

**Fig. 4.** Oxygen equilibrium curves of blood isolated from *Rana temporaria* (N = 10) submerged at low temperatures (3-4°C), and incubated at 1.5°C and 7.0°C and P₂CO₂ = 3.9 mmHg (similar to in vivo values). P₅₀ values were 8.11 and 12.3 mmHg at 1.5°C and 7°C, respectively, increasing to 13.0 and 15.3 mmHg at 7.8 mmHg CO₂. The Bohr coefficient (Δlog P₅₀/ΔpH) was more than twice as high at the lower temperature. Data from Tattersall (1998).

![Graph](image)

**Fig. 5.** Changes in plasma pH (A) and plasma lactate levels (B) as a function of time submerged at 3 °C in anoxic (filled circles, N = 9) and normoxic (open circles, N = 4) water in Canadian *Rana pipiens*. Values are means ± S.E. Some error bars are smaller than the symbol size. After Stewart et al. (2004).

Table 1. Changes in plasma [lactate] that produce a fall in plasma pH to 7.400 during submergence anoxia at 3 °C among anoxia-tolerant turtles (*Chrysemys picta bellii* and *Chelydra serpentina*), anoxia-intolerant turtles (*Graptemys geographica*, *Apalone spinifera*, and *Sternotherus odoratus*), and ranid frogs (*Rana pipiens* and *R. catesbeiana*). From Stewart et al. (2004).

<table>
<thead>
<tr>
<th>Species</th>
<th>Initial pH</th>
<th>Initial [lactate] (mmol l⁻¹)</th>
<th>[lactate] at pH 7.400</th>
<th>Lactate accumulation (mmol/day)</th>
<th>Δ[lactate]/ΔpH at pH 7.400</th>
<th>Days to reach pH 7.400</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chrysemys picta bellii</em></td>
<td>7.993</td>
<td>1.7</td>
<td>99.3</td>
<td>1.36</td>
<td>167.5</td>
<td>73</td>
</tr>
<tr>
<td><em>Chelydra serpentina</em></td>
<td>8.057</td>
<td>1.8</td>
<td>84.6</td>
<td>2.28</td>
<td>126.0</td>
<td>37</td>
</tr>
<tr>
<td><em>Graptemys geographica</em></td>
<td>8.010</td>
<td>1.7</td>
<td>69.4</td>
<td>3.86</td>
<td>111.0</td>
<td>18</td>
</tr>
<tr>
<td><em>Apalone spinifera</em></td>
<td>7.923</td>
<td>0.8</td>
<td>54.0</td>
<td>6.00</td>
<td>101.7</td>
<td>9</td>
</tr>
<tr>
<td><em>Sternotherus odoratus</em></td>
<td>7.958</td>
<td>0.7</td>
<td>38.0</td>
<td>7.60</td>
<td>66.3</td>
<td>5</td>
</tr>
<tr>
<td><em>Rana pipiens</em></td>
<td>8.058</td>
<td>3.2</td>
<td>38.2</td>
<td>9.55</td>
<td>34.7</td>
<td>4</td>
</tr>
<tr>
<td><em>Rana catesbeiana</em></td>
<td>8.071</td>
<td>2.6</td>
<td>35.6</td>
<td>11.87</td>
<td>49.2</td>
<td>3</td>
</tr>
</tbody>
</table>

but potentially exacerbate the problem of anoxia exposure by depleting cellular ATP stores faster than has previously been predicted. Overwintering frogs in anoxia, however, are capable of modulating the rate at which this enzyme operates (St-Pierre et al., 2000a). Under anoxic conditions, the F$_1$F$_0$-ATPase operates at much reduced levels compared to that under more highly oxygenated conditions, suggesting a modulatory capacity. This capacity translates into a much reduced rate at which ATP is consumed in anoxic tissues; however the small turnover of ATP must still be sufficient to maintain mitochondrial membrane potential. Although it is not clear why this ATP consumption still occurs, it is possible that maintaining the membrane potential can prevent mitochondrial damage and/or programmed cell death that would otherwise occur due to a collapsed membrane potential, while conserving the limited ATP present in the anoxic cell.

The above mechanisms for anoxia tolerance may be suitable for short-term anoxic exposures that may be encountered by overwintering frogs. Given, however, the more limited anoxia tolerance seen in many ranid frogs compared to turtles, it is likely that long-term hypoxia exposure is the more ecologically relevant scenario for most overwintering frogs, which is supported by the various mechanisms of hypoxia tolerance observed in overwintering frogs (Boutilier & St-Pierre, 2000). These mechanisms are discussed next.

(6) Physiological responses to submergence in hypoxic water

The requirement for sufficient dissolved oxygen while hibernating is an overriding one for frogs. Submergence in normoxic water for months is not a physiological challenge, while submergence in anoxia leads to death in a matter of days, so the question becomes one of what happens in between; that is, as $PO_2$ declines in the water, at what point does hypoxia become a challenge? Hence, most recent studies have dealt with the ability of frogs to tolerate considerable hypoxia over long periods, which is common in many cold-temperate aquatic overwintering environments.

A number of studies of hypoxic frogs have chosen a particular level of hypoxia for study, sometimes without evidence that the chosen $PO_2$ is stressful. A traditional approach to defining hypoxic stress has been to determine the critical $O_2$ tension ($P_c$), which is usually defined as that ambient $PO_2$ during progressive hypoxia at which an organism (often an aquatic one) can no longer maintain its standard metabolic rate (SMR), most often measured as the rate of $O_2$ consumption ($V_{O_2}$). This approach has been questioned by H.O. Portner & colleagues (Portner & Grieshaber, 1993; Portner, MacLatchy & Toews, 1991) who argue that the $P_c$ should be defined as the $PO_2$ at which anaerobiosis commences, as indicated by an increase in lactate levels. In their view, the appearance of lactate is a signal for the animal to seek a higher-$PO_2$ environment. In *Bufo marinus* at 20 °C, they reported an increase in $V_{O_2}$ (presumably due to escape activity) that preceded the eventual steady reduction in $V_{O_2}$ as progressive hypoxia continued. One might reason that the appearance of a decrease in $V_{O_2}$ and an increase in lactate concentration would be coincident, as the Pasteur effect comes into play in an attempt to make up the deficit in ATP production associated with the fall in $V_{O_2}$. The issue remains unsettled for overwintering frogs (Ultsch et al., 2004b), although here we use the traditional definition of $P_c$.

One point that has become evident is that the $P_c$ is a variable itself. Essentially there is an acute $P_c$ and a chronic $P_c$. If a frog is submerged immediately and exposed to a relatively rapid progressive hypoxia, the $P_c$ determined under these conditions is an acute $P_c$, and it will be higher than the $P_c$ exhibited if the exposure to hypoxia is gradual. A number of factors can contribute to a reduction of $P_c$, as illustrated in Fig. 6. If one starts with an air-breathing frog

![Fig. 6](image-url)
with a $P_e$ standardised to 100% of its value at 20°C in air, and lowers the temperature to 3°C, the relatively high $P_e$ will be substantially lower due to a decrease in the SMR. Even for a moderate Q10 of 2.0, this effect alone would reduce the $P_e$ to 31% of its original value. The actual effect on $P_e$ is likely even more pronounced, considering that Q10 values can be greater than 4.0 at low temperatures (Tattersall & Boutillier, 1997; see also Ultsch, 1989). Further reductions in metabolic rate of about 50% each have been reported due to prolonged diving (Donohoe & Boutillier, 1990) and to the presence of long-term hypoxia (Donohoe & Boutillier, 1998), which would further reduce the $P_e$ to 7.8% of the original. Finally, whole-body O2 conductance, the efficacy with which O2 is transported from the external environment to the mitochondria (Ultsch, Bradford & Freda, 1999), represented by the slope of relationship between $V_{O2}$ and PO$_2$ in the range of metabolic O2 conformity, is also subject to modification by a number of physiological and morphological changes associated with exposure to prolonged hypoxia (see below). Any of these changes that increase the O2 conductance will further reduce the $P_e$; however, the preponderant effects are those that lower metabolic rate. The overall effect of all changes is a profound lowering of the $P_e$, conferring an ability to remain aerobic under conditions of considerable hypoxia. The $P_e$ of Rana pipiens at 3°C is reported as 36 mmHg (Ultsch et al., 2004b), and that of R. temporaria at 1.5-3°C has been estimated to be =30-40 mmHg (Tattersall & Boutillier, 1997, 1999a). There are no convincing data for fishes at such low temperatures, but one would expect them to have a lower $P_e$ than submerged frogs, because the countercurrent exchange system in their ventilated gills is a more effective system for O2 uptake than cutaneous exchange through a relatively thick and poorly ventilated skin. The $P_e$ of carp (Cyprinus carpio) and rainbow trout (Oncorhynchus mykiss) at 5-10°C ranges from 11 to 21 mmHg (Ott, Heisler & Ultsch, 1980), so at 1-3°C a typical fish $P_e$ is likely less than half that of a submerged frog, which suggests that winterkill in frogs will precede that of fishes in the same habitat. Nevertheless, a PO$_2$ of 40 mmHg represents about a 75% reduction in O$_2$ availability compared to normoxic water, so frogs are well adapted to environmental hypoxia at relevant overwintering temperatures.

There are a number of mechanisms involved at both the morphological and physiological levels that are brought into play that enable a frog to function in a relatively severe hypoxia. Morphological effects that increase O2 delivery have been shown to be particularly important in the tadpole stage. When bullfrog tadpoles were exposed to four weeks of hypoxia in both the air and water phase (bullfrog tadpoles have functional lungs) at 75 mmHg at 20-23°C, aquatic $V_{O2}$ was enhanced by thinning of some regions of the skin, increases in the cutaneous capillary density, and enlargement of the gills due to increases in the size and number of gill filaments (Burggren & Mwalukoma, 1983). There were no corresponding morphological effects in adult bullfrogs, which used mainly physiological adjustments to enhance their O$_2$ delivery, including a reduction in the $P_{50}$ of the blood from 35 mmHg to 24 mmHg, and a doubling of the O$_2$ carrying capacity of the blood due to polycythemia (Pinder & Burggren, 1983). However, Boutillier, Glass & Heisler (1986) found that hypoxia exposure induced a redistribution of blood flow to the skin, which should result in capillary recruitment, and thus, an increase in gas exchange capacity. In addition, at 5°C, the cutaneous diffusing capacity for O$_2$ (DO$_2$) of adult bullfrogs doubled as the $P_{50}$ was decreased from 140 to 30 mmHg, presumably by capillary recruitment and by lowering the internal PO$_2$, thus preventing the PO$_2$ gradient across the skin from decreasing as rapidly as it would have if $P_{50}$ were the primary determinant of O$_2$ exchange rate (Pinder, 1987). This study reported a $P_e$ of 80 mmHg, which is considerably higher than reported for other submerged ranids at low temperature, and it is possible that the use of curarisation had some unknown effect, but it also possible that the large size of the frogs (mean mass 262 g), and the attendant lower surface area to volume ratio, may have resulted in a higher $P_e$. In any event, with an increase in DO$_2$ accompanying the same adaptive changes in respiratory variables of whole blood shown for adults at higher temperatures, and which would also be expected at lower temperatures, there is a clear ability to acclimate to hypoxia during overwintering.

(7) Biochemical responses to prolonged hypoxic submergence

Once O$_2$ transport has been maximised in hypoxia, further physiological adaptations relate to minimizing O$_2$ requirements at the cellular level, i.e., metabolic depression. The reduction is not immediate. Rana temporaria submerged at 3°C at a PO$_2$ of 60 mmHg initially accumulate plasma lactate from a control level of 1.27 mmol l$^{-1}$ up to 9.22 mmol l$^{-1}$ over a four week period, but a continuing reduction of metabolic rate allows the frogs to become completely aerobic between four and eight weeks of submergence, to the extent that they are able to metabolise the lactate and return it to pre-diving levels (Donohoe & Boutillier, 1998). Thus a PO$_2$ of 60 mmHg, while a considerable hypoxia, may represent only a transient challenge to an overwintering frog, due to a 'hypometabolic rescue' of aerobic capacity (Donohoe & Boutillier, 1998). At levels of hypoxia that are long-term challenges, several mechanisms have been proposed that would lower metabolic rate and also increase metabolic efficiency (see reviews of Boutillier, 2001a, b; Boutillier et al., 1997; Boutillier & St-Pierre, 2000, 2002). These include hypoperfusion of skeletal muscle in favor of more oxygen-dependent tissues (Donohoe & Boutillier, 1998; West et al., 2006), changes in mitochondrial function that reduce ATP “wastage” (Boutillier & St-Pierre, 2002), inhibition of protein synthesis (Bartrons et al., 2004; Boutillier, 2001a), ion channel arrest (Donohoe et al., 2000), and a decline in the electrochemical gradient that cells are maintaining (Donohoe et al., 2000), thus lowering the considerable expense of moving ions across membranes against electrochemical gradients. In concert, all these effect a profound metabolic depression that allows the cold, submerged frog to be tolerant of a substantial, but not unlimited, hypoxia.

Direct evidence for a hypoperfusion of muscle of overwintering frogs is lacking (West et al., 2006), although the redistribution of blood flow between the pulmonary and
cutaneous blood supplies during submergence observed by Boutilier et al. (1986) suggests that a similar oxygen-dependent redistribution occurs in the systemically perfused tissues during hibernation. Donohoe & Boutilier (1998) further emphasised that the oxyconformation response so characteristic of muscle metabolic requirements (West & Boutilier, 1998) could serve to reduce whole-animal metabolic requirements. Considering that a frog’s body mass consists of approximately 35% skeletal muscle (Putnam, 1979), a progressive reduction in blood supply to this tissue could account for the vast majority of the whole-animal metabolic suppression, effecting a substantial supply to this tissue could account for the vast majority of the metabolic savings. Interestingly, muscle function does not appear to deteriorate during the winter months, with normal power and force development unaffected by long-term normoxic or hypoxic submergence (West et al., 2006). The mechanism of this apparent atrophy resistance is unknown, although its relevance to the ecological realities that present themselves during the intense spring breeding season may dictate that muscle fibre function not be compromised during the overwintering period, despite the potential for major metabolic rearrangements that could effect metabolic savings.

In terms of metabolic efficiency, St-Pierre, Tattersall & Boutilier (2000) found that the isolated mitochondria from frog skeletal muscle have reduced State 3 respiration (i.e., the maximal respiration rate) and increases in oxygen affinity that take place during four months of simulated hibernation in normoxic water. Frogs submerging in hypoxic water also exhibited increases in mitochondrial oxygen affinity, with the most pronounced change observed during the first month of overwintering. These slow changes in mitochondrial efficiency are likely due to the fact that whole-animal metabolic rates also take over one month to readjust to lower levels (Donohoe & Boutilier, 1998; Donohoe et al., 1998), and thus the greatest increase in oxygen affinity occurs during the early, more intensely stressful period of overwintering. In other words, it appears to be adaptive for mitochondrial O₂ affinity to change prior to the whole-animal reduction in metabolism. The mitochondrial changes may not be adequate to prevent an initial anaerobiosis, as evidenced by the transient plasma lactate accumulation in hypoxic submerged frogs (Donohoe & Boutilier, 1998); this lactate disappears after one month of submergence (Donohoe & Boutilier, 1998) when total metabolic requirements and aerobic efficiency have been adjusted to a low enough level to obviate the need for anaerobic ATP production. Accompanying these changes in mitochondrial O₂ affinity are changes in other aspects of aerobic metabolism. For instance, the membrane potential across the inner mitochondrial membrane, which dictates the movement of protons and ultimately determines the rate of ATP synthesis through oxidative phosphorylation, is reduced during overwintering (St-Pierre, Brand & Boutilier, 2000b). This reduction indicates a decrease in the activity of the enzymatic components of the electron-transport chain, possibly to conserve the limited on-board fuels. A further advantage of the reduced proton motive force is the consequent reduction in proton leak (an inherent inefficiency of aerobic respiration) that occurs as a result of the lower membrane potential (St-Pierre et al., 2000b). So far, however, no evidence for a reduction in the mitochondrial proton leak pathways has been found in amphibian tissues undergoing significant metabolic suppression, suggesting a limitation on the biochemical re-organisation that can take place during hibernation or cellular re-organisation that accompanies metabolic change.

All of the studies of overwintering of frogs in hypoxic waters so far mentioned used one or two levels of hypoxia. Ultsch et al. (2004b) subjected submerged *Rana pipiens* at 3°C for two days to each of eight levels of progressive hypoxia above and below the *P*ₐ of 36.4 mmHg to determine at what degree of hypoxia various blood variables first started to change relative to values for frogs in shallow water with access to air (Fig. 3). Lactate started to increase at a *P*ₐ of 42 mmHg, although pH did not fall below control values until the *P*ₐ reached 10 mmHg. Even in anoxic *R. pipiens* submerged at 3°C for two days, the *PCO₂* was only 2.33 mmHg compared to a control *PCO₂* of 6.05 mmHg (Stewart et al., 2004), which constituted a respiratory alkalosis typical of cold, submerged frogs in normoxic water (Boutilier et al., 1997; Stewart et al., 2004); therefore the decrease in pH relative to controls caused by the developing metabolic lactic acidosis was delayed until the lower *P*ₐ was reached. Hyperglycaemia also first became evident at a *P*ₐ of 10 mmHg, presumably due to liver glycogen mobilisation. The initiation of the increase in lactate at a *P*ₐ above the *P*ᵣ is similar to the findings of Pörtner & Grieshaber (1993) and Pörtner et al. (1991) mentioned above, but the determination of the *P*ᵣ was not so precise that it can be irrevocably stated that lactate increased before *V*₀₂ started to decrease. Taken in concert, however, these data suggest that a *P*ₐ in the neighborhood of ≤30 mmHg that persists for a long period will be stressful to a hibernating frog, and is probably too low to permit the oxidation of fat stores, forcing the frog to switch to a largely carbohydrate metabolism, as witnessed by changes in respiratory quotients (Donohoe & Boutilier, 1999), with its attendant accumulation of lactate and a resultant acidosis.

The overall metabolic response of overwintering frogs to the combination of low temperature and submergence is illustrated in Fig. 7. A collation of data from previous studies of air-breathing *Rana temporaria* at higher ambient temperatures reveals a classic exponential temperature sensitivity to metabolic rate. When compared to the metabolic rates of cold, submerged frogs measured at 10°C and below, it is evident that submerged animals have significantly lower metabolic requirements, approximately 2-3 times lower than expected from the extrapolated values of air-breathing frogs at higher temperatures. The cause of this difference may be a submergence-induced quiescence that lowers metabolic rates, where submerged frogs exhibit less evidence of anaerobic recruitment than frogs with access to air. Finally, when submerged in normoxic water for long periods of time, frogs can lower metabolic rate up to 40% further, presumably via essentially biochemically driven readjustments at the cellular level.

(8) Water and ionic balance during normoxic and hypoxic submergence

The skin of amphibians is not only a respiratory organ, but also a major osmoregulatory organ. Along with the kidneys,
Due to the long period of time spent in the dilute aquatic environment, some degree of regulation of the water influx must occur in ranid frogs. Indeed, Parsons and Lau (1976) show from in vitro studies that there is a rapid decrease in skin water permeability from 15 to 5 °C (Q10 ≈ 2-2.6). More importantly, however, there is a seasonal reduction in the sensitivity of the skin to circulating antidiuretic hormone (ADH). Injections of ADH led to a 200% increase in water permeability of skin of summer frogs, whereas similar injections in winter frogs caused little change, supporting a role for this hormone in modifying seasonal water balance (Parsons & Lau, 1976). The altered set-point in osmotic balance can affect ionic balance as well. A more dilute tissue does not require the same energetic input to maintain ion gradients as a more concentrated one. In fact, submerged frogs kept at 4 °C and a reduced PO2 exhibit higher water retention than normoxic frogs (Christiansen & Penney, 1973), although this hypoxic response is not universal (cf. Donohoe et al., 2000).

While one might expect changes in the ion transport rates or ion permeabilities of the skin to occur in hibernating amphibians, there are few data to support or refute this supposition. Most of the experiments conducted on ion and water exchange have been conducted at temperatures outside those relevant to overwintering. One of the few studies examining skin permeability to ions at low temperatures (Koefoed-Johnsen & Ussing, 1974) showed that frogs acclimated to 5 °C had increased skin electrical resistance compared to 16 °C-acclimated frogs. The ionic pathway accounting for this increased resistance was determined to be a cellular chloride pathway, suggestive of some degree of ion channel suppression. To the best of our knowledge, this finding has not been followed up with in vivo studies, nor in studies involving submerged frogs. Sodium ion permeability of the skin has not been examined throughout a hibernation period at low temperatures, however Dinno and Nagel (1980) noted that the acute temperature effects on transcellular sodium ion transport in frogs are primarily a result of changes in apical and basolateral membrane permeabilities, rather than changes in active transport mechanisms, which is consistent with a relative ion channel suppression as observed for chloride movement. Ion transport, however, cannot be completely shut down, since water permeability is not zero. A cold, submerged frog will always have water entering via passive diffusion or via specific ion transport mechanisms that facilitate water movement across the cell membrane. In fact, it is likely that cutaneous epithelial ion transport costs are relatively high compared to other metabolic costs in overwintering frogs due to the constant influx of water that a submerged frog will experience. The skin, in addition to being involved in ionic and osmotic homeostasis, is the sole respiratory organ for submerged overwintering adults, and conditions which optimise oxygen uptake (such as capillary recruitment in the skin) will likely exacerbate passive ion loss and increase passive water influx (Parsons, 1978, 1994). Unfortunately, neither the ionic conductance across the skin nor its metabolic costs have been determined in frogs under simulated hibernation conditions.

The ionic balance of muscle in overwintering frogs has been examined and may reveal a cellular mechanism that...
permits increased metabolic efficiency of ion exchange, and has been proposed to be a putative mechanism underlying metabolic suppression. Donohoe et al. (2000) found altered ionic concentrations in submerged frog muscle; progressive decreases in Na\(^+\) influx rates and K\(^+\) efflux rates during overwintering submergence indicated a progressive shutdown of ion channels (i.e., the ion channel arrest hypothesis; Hochachka & Guppy, 1987). In addition, the electrochemical gradients for ion regulation in muscle cells are reduced in cold, submerged frogs (possibly correlated with, but not caused by, the water accumulation that is evident in many overwintering frogs). A 33% reduction in intracellular [K\(^+\)] and extracellular [Na\(^+\)] occurs within the first four weeks of cold submergence (Donohoe et al., 2000). These intracellular changes occur while extracellular [K\(^+\)] and intracellular [Na\(^+\)] remain constant, suggesting that the changes are not simply a tissue dilution effect. Curiously, water retention was reported as being greater in hypoxic animals than in normoxic animals (Christiansen & Penney, 1973), although Donohoe et al. (2000) did not report a similar relative increase in the oedematous state of their hypoxic submerged frogs. The reasons for these discrepancies are unknown, but could be related to differing oxygen levels in the two studies.

(9) Energetics of overwintering

Frogs enter hibernation with fat and glycogen stores, and a reasonable question is whether energetics limits hibernation time in normoxic water, and more so in hypoxic water. A simple test would be to see how long frogs can survive continuous submergence in cold water as a function of \(P_{O_2}\), and then to measure body energy stores at death, but no such data are available. However, Rana muscosa can survive at least 12 months of submergence at 4°C (Bradford, 1984), indicating that energy stores in that species are more than adequate to cover the maximal expected hibernation time. Some species hibernate as long as 8-9 months in cold climates (Bradford, 1983; Pasanen & Koskela, 1974) and still have enough on-board fuel reserves for reproduction before feeding. During hibernation, fats are the preferred substrate, producing more than twice the energy per unit mass of carbohydrates or proteins, but there is an \(O_2\) cost in that they require more \(O_2\) per gram for oxidation. The predominance of fat metabolism can be shown from the data of Pasanen & Koskela (1974), which can be used to calculate that fat metabolism supplied about 80% of the energy utilized during overwintering, presumably aerobically, in Rana temporaria in northern Finland. In normoxic water, although fat metabolism predominates, there is about a 30% reduction in total glycogen stores over the first eight weeks of submergence, followed by little change over the next eight weeks (Boutilier, 2001b), during which the frog is presumably metabolizing mainly fats. Donohoe & Boutilier (1998), found that there was a shift in the respiratory quotient (RQ) from 0.85 to 1.0 in R. temporaria submerged in hypoxic water at 3°C that indicated a shift from a mixed fuel metabolism to a mainly carbohydrate metabolism. If hypoxia inhibits fat metabolism, shifting to a reliance upon glycogen should shorten the time that a frog could remain submerged because of limitations imposed by the energetics of glycogen stores. Even in well-oxygenated environments, frogs (Rana pipiens) are often found dead with no obvious injuries or other indications of the cause of death (Cunjak, 1986; McAdam & Nagel-Hisey, 1998); in other cases both live and dead leopard frogs are found in spring in a given habitat, rather than only one or the other (Cunjak, 1986; Merrell & Rodell, 1968). It is possible that these dead frogs in either case had exhausted their energy reserves. It would be interesting to collect such frogs and determine their fat and glycogen stores.

Several lines of reasoning suggest that energetics may be more of a limiting factor during overwintering in northern frogs that it is in northern turtles. One is that the overwinter survivorship of turtles, normally >80-90% (Congdon, Dunham & van Loven Sels, 1994; Congdon et al., 2000; Galbraith & Brooks, 1987; Wilbur, 1973), tends to be considerably higher than that of frogs, which even when anoxia or severe hypoxia does not appear to be a factor, is often <50% (Elmberg, 1990). Thus something kills frogs while they are overwintering more frequently than turtles, and depletion of energy stores is a candidate. Furthermore, several species of turtles (Graptemys geographic, Apalone spinifera, and Chrysemys picta) have been kept submerged in normoxic water at 3°C for 217 days and recovered when given air access and warmed, indicating that their energy stores could last at least that long (G. Ultsch, unpublished data). As mentioned, there are no comparable data available on frogs to see if they would become energy-depleted over such a long-term submergence. However, if we theorise that there is a potential ability of turtles to tolerate submergence in normoxic water longer than frogs, based on energetic considerations, even when both are entirely aerobic, then one explanation might be that turtles are better able to depress their metabolic rates. The aerobic metabolic rate of radian frogs in normoxic water at 3-5°C, based on an average from four studies (Bradford, 1983; Pinder, 1987; Tattersall & Boutilier, 1997; Ultsch et al., 2004) is about 6 \(\mu\)mol O\(_2\) g\(^{-1}\) h\(^{-1}\). Unfortunately, there are no data on the aerobic metabolic rates of turtles submerged in normoxic water under similar conditions. However, Herbert & Jackson (1985) report the metabolic rate of the painted turtle (Chrysemys picta bellii) breathing air and water at 3°C to be 1.14 \(\mu\)mol O\(_2\) g\(^{-1}\) h\(^{-1}\) at a mean mass of 952 g. Since a significant portion of the mass of the painted turtle is shell (35%; Jackson, Crocker & Ultsch, 2001), if we assume that 65% of the total mass is doing most of the metabolizing, the metabolic rate of the non-shell tissues can be considered to be 1.75 \(\mu\)mol O\(_2\) g\(^{-1}\) h\(^{-1}\), and it is this metabolic rate that is most appropriately compared to that of the frog. This would be a conservative estimate of the aerobic metabolic rate of a submerged painted turtle that is meeting all of its metabolic requirements aerobically, as this species can do after several weeks of submergence (Jackson et al., 2001). Further, assuming that the mean size of the frogs is about 60 g, and that the slope of the log-log regression of metabolic rate against body size in turtles is 0.75, it follows that a 60 g turtle would have a metabolic rate of 2.53 \(\mu\)mol O\(_2\) g\(^{-1}\) h\(^{-1}\) for 39 g of metabolizing tissue,
which is only 42% that of a frog of the same size. Since the turtle’s metabolic rate is probably further depressed by denial of air access, it seems a fair estimate that the frog has at least twice the metabolic rate of the turtle, and therefore should be able to survive less than half as long if both species entered hibernation with similar relative energy stores.

Whether frogs are limited in their duration of overwintering in nature is still an open question. Boutillier et al. (1997) calculated that the fat stored in fat bodies, along with liver glycogen stores, of R. temporaria would last only 92 days at the metabolic rate of a normoxic air-breathing frog, 157 days at the reduced metabolic rate shown after 60 days of normoxic submergence at 3 °C, and 308 days at the metabolic rate associated with a $P_{\text{O}_2}$ of 50 mmHg. None of the estimates include muscle glycogen or other body fats, and since the fat in fat bodies is a minor portion of the total body fat (2-14% in R. clamitans and R. pipiens; Brenner, 1969; Brenner & Brenner, 1969), they are probably underestimated. Bradford (1983) calculated that a female R. muscosa hibernating for eight months would still have at least 1/3 of its total fat stores remaining, and as mentioned, total energy stores are sufficient for at least 12 months in submerged R. muscosa at 4 °C (Bradford, 1984). It is also unclear if overwintering stresses limit the northern ranges of frogs, be they limitations of energetics or otherwise. In turtles, reproductive limitations apparently set the northern ranges, rather than tolerance of the rigours of hibernation, since turtles lay shelled eggs that take months to incubate and the eggs must hatch in the same season they are deposited, which limits the time available for successful reproduction in northern climates with short summers (Obbard & Brooks, 1981; Ultsch, 1989, 2006). Among frogs, eggs are usually laid earlier than those of turtles, hatch much more quickly, and tadpoles can overwinter, so reproductive success may not be the northern range-limiting factor; however the ability to find enough food to lay down energy stores during a short summer season could be of paramount importance.

The energy stores needed for overwintering are not always the only consideration. Some ranid frogs breed soon after emergence from hibernation (Rana pretiosa, R. pipiens, R. temporaria), but others do not breed immediately after emergence from hibernation and prior to feeding, so the generalisation that enough energy must be stored not only for overwintering, but also for reproduction after emergence, is not valid. Green frogs in Michigan hibernate over a three-month period from mid-May to mid-August (Martof, 1956), bullfrogs in Pennsylvania (Hulse et al., 2001) and in Missouri (Willis et al., 1956) breed mostly from May to July, and in Maine mink frogs (R. septentrionalis) breed from late June to early August (Hunter, Calhoun & McCollough, 1999). Brenner (1969) found that both the terrestrial hibernating cricket frog (Acris crepitans) and the aquatically hibernating green frog (R. clamitans) fed during the post-hibernation season and deposited fat prior to reproduction. However, for those that do breed before feeding, energy storage prior to overwintering should be more of a crucial factor than in those that feed before breeding. For example, in northern Finland, R. temporaria enter hibernation toward the end of September, hibernate until at least the end of April, migrate to spawning areas and finish spawning about mid-May; and do not feed until spawning is completed (Pasanen & Koskela, 1974).

Therefore energy stores are highest when entering hibernation and lowest after spawning. During hibernation liver glycogen content was reduced by 51% in males and 56% in females, leaving a significant amount available as an energy supply for breeding. However, the combined lipid stores of the liver and fat bodies were reduced by 84% (93% for fat bodies and 70% for liver fats), leaving only 16% available for breeding purposes (Pasanen & Koskela, 1974). Because of the higher energy content of lipids, the energy available to fuel breeding activities in the forms of glycogen and lipids after hibernation can be calculated to be roughly equivalent. It is possible that the fat bodies in mature females are utilised primarily in provisioning energy for the developing gonads that occurs during the late autumn and winter, whereas in males, the fat bodies are required for the early spring calling and breeding activity. Many immature frogs (Rana temporaria) had depleted their fat bodies almost completely by the end of winter (Pasanen & Koskela, 1974). In immature frogs, the fat bodies appear to contribute significantly to surviving the prolonged winters, rather than oogenesis, spermatogenesis, or spring reproductive behaviours. In adults, however, fat bodies alone might not be a reliable indicator of the energy stores available for normal metabolic support during the winter, as they constitute less than 25% of total carcass fats.

Biogeographical evidence for the potential importance of glycogen energetics to overwintering success in frogs was first noted by Pasanen & Koskela (1974). These authors observed, from a collection of previous studies on liver glycogen content in Rana temporaria, that the values were more highly elevated the further north the frogs were collected. Indeed, at 64°N, prehibernation liver glycogen content was double (~15% of liver mass) that of frogs at 37-38°N (7-8% of liver mass), with values from middle-range latitudes falling in between. As mentioned, fats are the preferred substrate if oxygen is not limiting, and were the source of at least 80% of the energy used during hibernation. In addition to their 20% contribution to the energetics of hibernation, glycogen stores can be viewed as an energy reserve during periods of transient hypoxia or anoxia during hibernation, as well as during the non-feeding period following emergence in spring. These authors point out that the same does not hold true for glycogen content of the muscle, likely because muscle glycogen serves a less important role in fueling whole-animal metabolism in the winter, particularly if frogs remain quiescent throughout, and adopt a metabolic suppression in muscle tissue induced by hypoperfusion. Similar results in geographic variation in energetics being associated with severity of winter have been observed in freeze-tolerant anurans (Irwin & Lee, 2003), where a high liver glycogen would support the rapid production of cryoprotectants like glucose and would support anaerobic glycolysis during tissue freezing.

Metabolic depression during overwintering has typically been viewed as an energy-sparing adaptation among frogs,

but that may not be its only purpose. Since frogs are very intolerant of anoxia, and perhaps in particular of the resultant lactic acidosis, another way of viewing the function of metabolic depression is that it permits the frog to remain aerobic in a hypoxic environment, thus avoiding acidosis. Clearly, more studies will be needed to determine the importance of energetics in the overwintering of frogs.

(10) Behavioural responses to temperature and oxygen gradients

Beyond the physiological and biochemical changes that serve to promote O2 homeostasis in overwintering frogs, there are behavioural mechanisms that may come into play. Most obvious are changes in body temperature via behavioural thermoregulation. These may play an important role in allowing frogs to manipulate their metabolism in order to enter into and out of a transient temperature-induced metabolic suppression. Frogs in the wild were long assumed to hibernate in an inactive state while burrowed in the mud bottom of ice-covered lakes and ponds (Hutchinson & Dady, 1964; Pinder, Storey & Ultsch, 1992). However, field studies conducted on radio-tagged bullfrogs reveal that substantial underwater movements occur throughout the winter, which appear to demonstrate, at the very least, an avoidance of anoxic mud (Stinner et al., 1994). Many overwintering habitats develop large spatial and temporal fluctuations in temperature and oxygen levels. As a result, one would expect that frogs living in these complex environments would evolve behavioural mechanisms that optimize survival by avoidance of extremes. However, there are few direct measurements of thermoregulatory or other behaviours among naturally overwintering frogs, so we must examine the few laboratory measurements that can shed light on the natural situation.

Laboratory studies show that frogs in hypoxic water in a temperature gradient will move to a colder temperature, which has been termed behavioural hypothermia (Wood & Malvin, 1991) or anapyrexia (Steiner & Branco, 2002). The result is a lowered metabolic rate and an increased O2 affinity of the blood, which thereby decreases their P50 (Boutilier, Tattersall & Donohoe, 2000; Tattersall & Boutilier, 1997). However, frogs will also tend to move to areas with a higher PO2 at a constant temperature (Tattersall & Boutilier, 1999a). Whether such behavioural responses involve the use of O2 sensors is uncertain; perhaps the frogs simply respond to some internal cue associated with hypoxia (e.g., lactate, low pH, adenosine, or nitric oxide; Branco et al., 2000; Steiner & Branco, 2002) and move randomly until an internal stress is alleviated. Behavioural hypothermia reduces metabolic demands, increases blood oxygen saturation, and decreases the cardiovascular costs associated with hypoxia, thereby extending the survival time during periods of hypoxic stress (Wood & Gonzalez, 1996). Tattersall & Boutilier (1997) showed that when cold-submerged frogs make behavioural decisions, and reduce their preferred temperature by approximately 5° C, they effect a metabolic savings of over 70%. This decrease in overall metabolism eliminates or reduces the requirement for anaerobic metabolism to make up the shortfall in ATP requirements that aerobic metabolism can no longer provide. While behavioural hypothermia in response to environmental hypoxia has been convincingly demonstrated in the laboratory, and its potential adaptive value can be logically argued, there are no field data to suggest that it occurs naturally. In fact, at least in overwintering environments with a relatively high PO2, the frogs seem to move to the warmest temperature available, which they also do in a laboratory setting (Tattersall & Boutilier, 1997). Preference for warmer water was mentioned above for overwintering *Rana luteiventris*, but in that case the warmer areas also had a higher PO2, which confounds conclusions. Stinner et al. (1994) found hibernating bullfrogs to prefer areas of their study pond the authors presumed to be warmer, although they do not give comparative temperature data, and they argued that these areas were also likely to have a higher PO2. What advantage accrues from a preference for warmer water is not obvious, especially if the frogs do not feed in the winter, which they apparently do not. It would seem that moving to warmer water would only raise metabolic rate and hasten the depletion of energy reserves. Moving to warmer water might mean moving to an area that is not covered by ice (which was the case for *R. luteiventris* above), and therefore prevent air-breathing, but air-breathing is not necessary for aerobic respiration at low temperature if the *P*50 is sufficiently high; it would, however, prevent lung collapse (Ultsch et al., 2004a). Since lung collapse must normally occur during prolonged submergence, its prevention may not be an issue; perhaps the frogs move to warmer water because this is what they would do in springtime, and they are simply responding to such a cue. Or it may be that the preferred temperature is always above usual northern winter water temperatures (0-5° C), regardless of how long the frogs have been cold; that is, while frogs can function at temperatures near 0° C, they would prefer to be warmer. In aquatic overwintering frogs, selective pressures to avoid the potential for freezing are paramount, and thus, a natural tendency to prefer warmer temperatures may result from a number of reasons: (1) it would minimise the chances of freezing since warmer waters will freeze last; (2) warmer waters would be indicative of open waters in the spring and provide orientation for the frog to leave the overwintering environment; and (3) warmer waters would allow the frog to be better able to respond to external risks (Tattersall & Boutilier, 1999c). Field studies of PO2, temperature, and the location of overwintering frogs in a heterogeneous oxygen and temperature environment will be needed to determine the relevance of the laboratory studies to the actualities of overwintering behaviour.

The one field study that sheds some light on these laboratory studies demonstrated that bullfrogs appear to select microhabitats very close to the ice, in the colder, shallower waters toward the end of winter (Stinner et al., 1994). If they are responding to a progressive decline in oxygen levels by selecting lower temperatures, then they may be exploiting high Q10 values so as to suppress metabolism. Alternatively, they may be following oxygen gradients, and selecting oxygen-rich waters near the surface. Although they cannot easily adjust their height in the water.
column by adjusting buoyancy since their lungs are most likely collapsed, it is conceivable that they use differences in water depth as proximate cues for finding colder or more oxygenated water. Submerged frogs can, to a limited extent, respond as if they detect severe hypoxia; they do not spend more than 30% of their time below levels of oxygen which would be limiting to aerobic metabolism, and ultimately, survival (Tattersall & Boutilier, 1999a). The fact that ambient hypoxia is often slow to develop in nature supports the notion that this behavioural detection of oxygen may be important to survival. A further point to consider is that the anoxia tolerance exhibited by these submerged frogs would allow them to inhabit temporarily the oxygen-depleted detritus in an effort to escape from or confuse predators.

A final confounding problem for frog behaviour during the winter is the risk of predation (Emery et al., 1972). Frogs cannot tolerate prolonged anoxia, (Boutilier et al., 1997; Christiansen & Penney, 1973) so they are unlikely to burrow into the mud to avoid detection for more than a day or two. This suggests that submerged frogs are unable to remain continuously buried in the mud during the winter, and instead may evade discovery by remaining active and selecting microhabitats that can support an aerobic metabolism while still providing some cover. Predation risk may disrupt or even prevent normal thermoregulatory behaviour during the winter, and frogs that escape predation would still require long periods of time to recover fully. Frogs transiently prefer cooler temperatures after intense activity, suggesting a role for behavioural thermoregulation following internally derived hypoxia and metabolic disturbances (Tattersall & Boutilier, 1999a). The contribution of activity and exercise toward changing thermoregulatory behaviour during the winter is poorly appreciated, and may play an important part in overwintering survival.

In summary, behaviour can be of great importance in maintaining body oxygen stores in overwintering frogs, as air-breathing is not an available option for maintaining oxygen uptake if ice is present. The behavioural detection of oxygen or temperature is the only immediate means by which frogs can adjust either the supply or the demand of oxygen, and thus maintain metabolic homeostasis aerobically (Tattersall & Boutilier, 1999a). Most importantly, these behavioural decisions must be made within the natural setting of the overwintering environment, where external influences, such as environmental change, predation, and aggregation may conflict with the ability of the frog to exhibit a physiologically appropriate environmental preference.

V. CONCLUSIONS

(1) Both adults and larval frogs will die in a matter of days if exposed to submergence anoxia in cold water.
(2) Tadpoles are more tolerant of submergence hypoxia than are adults, and in some habitats can serve as a population reservoir during a harsh winter.
(3) The presence of dissolved O₂ is a necessary requirement of a hibernaculum, and will override other considerations, such as concealment.
(4) Sites that can provide access to air are sometimes used for overwintering, and may be preferred when available.
(5) Even at water temperatures near 0°C, frogs are not strictly torpid and can, and sometimes do, move about both on land and in water.
(6) Frogs may migrate overland to suitable hibernation sites, or they may overwinter in their summer habitats.
(7) North American ranids typically hibernate underwater, but terrestrial overwintering can occur in suitable microhabitats; terrestrial hibernation occurs more commonly in some European ranids.
(8) Overwintering mortality, at least among adults, is highly variable (reaching 100%), depending upon the location of the overwintering site, the microenvironment of the hibernaculum, and the harshness of the winter.
(9) High mortality during overwintering is offset by high fecundity of remaining females, survival of overwintering tadpoles in species where larval overwinter, and immigration from nearby populations.
(10) Frogs may choose to breathe air in the cold; however, if water depth is too great they will remain submerged and utilise only cutaneous respiration. This behaviour is well supported from physiological measurements showing that in cold normoxic water frogs can meet all of their gas exchange requirements via extrapulmonary avenues.
(11) Frogs submerged in normoxic water have very low blood PO₂ values which agree well with their very low blood P½ values, meaning that the haemoglobin oxygen saturation in vivo should fall within the range appropriate for loading and unloading O₂.
(12) Ranid frogs can only survive an absence of oxygen for 2–4 days at overwintering temperatures. Few ranid frogs have the capacity to survive prolonged freezing.
(13) Cutaneous gas exchange can be maintained in hypoxic water via recruitment of under-perfused cutaneous capillaries, as well as through behaviours that act to disrupt the hypoxic boundary layer over the skin.
(14) Changes to Pc and metabolic turnover can be effected through changes in morphology, preferred temperature, or metabolic suppression at the cellular level.
(15) Metabolic suppression is a necessity to survive levels of oxygen at or below the normal Pc. Numerous biochemical and physiological mechanisms are proposed to enable metabolic depression and maintenance of O₂ homeostasis, including alterations in mitochondrial affinity and proton motive force, redistribution of blood flow away from oxy-conforming tissues to oxygen-sensitive tissues, cellular ion-channel arrest, and a reduction in cellular membrane potential via a dilution of key intracellular and extracellular ions.
(16) Behavioural responses to temperature gradients and to low levels of dissolved oxygen are paramount to overwintering frogs and allow them to exhibit preferences for temperature and oxygen that coincide with their physiological tolerances. Remaining inactive or unresponsive to the environment when overwintering is not a likely survival strategy.
(17) It is adaptive for overwintering ranid frogs to remain submerged where they can exhibit an aerobic metabolism,
effectively precluding them from burrowing into anoxic mud, while incorporating a reduced metabolic turnover at the tissue level to conserve energy stores.

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**VII. REFERENCES**


Overwintering in ranid frogs


