

## BALANCING HYPOXIA AND HYPOTHERMIA IN COLD-SUBMERGED FROGS

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*Accepted 11 November 1996*

### Summary

Many animals respond to hypoxic stress by selecting cooler environments, the so-called 'behavioural hypothermia' response. Amphibians overwintering in ice-covered ponds and lakes offer an ecologically relevant test of this response since they must choose between the confounding metabolic effects of profound hypothermia or hypoxia; thermal and chemical conditions can vary from 0 °C and normoxic at the ice–water interface to 4 °C and markedly hypoxic at depths of 2–4 m. To mimic such environmental conditions, we constructed an experimental chamber that enabled continuous electronic surveillance of an animal's movement along a thermal gradient. When *Rana temporaria* pre-acclimated to 3.5 °C were placed in a normoxic thermal gradient ranging from 0.8 to 8 °C, they

invariably favoured the warmer end of the chamber. Upon exposure to hypoxia, however, their preferred temperature shifted from a median of 6.8 °C ( $P_{O_2}$ =158 mmHg; 1 mmHg=0.133 kPa) to 1.9 °C ( $P_{O_2}$ =25 mmHg). Metabolic rate measurements from animals exposed simultaneously to acute changes in water temperature and  $P_{O_2}$  suggest that movement to colder conditions in hypoxia effects the greatest metabolic savings and prolongs the onset of a plasma lactacidosis.

Key words: *Rana temporaria*, frog, oxygen, overwintering, hibernation, behavioural hypothermia, acid–base balance, temperature.

### Introduction

Every year, numerous amphibian species in the northern hemisphere spend the entire winter (3–9 months) submerged in small lakes and ponds (Willis *et al.* 1956; Emery *et al.* 1972; Juszczak *et al.* 1984; Bradford, 1983; Cunjak, 1986). As ice and snow form on the surface, these bodies of water become effectively 'closed' to atmospheric oxygen. The reduction in light penetration means that photosynthetic production of oxygen is low, and the resident organisms, particularly those in the decaying detritus layer on the bottom, consume enough oxygen to lower the water oxygen tension to near-anoxic levels (Greenbank, 1945). Both the temperature-dependence of water density and the low convection rates cause the waters to become stratified for temperature and oxygen, resulting in warm temperatures (4 °C) and low oxygen tensions (7–20 mmHg) at the bottom, and cold temperatures (0 °C) and higher oxygen tensions (60–85 mmHg) just below the ice (Bradford, 1983; Friet, 1993). Low oxygen levels have been implicated in the massive 'winterkill' of many fish and amphibians in small, ice-covered ponds and lakes, making these systems the focus of numerous ecological studies (Barica and Mathias, 1979; Bradford, 1983; Greenbank, 1945).

Less well understood are the behavioural and physiological responses of animals that find themselves constrained within such narrow ranges of temperature and oxygen tensions, where movements within the stratified layers become the only option

for thermoregulation. While behavioural thermoregulation and temperature selection are well documented for amphibians living at higher temperatures (Brattstrom, 1979; Lillywhite, 1970, 1971; Lucas and Reynolds, 1967; Shoemaker *et al.* 1989; Whitford and Massey, 1970), no such studies have been performed on animals living in the cold. Moreover, even though the hypoxia-induced selection of decreased environmental temperature (e.g. 25–15 °C) has been demonstrated in amphibians breathing air (Wood and Malvin, 1991), low atmospheric partial pressures of oxygen are normally encountered only at high altitudes where temperature and oxygen gradients are not very pronounced. Because ice-covered ponds have naturally occurring temperature and oxygen gradients, overwintering amphibians provide us with an ecologically relevant model for the study of behavioural hypothermia. In such conditions, animals must choose between the confounding effects of hypothermia and hypoxia (i.e. between the deleterious effects of low temperature on activity *per se* during normoxia *versus* substrate limitation and harmful anaerobic end-product accumulation during hypoxia). Considering that  $Q_{10}$  values for many metabolic processes are often quite high at very low temperatures (Hochachka and Guppy, 1987), it is probable that even small changes in the selected temperature in this range could have profound effects on the mechanisms of physiological homeostasis. The

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physiological significance of hypoxia-induced behavioural hypothermia has previously been demonstrated in terrestrial toads selecting aerial temperatures between 25 and 15 °C (Wood and Malvin, 1991). Specifically, arterial oxygen saturation is maintained at the lower  $P_{O_2}$  levels through a temperature-induced decrease in  $P_{50}$  (blood oxygen partial pressure at 50% blood oxygen saturation) and aerobic metabolic rate is decreased (Boutilier *et al.* 1987; Wood and Malvin, 1991), thus ameliorating the potentially deleterious effects of reduced environmental  $P_{O_2}$  on overall oxygen homeostasis. While ectotherms at higher temperatures in aerial environments may utilise physiological mechanisms such as controlled evaporative water loss or peripheral vasodilation/constriction to effect small changes in body temperature (Lillywhite, 1970, 1971; Shoemaker *et al.* 1989), animals submerged in cold water are at the mercy of their cold environment, and behaviour is the only mechanism by which body temperature can be adjusted.

To study behavioural thermoregulation and temperature selection in cold environments, we constructed a horizontal temperature gradient ranging from 0.8 to 8 °C, which mimics the temperatures found in lakes during late autumn, winter and early spring in the northern hemisphere. We hypothesised that submerged frogs would tend to select higher temperatures when exposed to normoxic water, but that animals exposed to hypoxia would select the cooler end of the chamber. Following these experiments, we measured routine aerobic metabolic rates, blood acid–base status and lactate concentrations in animals subjected to the same regime of temperature and oxygen as they had chosen voluntarily in the thermal gradient. This series of physiological experiments enabled us to estimate the energetic costs and benefits of temperature selection in normoxic and hypoxic environments.

## Materials and methods

### *Maintenance of experimental animals*

Frogs [*Rana temporaria* (L.), 20–30 g] were obtained from commercial suppliers (Blades Biological Co., UK), who collected the animals from natural populations in Ireland in the autumn of 1995. The animals were kept in aerated water at 3.5 °C with access to the surface for air-breathing for approximately 1 month before use. The frogs were housed in a darkened room for the duration of their acclimation and throughout the experiments themselves, to mimic the reduced light levels that characterise ice-covered ponds in nature. Behavioural experiments were conducted during the months of December and January, and metabolic rate experiments and metabolite studies on a separate group of frogs at the end of January.

### *Design of the thermal gradient*

A continuous flow-through temperature-gradient chamber (87 cm × 12 cm × 40 cm) was fashioned from acrylic and was similar to the design of Kinney *et al.* (1981). Two reservoirs of water, maintained at  $0.8 \pm 0.2$  °C and  $8 \pm 0.2$  °C (Churchill and

Haake thermoregulators) were each connected to the bottom of the chamber by submersible pumps delivering water at a flow rate of  $11 \text{ min}^{-1}$ . Waters of known temperature entered each horizontal side of the chamber through opposing pairs of seven adjustable valves built into the chamber bottom. The valves were adjusted so that the rate of flow through each successive port either increased or decreased along the horizontal length of the chamber, depending on the desired temperature. Water flowing into each side of the chamber moved upwards into seven separate compartments until being allowed to mix at a height of 25 cm. The thermal gradient was measured using mercury thermometers placed immediately above the point where mixing occurred and where frogs were confined to a 4 cm high space. Water flowed out the chamber through ports built into the top of the tank, aligned directly above the input valves in order to facilitate laminar flow. The outflowing water was returned to the temperature-controlled reservoirs, thereby maintaining a closed system. The oxygen partial pressure ( $P_{O_2}$ ) of the chamber water could be adjusted by bubbling the reservoirs with either air or nitrogen.

Animal location in the chamber was determined by a series of 20 infrared detectors, spaced 4 cm apart, mounted horizontally along the thermal gradient. Each detector was coupled to an infrared emitter on the opposite side of the chamber. The detectors and emitters were wired into a common circuit where each pair had an adjustable output voltage. Diodes connected to each circuit ensured the independence of each voltage output, except that higher voltages took precedence over lower voltages. Only one or two adjacent detectors were activated at any given time, owing to the small size of the frogs (6 cm long). Animal location in the gradient was then calculated from the regression of temperature  $T$ , (°C) versus distance ( $d$ , cm) along the gradient ( $T = 0.07 + 0.087d$ ,  $r^2 = 0.97$ ; Fig. 1).

### *Series I: temperature-selection experiments*

Frogs ( $N = 6$ ) were initially placed in the centre of the thermal gradient in normoxia (158 mmHg) and their location was monitored every 4 min by recording the output from the infrared detectors using a pen recorder and a Squirrel data logger (Grant Instruments Cambridge Ltd). The first 4 h were discounted as the frogs were usually found to be investigating the thermal gradient (see Results). Activity recordings were subsequently made on frogs left in normoxia for approximately 24 h, after which time the  $P_{O_2}$  of the chamber water was lowered to approximately 25 mmHg (measured range 20–30 mmHg). This oxygen level was determined, in preliminary experiments, to elicit a temperature-selection response within 6 h. All experiments (40 h in duration) were carried out on animals kept in the dark, and began at the same time of day, to account for any effects of entrained photoperiod. Since frog location (not body temperature) was monitored, control experiments at a constant horizontal temperature of 4 °C were conducted to look for edge bias. For unknown reasons (possibly rheotactic), frogs showed a bias for one particular end of the tank (Fig. 1A). However, when the

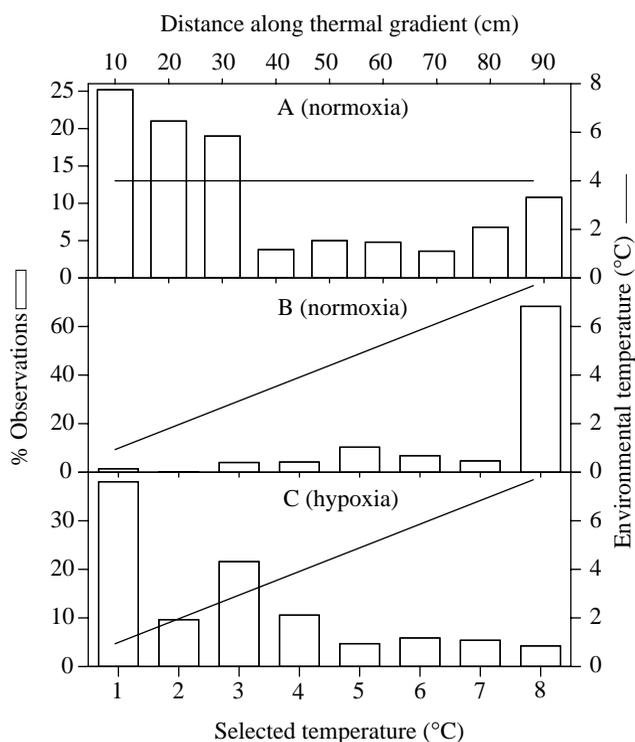


Fig. 1. Proportion of time spent at various positions and temperatures by six frogs in a thermal gradient. (A) The edge bias present when the temperature was constant across the entire chamber in normoxia (158 mmHg). (B,C) The changes in selected locations, and thus selected temperatures, during normoxia (B) and hypoxia (25 mmHg) (C) with a thermal gradient (solid line) present (regression equation for thermal gradient:  $T=0.07+0.087d$ , where  $T$  is temperature and  $d$  is distance). All graphs represent summarised data from approximately 6 h of observations on six frogs.

thermal gradient was initiated, this bias was overcome as frogs in normoxia invariably chose the end of the tank corresponding to the highest temperature (Fig. 1B).

#### Series II: aerobic metabolic rate experiments

Measurements of whole-animal oxygen consumption ( $\dot{M}_{O_2}$ ) were carried out using closed-system respirometry on animals sealed in darkened 350 ml glass flasks. Water samples were taken into gas-tight 1 ml syringes and the oxygen partial pressures were measured using Radiometer E5046 electrodes and meters. Repeated measurements of metabolic rates showed less than 10% variation and, although no quantitative measures of activity were obtained, the metabolic rates probably reflect very close to minimal routine requirements. Metabolic rates ( $N=6$ ) were measured at 1.5 and 5.5 °C and at three different oxygen tensions (158, 60 and 25 mmHg) to examine short-term changes in metabolic rate response to temperature and oxygen levels. Frogs acclimated for 1 month to 3.5 °C were placed in the respirometers (with a flow-through water supply) for 3 days prior to measurements. Metabolic rates were estimated by measuring the difference in  $P_{O_2}$  before and after sealing off the respirometers for 3–4 h.

Following the temperature-selection experiments, metabolic rates were measured in frogs subjected to the same sequence of temperature and oxygen fluctuations they had freely chosen in the thermal gradient: i.e. an initial 24 h at 7 °C in normoxia, followed by 3 h at 1.5 °C in hypoxia ( $P_{O_2}=25$  mmHg), a further 3 h at 1.5 °C in hypoxia, a 1 h normoxic recovery at 1.5 °C, and 16 h of normoxic recovery at 1.5 °C. One additional group of animals was exposed to 3 h of hypoxia ( $P_{O_2}=25$  mmHg) at 7 °C to determine the extent of the metabolic rate response had behavioural hypothermia not occurred.

#### Series III: acid-base and blood metabolite experiments

Six groups ( $N=36$ ) of six cold-acclimated frogs were exposed as described above to the same conditions selected by the frogs in the thermal gradient (see above). Animals were then rapidly anaesthetised in a solution of 0.2% MS-222 and 1.2%  $NaHCO_3$  administered at the appropriate oxygen tension and temperature. Upon complete anaesthesia (10 min), the heart was exposed quickly, and a blood sample of 200  $\mu$ l was taken immediately from the aortic arch into heparinised capillary tubes. Blood pH was subsequently measured using a water-jacketed G297/G2 capillary pH electrode and PHM84 Research meter (Radiometer, Copenhagen) thermostatted to the respective experimental temperature. True plasma (50  $\mu$ l) was drawn anaerobically into a Hamilton syringe, and total  $CO_2$  concentration was measured using a Corning 965  $CO_2$  analyser. Plasma bicarbonate concentration and  $CO_2$  partial pressure ( $P_{CO_2}$ ) were calculated as described in Boutilier *et al.* (1993) with  $\alpha_{CO_2}$  and apparent pK values taken from the empirical formulae of Heisler (1989).  $P_{CO_2}$  isopleths were derived using the same constants incorporated into recursive calculations using a computer spreadsheet.

Blood not analysed immediately was centrifuged (15 800 g) for 2 min and the plasma removed and stored at  $-80$  °C until analysed for lactate concentrations using a Elx 800 UV Biotek Instruments plate reader and a standard enzymatic assay (Passonneau and Lowry, 1993) with chemicals and enzymes purchased from Sigma Chemicals, UK. Prior to the lactate assay, plasma samples were extracted using 7% perchloric acid and neutralised with 2 mol l<sup>-1</sup> KOH plus 0.4 mol l<sup>-1</sup> sodium imidazole to prevent protein contamination of the assay.

#### Statistical analysis

The temperature-selection experiments were analysed using two-way analysis of variance (ANOVA) for repeated measures (factors: oxygen level and time). Since the time data showed serial correlation, every tenth time point was subsampled (thus eliminating the correlation) and analysed as described above. For the metabolic rate experiments, one-way ANOVA and Tukey's tests were performed. All other comparisons were made using Dunnett's multiple-comparisons and with the 7 °C normoxic value as the control value. All results, unless otherwise indicated, were considered significant at  $P<0.05$  and are presented as mean  $\pm$  S.E.M.

## Results

### Series I: temperature-selection experiments

Two-way ANOVA revealed significant effects of oxygen partial pressure, time and their interaction on selected temperature ( $P < 0.01$ ). During the first 4 h in the thermal gradient in normoxia ( $P_{O_2} = 158$  mmHg), all frogs performed considerable searching behaviour, and these data were not utilised in the analysis. After this initial period of activity, excursions to different regions of the gradient were made, until each individual selected a temperature at the warmer end of the tank (average median selected temperature of six frogs =  $6.8^\circ\text{C}$ ; Figs 1, 2). Upon exposure to hypoxia ( $P_{O_2} = 25$  mmHg), activity increased, and a significantly lower ( $P < 0.01$ ) temperature was selected (median =  $1.9^\circ\text{C}$ ), at which the animals remained. Hypoxic conditions were maintained for a total of 6 h, during which time the frogs did not attempt to select a higher temperature. Upon returning to normoxia, all frogs remained at the cold extreme of the thermal gradient for up to 24 h (data not shown), at which point they were removed.

### Series II: aerobic metabolic rate experiments

Results from a series of metabolic rate experiments conducted prior to the behavioural experiments are shown in Fig. 3. The effect of temperature on metabolic rate ( $Q_{10} = 4.31$ ; range 2.6–6.6) yielded a theoretical metabolic saving of 14% for every degree Celsius that body temperature was lowered. The effect of oxygen partial pressure on metabolic rate was

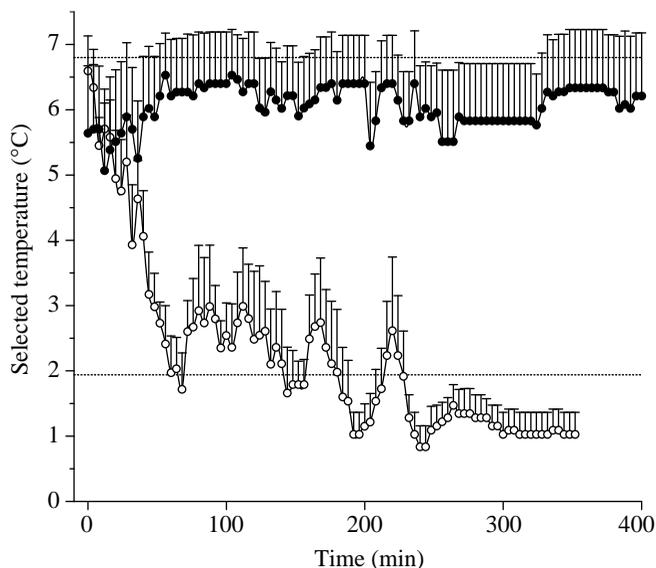


Fig. 2. Time course of temperature selection by cold-submerged frogs free to move in a thermal gradient from  $0.8$  to  $8^\circ\text{C}$ . Frogs ( $N=6$ ) were placed in the centre of the normoxic ( $P_{O_2} = 158$  mmHg) thermal gradient and the selected temperature was recorded (filled circles) beginning after 4 h of searching behaviour. Subsequently, the thermal gradient was made hypoxic ( $P_{O_2} = 25$  mmHg; open circles). The hypoxic frogs selected a significantly ( $P < 0.01$ ) lower temperature (median selected temperatures,  $6.8^\circ\text{C}$  and  $1.9^\circ\text{C}$ , for normoxic and hypoxic frogs, respectively, are shown as horizontal dotted lines) than the normoxic frogs. Values are means + S.E.M.

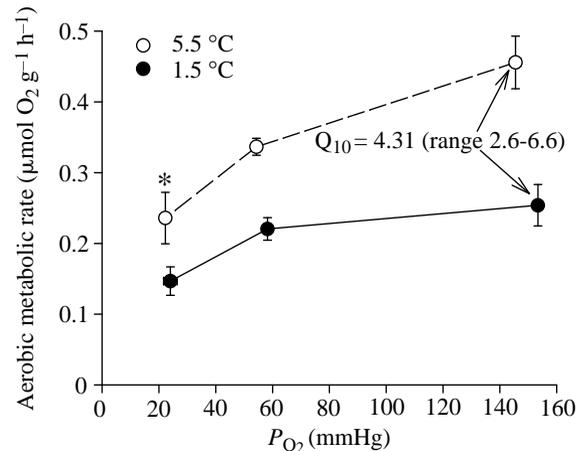


Fig. 3. Aerobic metabolic rates of submerged frogs pre-acclimated to  $3.5^\circ\text{C}$  and exposed to short-term changes ( $< 24$  h) in temperature and oxygen partial pressures ( $P_{O_2}$ ). An asterisk denotes a significant effect of oxygen tension on metabolic rate compared with the normoxic ( $158$  mmHg) value. Each point is the mean  $\pm$  S.E.M. of six measurements on the same six animals ( $N=6$ ).

most evident at the higher temperature ( $5.5^\circ\text{C}$ ), where extreme hypoxia yielded a significantly lower metabolic rate than the normoxic value. Frogs at  $1.5^\circ\text{C}$  appeared less sensitive to oxygen tension, as the metabolic rates of neither of the hypoxic groups were significantly lower than the normoxic value, although aerobic metabolic rate did show a tendency to decrease with decreasing  $P_{O_2}$ .

Following the behavioural experiments, additional metabolic rate measurements were carried out so as to provide data corresponding to the same time course and temperature regime as that chosen by the freely moving frogs. An additional group of frogs exposed to 3 h of hypoxia at  $7^\circ\text{C}$  had reduced metabolic rates representing 40% of the normoxic level (Fig. 4). After reducing the temperature to  $1.5^\circ\text{C}$  in hypoxia, the frogs further reduced their metabolic rate to 14–17% of the normoxic level at  $7^\circ\text{C}$ . Following the return to normoxia at  $1.5^\circ\text{C}$ , metabolic rate approximately doubled, increasing to 29–32% of the normoxic value at  $7^\circ\text{C}$ .

### Series III: acid-base and blood metabolite experiments

A third series of experiments was designed to apply to six groups of animals ( $N=6$  per group) under the same conditions as were chosen voluntarily by animals in the temperature-selection experiments. The blood acid-base balance and plasma lactate concentrations of normoxic animals at  $7^\circ\text{C}$  are shown in Figs 5 and 6 respectively. Upon exposure to  $1.5^\circ\text{C}$  and hypoxia for 3 h, a significant respiratory alkalosis resulted, due primarily to the significant effect of temperature on blood pH (Fig. 5). During a further 3 h of continued hypoxic exposure at  $1.5^\circ\text{C}$ , there was no significant change in blood pH; however, after 1 h of recovery in normoxia at  $1.5^\circ\text{C}$ , there was a slight, although non-significant, decrease in blood pH. At the end of 16 h of recovery at  $1.5^\circ\text{C}$ , frogs exhibited another slight increase in blood pH, presumably as homeostasis was

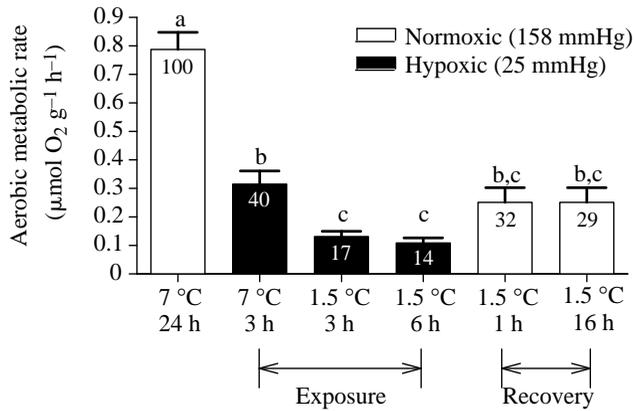


Fig. 4. Aerobic metabolic rates of submerged frogs pre-acclimated to 3.5 °C. The frogs were exposed to short-term changes in temperature and oxygen partial pressure following the same time course as experienced by the freely moving frogs in Figs 1 and 2. This was 24 h at 7 °C in normoxia, 3 h of hypoxia at 1.5 °C, 6 h of hypoxia at 1.5 °C, 1 h of normoxia at 1.5 °C and 16 h of normoxia at 1.5 °C. One additional measurement was made at 7 °C and hypoxia. Letters at the top of the bars denote statistical similarity ( $P>0.05$ ) and numbers in bars are percentage values of the metabolic rates compared with the 7 °C normoxic values. Each point is the mean + S.E.M. of six measurements on the same six animals ( $N=6$ ).

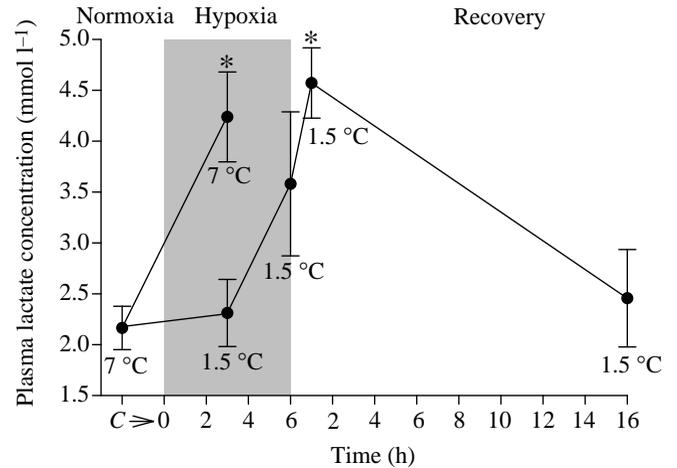


Fig. 6. Plasma lactate concentrations from submerged frogs exposed to the same short-term changes in temperature and oxygen level as experienced by the freely moving frogs (see Figs 1 and 3 for details). Recovery refers to the return to normoxia, although temperature was maintained at 1.5 °C. An asterisk denotes a significant difference ( $P<0.05$ ) from the 7 °C normoxic value. Each point is the mean ± S.E.M. of six independent measurements on six individual animals ( $N=36$ ). C, control conditions.

being restored. Exposure of frogs to hypoxia at 7 °C also resulted in a significant increase in blood pH ( $P<0.05$ ).

When frogs at 7 °C were exposed to hypoxia ( $P_{O_2}=25$  mmHg), their plasma lactate concentrations

approximately doubled within 3 h (Fig. 6). If, however, over the same 3 h time course, the animals were exposed to hypoxia at 1.5 °C, plasma lactate concentrations remained unchanged. Continued exposure to hypoxia at 1.5 °C for up to 6 h, as in the

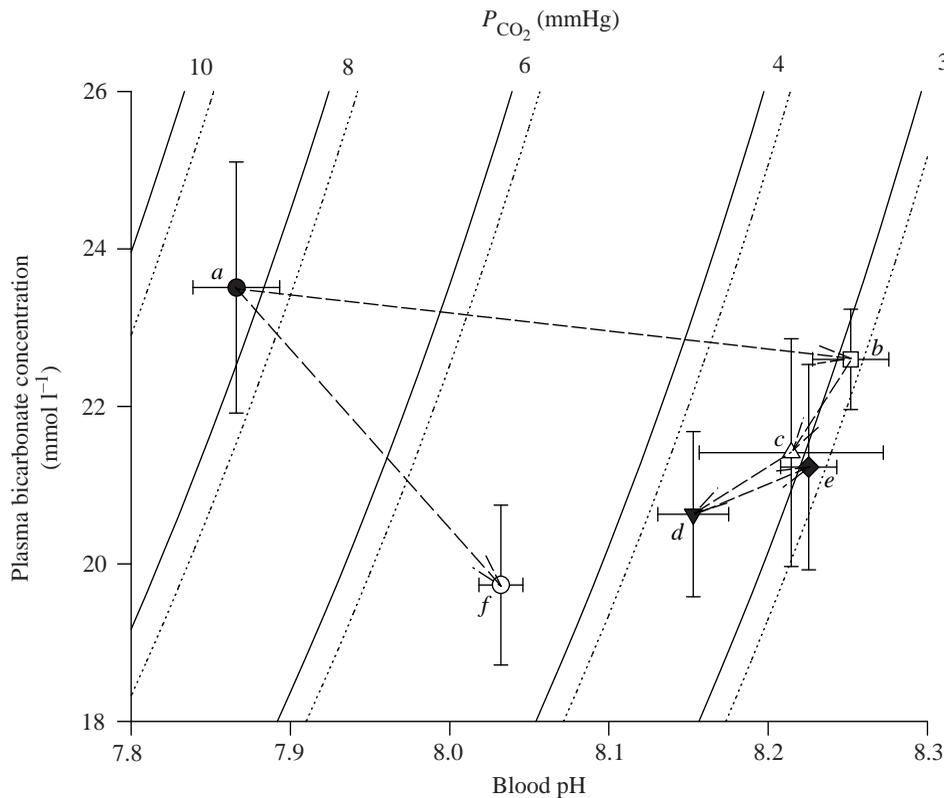


Fig. 5. Blood pH and plasma bicarbonate concentration from submerged frogs exposed to the same short-term changes in temperature and oxygen level as experienced by the freely moving frogs (see Figs 1 and 3 for details). All experimental pH values are significantly higher than the 7 °C normoxia values. Plasma bicarbonate concentration was unaffected ( $P>0.05$ ). CO<sub>2</sub> isopleths are derived from the empirical formulae of Heisler (1989) at 1.5 °C (solid lines) and 7 °C (dotted lines). Each point is the mean ± S.E.M. of six independent measurements on six individual animals ( $N=36$ ). a, 24 h of normoxia at 7 °C; f, 3 h of hypoxia at 7 °C; d, 1 h of recovery at 1.5 °C; c, 6 h of hypoxia at 1.5 °C; e, 16 h of recovery at 1.5 °C; b, 3 h of hypoxia at 1.5 °C.

temperature-selection experiments, resulted in increases in some but not all frogs (note the high variance at 6 h in hypoxia; Fig. 6). After 1 h of recovery in normoxia at 1.5 °C, plasma lactate concentrations had increased significantly to 4.5 mmol l<sup>-1</sup> relative to the 7 °C normoxia value of 2.2 mmol l<sup>-1</sup>, but after 16 h of recovery, the levels were not significantly different from those seen prior to the exposure to hypoxia.

### Discussion

When winter-acclimatised *Rana temporaria* are presented with a normoxic thermal gradient ranging from 0.8 to 8.0 °C, they respond by consistently seeking out warmer temperatures (median=6.8 °C). However, when the same cold-submerged frogs are confronted with hypoxic water, characteristic of the conditions in their overwintering habitat (Bradford, 1983), they invariably select the cooler end of the thermal gradient (median=1.9 °C) where they remain even when normoxic levels are restored for up to 24 h. In nature, the wintering sites of ranid frogs are typically on the bottom of ponds, either in shallow depressions in the mud or under rocks and ledges (Emery *et al.* 1972; Cunjak, 1986). Although it has often been assumed that such animals remain in a dormant or torpid state throughout the winter (Hochachka and Guppy, 1987), recent field studies on hibernating bullfrogs (*Rana catesbeiana*), whose positions under the ice were monitored by telemetry, indicate that shallow, colder and more oxygenated waters are preferred after an initial period in the deeper, warmer and more hypoxic waters (Friet, 1993).

The ability to remain periodically active throughout the winter means that the animals can take advantage of the thermally and chemically stratified waters in a number of ways. Frogs might, for example, move into zones of low oxygen level in order to avoid predation, hiding themselves away at the bottom of ponds where hypoxia-sensitive predators will not venture. Some truly anoxia-tolerant species such as the freshwater turtle *Chrysemys picta bellii* (e.g. Ultsch and Jackson, 1982) appear to enter a deep hypometabolic state and remain entirely inactive throughout the overwintering period. This might be the only sensible strategy for an animal with limited extrapulmonary gas exchange. Frogs, however, cannot tolerate such prolonged periods of anoxia (Pinder *et al.* 1992), and so individuals must retain the option of moving into more oxygenated areas (Friet, 1993) and capitalise on their powers of cutaneous oxygen uptake (Boutilier *et al.* 1986; Pinder, 1987) if they are to survive. Indeed, cutaneous gas exchange can accommodate all the aerobic metabolic requirements of frogs living at temperatures of 7 °C and below (Pinder, 1987; Figs 3, 6). At these temperatures, there is no need to rely on anaerobic metabolism as long as the dissolved levels of oxygen remain high; and, as we have seen, normoxic animals tend to select temperatures at the higher end of the temperature range in which they are confined (Figs 1, 2). Our results indicate, however, that as the levels of dissolved oxygen fall, the animals' movement to lower temperatures may be a response that is aimed ultimately at the conservation of metabolic energy.

The results of the present study suggest that frogs select

microhabitats under the ice in order to optimise energetic savings. The response of the frogs at 7 °C to aquatic hypoxia resulted in a reduction in their metabolic rate by some 60 %, an energy-saving response in its own right. Their ability to select lower and lower temperatures during hypoxia (Figs 1, 2) allows even greater metabolic savings (Fig. 4), owing to the large  $Q_{10}$  effect at these low temperatures (Fig. 3). Movement to a lower temperature in response to hypoxia also delays the onset of anaerobiosis (Fig. 6), thereby conserving fuel and improving the overall energy yield of metabolic substrates. This may have profound metabolic consequences for animals hibernating over prolonged periods during the winter. For example, *Rana temporaria* submerged in aerated water at 4 °C progressively suppress their metabolic rate by up to 65 % over a period of 2 months (Donohoe *et al.* 1996). It is not known whether animals in such depressed states of metabolism would continue to behave in exactly the same manner as observed in the present study (Figs 1, 2), but we have no reason to suggest otherwise. Field studies in which the movements of overwintering bullfrogs were tracked by telemetry reveal that, while the greatest levels of activity occurred over the initial 2–4 weeks under the ice, frogs continued to make occasional readjustments in their location throughout the winter months (Friet, 1993).

The physiological responses of *R. temporaria* to cold submersion at 7 °C are very similar to those seen in the cold-submerged bullfrog *Rana catesbeiana* (Pinder, 1987). Compared with animals breathing air, cold-submerged frogs effect a reduction in blood  $P_{CO_2}$  which is almost certainly the result of increased skin perfusion associated with enhanced extrapulmonary uptake of oxygen. The increased cutaneous diffusing capacity during cold submersion in bullfrogs is thought to occur through the combined effects of an increase in the functional surface area for skin gas exchange (i.e. capillary recruitment; Burggren and Moalli, 1984; Feder and Burggren, 1985) and a decrease in the  $P_{O_2}$  of blood entering the skin (i.e. increasing the transcutaneous  $P_{O_2}$  gradient; Pinder, 1987; Pinder *et al.* 1992). As a consequence, animals submerged in cold normoxic waters can satisfy their metabolic requirements entirely through aerobic metabolism; i.e. lactate levels in cold-submerged frogs (Fig. 6) are similar to those measured in frogs and toads breathing air (Boutilier *et al.* 1987; Pinder *et al.* 1992; Pörtner *et al.* 1994).

However, when *R. temporaria* at 7 °C are exposed to aquatic hypoxia (water  $P_{O_2}$  of 25 mmHg), the onset of a lactacidosis within 3 h (Figs 5, 6) indicates that at this temperature they are no longer able to meet all of their metabolic demands aerobically, even with the hypoxia-induced 60 % reduction in aerobic metabolic rate (Fig. 4). In terms of acid–base balance, the lactacidosis at 7 °C is overshadowed by a large respiratory alkalosis during which blood  $P_{CO_2}$  levels are approximately halved and blood pH increases (Fig. 5). This hypoxia-induced decrease in blood  $P_{CO_2}$  is presumably linked to a further increase in cutaneous diffusing capacity, driven by the need to facilitate even greater extrapulmonary uptake of oxygen as ambient  $O_2$  levels decline. Under these circumstances, an increase in cutaneous diffusing capacity is probably brought

about by further increases in capillary blood flow to the skin and by further elevation of the transcutaneous  $P_{O_2}$  gradient, the latter being facilitated by the internal hypoxia *per se* as well as by an alkalosis-induced decrease in the  $P_{50}$  of the blood (Maginniss *et al.* 1980; Boutilier *et al.* 1992). Given that the frogs at 7 °C cannot remain entirely aerobic when confronted with the level of hypoxia we imposed, their ability to select lower environmental temperatures voluntarily (i.e. the so-called behavioural hypothermia response) has a number of clear advantages. First, the high  $Q_{10}$  of aerobic metabolism within this narrow temperature range (Fig. 3) allows the animal, through its thermal selection behaviour, to make large adjustments in metabolic rate for relatively small changes in ambient temperature. Thus, by progressively selecting lower and lower temperatures and thereby reducing aerobic metabolic rates, the animals are able to match their demands for oxygen with the availability of  $O_2$  in their environment (Fig. 3), effectively delaying the onset of anaerobiosis (Fig. 6). The selection of colder temperatures in hypoxia has the added advantage of raising blood pH according to the principles of constant relative alkalinity (e.g. Rahn and Baumgardner, 1972), decreasing blood  $P_{50}$  (e.g. Boutilier *et al.* 1987, 1992) and thereby facilitating an increase in the  $P_{O_2}$  gradient between the ambient water and skin capillary blood.

It seems clear that a behavioural hypothermia response such as that reported here can confer important advantages to overall oxygen homeostasis during acute exposures to hypoxia. The long-term responses are, however, unlikely to be as simple. Overwintering hypoxia in nature is normally slow to develop (e.g. Bradford, 1983), during which time large depressions of aerobic metabolic rate, hypoperfusion of non-essential organs and overall decreases in animal activity may be taking place (Boutilier *et al.* 1992; Pinder *et al.* 1992; Donohoe *et al.* 1996; West *et al.* 1996). The fact that overwintering frogs in nature eventually prefer shallow, cooler and more oxygenated regions of ponds after spending long periods in deeper, more hypoxic, regions (Friet, 1993) also suggests that in the latter stages of winter the animals might not only be selecting colder temperatures but also following gradients of increased oxygen availability.

A number of endogenous stimuli and mediators of behavioural hypothermia have been proposed previously, including various hormones and metabolites (arginine vasotocin, adenosine) as well as stresses due to infection or disease (Wood, 1995; Wood and Gonzales, 1996). Recently, lactate has been proposed to elicit behavioural hypothermia in terrestrial toads *Bufo marinus* (Pörtner *et al.* 1994). The plasma lactate levels measured in our normoxic, submerged frogs compare well with previous values for submerged *Rana pipiens* at 4 °C (Christiansen and Penney, 1973) and with values for normoxic toads at higher temperatures (Pörtner *et al.* 1994). However, the plasma lactate concentrations measured in our hypoxic frogs at 7 °C are considerably lower than those at which behavioural hypothermia was initiated in toads (Pörtner *et al.* 1994). Considering that no frog in our study chose voluntarily to remain at 7 °C during hypoxic exposure (Figs 1, 2), the plasma lactate concentrations at which preferred temperatures

became lowered could not have been much higher than those of normoxic frogs. This suggests that some other stimulus (e.g. oxygen itself) operates to trigger the behavioural hypothermia response in cold-submerged *R. temporaria*.

Frogs in the thermal gradient remained at less than 1 °C for the entire 16 h recovery period in normoxia, effectively 'locked' in position at the cool end of the chamber. It is well known that low temperature (0–1 °C) reduces overall activity in amphibians (Putnam and Bennett, 1981) and it has been shown to eliminate motor activity entirely in juvenile bullfrogs (Lotshaw, 1977). In the present study, however, *R. temporaria* moved freely at all temperatures that they encountered in the normoxic gradient and became 'behaviourally immobilised' only after their hypoxia-induced voluntary movement to low temperatures. Whether they are exhibiting a form of dormancy brought on by the stress of a previous metabolic acidosis (Figs 5, 6) or are simply favouring the higher oxygen concentrations at the lower temperatures remains to be shown. Numerous studies on fish in laboratory oxygen gradients have shown that low oxygen levels are avoided when a choice of oxygen tensions at a constant temperature is available (Jones, 1952; Whitmore *et al.* 1960). Considering that oxygen concentration varies by approximately 13% with the 4.9 °C change in preferred temperature in the present study, it is conceivable that oxygen itself may play a role in microhabitat selection in hibernating frogs, effectively 'resetting' the preferred temperature from 7 to less than 1 °C.

In conclusion, behavioural hypothermia, which is found ubiquitously among ectotherms and neonatal endotherms, is of ecological importance to amphibians hibernating in ice-covered ponds. This response is beneficial since it results in a reduction in oxygen consumption in an oxygen-limiting environment, prolongs the onset of lactacidosis and presumably increases haemoglobin oxygen-affinity as a result of the combined effects of lowered temperature and increased blood pH. This behavioural response may therefore enhance survival in hypoxia (Wood, 1995) and may be an important winter strategy by which anoxia-intolerant species can choose favourable hibernating sites under the ice. It remains to be determined whether anoxia-tolerant species (e.g. turtles) will also use this strategy when submerged in the cold, given that their physiological tolerance to anoxia is so great and their ability to extract dissolved environmental oxygen is limited.

The authors thank T. G. West for his support and assistance with experiments, A. W. Pinder for his ideas and input, D. M. Webber for sharing his knowledge of electronics and S. C. Wood for his helpful discussions. This research was funded by a BBSRC grant to R.G.B. and a 1967 Centennial NSERC Scholarship to G.J.T.

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