ABSTRACT

Rates of O₂ consumption (\(\dot{V}O₂\)) were determined for adult northern leopard frogs (\(Rana pipiens\)) submerged at 3 °C at water PO₂ (\(P_{w}O₂\)) ranging from 0–160 mmHg. The critical O₂ tension (\(P_c\)) was 36.4 mmHg. Hematocrit and blood levels of PO₂, glucose, lactate, pH, [Na⁺], [K⁺], and osmolality were determined for frogs submerged for two days. Above a \(P_{w}O₂\) of 50 mmHg, blood PO₂ ranged from 1–7 mmHg, which was sufficient to allow the frogs to function entirely aerobically. Plasma [lactate] increased as \(P_{w}O₂\) fell below 50 mmHg, the increase preceding significant changes in any other variable, and apparently preceding a fall in \(\dot{V}O₂\). Most other variables showed little or no change from those of air-breathing control animals, even during anoxia. We present an analysis of the importance of a large decrease in \(P_c\) in permitting frogs to successfully overwinter in icebound ponds and of the factors that contribute to that decrease.


INTRODUCTION

Most ranid frogs in north-temperate climates hibernate underwater, which means that gas exchange occurs via extrapulmonary surfaces, primarily the integument (Pinder et al., ’92). When submerged in normoxic water at low temperature, frogs can function essentially as aerobic aquatic animals for at least four to five months (Donohoe and Boutilier, ’98; Donohoe et al., ’98; Stewart et al., 2003). Many hibernate in situations where the water PO₂ is near normoxia throughout the winter, such as in moving water (Cunjak ’86; Ultsch et al., 2000). However, in some aquatic environments in which frogs hibernate, varying degrees of hypoxia, and perhaps occasional anoxia, can occur during overwintering, both temporally and spatially (Bradford, ’83; Friet, ’93; Emery et al., ’72). Frogs, even at low temperatures, cannot tolerate anoxia for more than a week, usually no more than three to four days (Christiansen and Penney, ’73; Stewart et al., 2003), but they can tolerate considerable hypoxia.

With regard to hibernation in hypoxic environments, questions of interest include: How severe a hypoxia can submerged frogs tolerate, for how long can they tolerate hypoxia, and what are the mechanisms involved in hypoxia tolerance? These questions have received considerable attention lately, largely in studies on \(Rana temporaria\) (see reviews of Boutilier, 2001; Boutilier and St-Pierre, 2002; Boutilier et al., ’97, ’99, 2000). Most of these studies have dealt with one or two levels of hypoxia, typically 30 and/or 60 mmHg. It has emerged that the major adaptation is metabolic depression, induced both by submergence and by hypoxia, which allows the frogs to remain aerobic at low water PO₂ (\(P_{w}O₂\)). Anaerobiosis plays a minor role, and frogs that accumulate considerable amounts of lactate will die; hence the limited time of survival in anoxic water of submerged frogs, even at low temperature.

We have recently detailed some physiological responses of northern leopard frogs (\(Rana pipiens\)) during simulated hibernation while submerged in normoxic and anoxic water at 3°C (Stewart et al., 2003). Here we report physiological responses, after two days of submergence at 3°C, to seven...
levels of hypoxia between these extremes to determine the critical \( P_{\text{wO}_2} \) for initiating any changes in several physiological variables.

We find that the rate of \( O_2 \) consumption (\( \dot{V}O_2 \)) of submerged frogs can be maintained down to a \( P_{\text{wO}_2} \) of \( \approx 36 \text{ mmHg} \). Plasma [lactate] starts to increase at a higher \( P_{\text{wO}_2} \) (\( \approx 42.2 \text{ mmHg} \)), while other variables we measured either do not change, or do not change until the \( P_{\text{wO}_2} \) is 0–10 mmHg. We conclude that the ability to tolerate severe hypoxia requires an equally severe metabolic depression and concomitant reduction in the critical oxygen tension (\( P_c \)), which has at least four components that have been previously reported: 1) a depression associated with cold (Q10 effect); 2) a depression associated with acute diving; 3) a depression associated with prolonged diving and hypoxia; and 4) an increase in the ability of the animals to transport \( O_2 \) at low \( P_O\).

**MATERIAL AND METHODS**

**Animals**

Northern leopard frogs (\( Rana p. \)) from Vermont, Minnesota, and southern Manitoba were purchased from commercial suppliers during autumn over three years. In order to minimize the number of animals used, data for frogs (from Manitoba) submerged in anoxic water for two days and for normoxic air-breathing frogs are from a previous study (Stewart et al., 2003). The additional data reported here for seven levels of hypoxia are for Minnesota and Vermont frogs, which had been kept by the suppliers at 4 \( ^\circ\text{C} \) and shipped overnight. Upon receipt, these frogs were placed in shallow 18–22 \( ^\circ\text{C} \) water in a cold room maintained at 3 \( ^\circ\text{C} \). The water cooled to ambient overnight, and the frogs were kept at this temperature for a minimum of two weeks before use in any experiments. Protocols were approved by the University of Alabama Institutional Animal Care and Use Committee (IACUC # 185).

**Metabolic rate (\( \dot{V}O_2 \)) and critical oxygen tension (\( P_c \)) during submergence**

Frogs were submerged at 3 \( ^\circ\text{C} \) in air-equilibrated water (\( P_{\text{wO}_2} \approx 158 \text{ mmHg} \)) for three days and then transferred underwater individually into Erlenmeyer flasks of 1 or 2 L, which were then sealed with a rubber stopper pierced by two 18-gauge syringe needles. The flasks were submerged in a water bath at 3 ± 0.2 \( ^\circ\text{C} \). The initial \( P_{\text{wO}_2} \) of the flask water had been adjusted according to the level of hypoxia desired by bubbling with nitrogen. Two hours were allowed for a frog to habituate to the chamber. Two-ml water samples were withdrawn until two subsequent measurements agreed within <1 mmHg (Radiometer BMS/MK2 Blood Micro System, thermostatted at 3\( ^\circ\text{C} \), and PHM73 pH/Blood-gas Monitor). Subsequent \( P_{\text{wO}_2} \)s were similarly determined after periods that were long enough (typically several hours) to be associated with at least a 10 mmHg decrease. The water volume of the stoppered flask (including 4 cc of water in the two connected glass syringes) was calculated as flask volume (ml) minus frog mass (g).

Using an \( O_2 \) solubility in water of 0.04512 ml \( O_2 \)/ml \( H_2O \), \( \dot{V}O_2 \) was calculated from the fall in \( P_{\text{wO}_2} \) as \( \mu l \cdot O_2 \cdot g^{-1} \cdot h^{-1} \). The \( \dot{V}O_2 \) was considered to have occurred at the midpoint of the associated \( P_O \) interval. The 108 metabolic rates determined over a range of average \( P_{\text{wO}_2} \) of 6.9–160 mmHg were adjusted to the mean body mass of 58.1 g (range 38.0–82.0 g) according to the method of Ultsch (’95).

\( P_c \) was determined with the method of Yeager and Ultsch (’89), which assumes that a two-segment line provides a reasonable approximation of the relationship between \( \dot{V}O_2 \) and \( P_{\text{wO}_2} \). Briefly, the method calculates the \( P_c \) as the intersection of the two best-fit linear regression lines over the ranges of metabolic \( O_2 \) regulation and metabolic \( O_2 \) conformity. The two best-fit lines are those of all possible combinations of two lines fitted to the data that give the minimal combined residual sums of squares.

**Determinations of blood variables during air access and submergence in hypoxic and anoxic water**

For studies of submergence in hypoxic water, frogs were first submerged in normoxic water for 3 d to assure depletion of lung \( O_2 \). They were then transferred underwater to a 20 L aquarium filled with normoxic water. A grating separated the frogs below from the water above, which was continually bubbled with a hypoxic gas mixture of known \( P_O \) from a Wösthoff M301/a-F gas mixing pump. A plastic cover on the aquarium ensured that all gas movement was out of the tank. About 2 h was required for the water to reach the target \( P_{\text{wO}_2} \). Blood was sampled from the frogs after 2 d of submergence at constant \( P_{\text{wO}_2} \).
Blood sampling closely followed the methods of Stewart et al. (2003). Briefly, blood was withdrawn anaerobically from doubly-pithed frogs into a heparinized syringe (these procedures were conducted underwater to prevent submerged frogs from breathing), via cardiac puncture. Whole-blood PO\textsubscript{2} was determined with a Radiometer BMS3/MK2 Blood Micro System thermostatted at 3°C and a PHM73 pH/blood-gas monitor; 0.5 mmHg was subtracted from the measured PO\textsubscript{2} as a correction for O\textsubscript{2} contamination during the sampling process, based on measurements of samples known to be anoxic. Plasma pH (determined in whole blood samples) was measured with a Ross combination pH semi-micro electrode, maintained at 3°C, placed into a flow-through plastic chamber designed to snugly fit the electrode into a sample well that was filled with blood from the bottom; the meter was a Radiometer MeterLab PHM 240 pH/ion meter calibrated with Radiometer pH precision buffers. Additional blood was collected by elevating the frog and allowing the heart to pump blood via a catheter into microcentrifuge and microhematocrit tubes. This blood was used for determinations of hematocrit, and plasma was obtained by centrifugation of blood at 13,000 g for 4 min. Plasma [lactate] and [glucose] (YSI 2300 Stat-Plus Analyzer), osmolality (Precision Systems \textregistered Osmette 5004), and [Na\textsuperscript{+}] and [K\textsuperscript{+}] (Instrumentation Laboratories 943 flame photometer) were then determined.

**Statistics**

Statistical analyses were performed using STATISTICA for Windows, 1999 edition (StatSoft, Inc.). Comparisons used Kruskal-Wallis ANOVA median rank tests; post-hoc comparisons used an LSD test. Significance was accepted at \( \alpha < 0.05 \). Values are reported as mean ± S.E.

**RESULTS**

\( \text{VO}_2 \text{ and } P_c \)

There was a significant positive effect of \( P_{wO2} \) on mass-adjusted \( \text{VO}_2 \) between 45 (4.0 \( \mu_l \) O\textsubscript{2} g\textsuperscript{-1} h\textsuperscript{-1}) and 160 mmHg (5.5 \( \mu_l \) O\textsubscript{2} g\textsuperscript{-1} h\textsuperscript{-1}, Fig. 1). Between 40 and 0 mmHg, the \( \text{VO}_2 \) fell much more rapidly; the \( P_c \) was 36.4 mmHg.

**Blood variables**

Blood PO\textsubscript{2} fell during submergence from the air-access control value of 22.3 mmHg, and was variable at 0–6.4 mmHg in frogs in hypoxic water (Fig. 2A). While there was a tendency toward a reduction of blood PO\textsubscript{2} at lower \( P_{wO2s} \), there was no statistical significance among the levels of hypoxia; anoxic frogs had a PO\textsubscript{2} of zero. Relative to that of air-access control frogs, plasma [glucose] rose at low \( P_{wO2} \), the increase being first evident
at 10.1 mmHg (Fig. 2B); hematocrit was unchanged (with a tendency to increase in anoxic frogs, Fig. 2C). [Lactate] first increased at 42.2 mmHg, and continued to increase as $P_wO_2$ fell (Fig. 3A), while pH decreases lagged behind lactate increases, and were not significant until $P_wO_2$ fell to 10.1 mmHg (Fig 3B). There were no significant changes in $[Na^+]$, $[K^+]$, or osmolality (Fig. 4A–C).

**DISCUSSION**

**Metabolic rate while submerged**

At a $P_wO_2$ of 158 mmHg (normoxic water), the $VO_2$ of submerged frogs was about 5.5 $\mu$L O$_2$ g$^{-1}$ h$^{-1}$, corrected to a mass of 58.1 g. Tattersall and Boutilier (’99) report a $VO_2$ of about 6.3 $\mu$L O$_2$ g$^{-1}$ h$^{-1}$ for submerged *R. temporaria* in normoxic water at 1.5°C and 15.2 $\mu$L O$_2$ g$^{-1}$ h$^{-1}$ for 7°C; they give a mass range of 20–30 g. Assuming a mass of 25 g, weight-correction of their reported $VO_2$s (using the relationship we found here for mass vs. $VO_2/W$) to 58.1 g would give routine metabolic rates of 3.2 and 7.7 $\mu$L O$_2$ g$^{-1}$ h$^{-1}$ at 1.5 and 7°C, respectively, which bracket our results at 3°C.

**Critical O$_2$ tension as determined from $VO_2$**

$P_c$ has been typically defined as the $O_2$ at which an animal switches from being a metabolic $O_2$ regulator ($VO_2$ independent of ambient $O_2$) to being a metabolic $O_2$ conformer ($VO_2$ falls as ambient $O_2$ falls). In practice, the definition can be expanded to state that the $P_c$ occurs at that $O_2$ when the relationship of $VO_2$ and $O_2$ undergoes an abrupt change, as results sometimes show a slight slope of the regression within the range of “conformity,” as seen here (Fig. 1), especially when using a closed-respirometry technique that does not allow one to factor out such effects as variable activity (Ultsch et al., ’80). Bradford (’83) reported the $P_c$ of submerged *R. muscosa* as about 30 mmHg at 4°C and Tattersall and Boutilier (’99) reported a $P_c$ of 41 mmHg for *R. temporaria* at 1.5°C, values similar to the 36.4 mmHg we find for *R. pippens*. Pinder (’87) reported a much higher $P_c$ for bullfrogs of about 80 mmHg at 5°C; the larger size of the frogs (mean of 262 g) and the fact that

![Fig. 3](image1.png) **Fig. 3.** Plasma [lactate] (A) and pH (B) as a function of ambient $O_2$, depicted as in Figure 1 (B and C).

![Fig. 4](image2.png) **Fig. 4.** Plasma $[Na^+]$ (A), $[K^+]$ (B), and osmolality (C), depicted as in Figure 1 (B and C).
they were curarized likely contributed to this rather high value.

Adult frogs often successfully overwinter in ponds that are likely to become quite hypoxic during the winter, although hibernacula PO$_2$ data adjacent to frogs for such situations are lacking, so their P$_c$ is undoubtedly quite low during hibernation compared to that of an air-breathing frog in summer. How such a fall in P$_c$ may occur is illustrated in Fig. 5. For illustrative purposes we assume an initial P$_c$ for air-breathing frogs substantially below normoxia. We also assume linear relationships between VO$_2$ and PO$_2$ over the ranges of 0 to P$_c$ and P$_c$ to normoxic PO$_2$. Any depression in VO$_2$ will then reduce the P$_c$ as shown, other factors being constant. Such metabolic depression can result from at least three known mechanisms. The first is due to decreased temperature; the Q$_{10}$ effect. Q$_{10}$ values are usually higher at lower temperature ranges, but we conservatively use a Q$_{10}$ of 2 as temperature declines from a typical northern summer water temperature of 20°C to an overwintering value of 3°C. This effect would reduce the P$_c$ to 31% of its original value for an air-breathing frog. Since the frog hibernates underwater, there is a second depression due to prolonged diving; this has been reported as 50% for R. temporaria submerged in normoxic water at 3°C (Donohoe et al., ’98), which here reduces the P$_c$ to 15% of its initial value. There is an additional 50% depression due to prolonged submergence in hypoxia (Donohoe and Boutilier, ’98), likely due to cellular mechanisms such as channel arrest (Boutilier, 2001); this effect further reduces the P$_c$ to 7.8% of the original value. Finally, P$_c$ can be lowered at any given VO$_2$ by changes in the whole-body O$_2$ conductance, the rate at which oxygen can be transferred from the external environment to the cell (Duke and Ultsch, ’90; Ultsch et al., ’99a) for a given ΔPO$_2$ (equivalent to the slope of the relationship between VO$_2$ and PO$_2$ in the range of metabolic O$_2$ conformity). Factors that would increase whole-body O$_2$ conductance include a left-shift of the O$_2$ dissociation curve due to lowered temperature and to reduced PCO$_2$, increased O$_2$ carrying capacity of the blood, reduction of the PO$_2$ boundary layer between the skin and the water by movement of either the animal or the water (Burggren and Feder, ’86), and recruitment of cutaneous capillaries coupled with cutaneous vasodilation (Burggren and Moalli, ’82; Pinder, ’87). The absolute decreases in P$_c$ that would result from increases in whole-body O$_2$ conductance are progressively diminished at lower VO$_2$s, but when all factors are considered, the original P$_c$ has been reduced by 93.2%, concomitant with a reduction in VO$_2$ of 92.3% relative to air-breathing animals at 20°C. While our analysis is hypothetical, a reduction in VO$_2$ of this magnitude is not unreasonable. Gatten et al. (’92) cite four values of VO$_2$ for air-breathing R. pipiens at 20°C that average 77 μl O$_2$ g$^{-1}$ h$^{-1}$. Our value for frogs in normoxic water of 5.5 μl O$_2$ g$^{-1}$ h$^{-1}$ represents

![Fig. 5. An analysis of factors that can contribute to a fall in P$_c$ in an overwintering submerged frog. Starting assumptions are for an air-breathing frog at 20°C with a P$_c$ of 80 mmHg. See text for details.](image-url)
a 93% reduction in metabolic rate, and VO₂ would very likely have further decreased had the measurements been taken on frogs that were submerged for months rather than hours. Similarly, Herbert and Jackson ('85) found that the aerobic VO₂ of the western painted turtle (Chrysemys picta bellii) fell by 94% between 20 and 3°C; the reduction in this case due to temperature alone, as the turtles were breathing air.

**Effects of hypoxia upon blood variables**

The discussion of Pₜ above is based on the common definition of Pₜ that relates changes in VO₂ to changes in ambient PO₂. In contrast, Pörtner et al. ('91, '94; reviewed by Pörtner and Grieshaber, '93) have suggested that a more physiologically relevant definition of Pₜ is the PO₂ at which anaerobiosis commences. Since they found that lactate in *Bufo marinus* breathing progressively hypoxic gas mixtures at 20°C starts to accumulate before VO₂ decreases, and often is associated with a transient increase in VO₂, Pₜ's determined using this definition will be higher that those using the traditional definition.

There are three possibilities concerning when lactate increases relative to a decrease in the routine or standard VO₂. In Fig. 6 we depict an animal with a Pₜ of 40 mmHg, using the traditional definition of Pₜ. Lactate production can increase at a lower PO₂ than Pₜ if there is a metabolic depression that lowers VO₂ as Pₜ is approached (A), thus effectively shifting Pₜ to the PO₂ associated with point A. The traditional approach to Pₜ would suggest that lactate should start to increase at the same PO₂ that VO₂ starts to fall (i.e., at Pₜ, point B), the Pasteur effect. The results of Pörtner et al. ('91; '94) support an increase in lactate starting at point C. In this scenario, lactate is viewed as a physiological signal that results in an increase in VO₂, perhaps by catecholamine release that increases the activity of the animal as it seeks a higher ambient PO₂. Our data support Pörtner et al.'s ('91, '94) hypothesis, since increases in lactate first occurred at 42.2 mmHg, while the Pₜ was 36.4 mmHg. However, we are tentative in this conclusion for several reasons. Firstly, we cannot put a statistical confidence limit on our Pₜ as derived, although inspection of Fig. 1 suggests that there is no decrease in VO₂ until approximately 30 mmHg. Secondly, we did not find an increase in VO₂ above the routine VO₂ prior to a decrease in routine VO₂, as did Pörtner et al. ('91; '94) in their studies with *B. marinus*. However, we were studying frogs submerged at a low temperature, and the depressed VO₂ may have made it difficult to see such an effect if it was present, especially since our approach pooled data from a number of individuals; it is also possible that the effect is not present at such low metabolic rates.

We can only hypothesize why increases in [lactate] were not accompanied by corresponding decreases in pH until the Pₜ had fallen to 10 mmHg. The most likely explanation is that the metabolic depression resulted in a decrease in plasma PCO₂ with the resultant respiratory alkalosis offsetting the lactacidosis until the [lactate] became high enough to cause the fall in pH seen in frogs in water with a Pₜ of 0–10 mmHg. Support for this contention is found in our earlier study (Stewart et al., 2003), where *R. pipiens* submerged at 3°C in normoxic water for 125 d, and in anoxic water for 2 d, both exhibited a significant fall in plasma PCO₂ relative to air-breathing frogs. Submerged hypoxic (25 mmHg) frogs have also been shown to reduce their blood PCO₂ (*R. temporaria* at 3°C; Boutilier et al., '97), as have submerged turtles (softshell turtles, *Apalone spinifera*, at 3°C, Reese et al., 2003).

Most other blood variables measured did not change in hypoxic frogs. This may be, in part, because frogs were submerged for only two days. However, since we knew that anoxic frogs could only remain responsive for four days, we chose two days as the halfway point. The large increases in lactate, and the mobilization of glucose at 10 mmHg and below, suggest that severe hypoxia is limiting energy production.

![Fig. 6](image-url)
Ecological considerations

Ranid frogs that hibernate underwater are found in some rather cold climates at high altitudes and latitudes. Therefore, they may spend up to nine months under ice (Bradford, ’83). Overwintering survival is highly variable (Bradford, ’83; Anholt et al., 2003), and occasional winterkills do occur (Manion and Cory, ’52; Bradford, ’83). If adults die, either tadpoles must successfully overwinter, or immigration must occur, for a habitat to harbor frogs in the subsequent year. Adults can survive by either selecting hibernacula with a high PO2 and/or moving water (to reduce the boundary layer, Pinder and Feder, ’90) and by reducing the Pd to low levels as described above. They can also move about in a heterogeneous O2 environment, but only horizontal PO2 gradients will be of any ecological importance, since the frogs remain on the bottom and cannot exploit vertical gradients, as can free-swimming animals such as fishes. Such horizontal gradients do occur (Friet, ’93), and cold frogs are quite capable of considerable mobility (Ultsch et al., 2000). However, it is uncertain if the frogs are capable of sensing ambient oxygen and moving in an appropriate direction, or if they simply respond to some factor such as increased [lactate] and start to move randomly (Tattersall and Boutilier, ’99).

Tadpoles, being aquatic animals with gills as well as having a more gas-permeable skin and a more favorable surface to volume ratio for aquatic gas exchange than adults, can be expected to have a lower Pd than adults. A 23°C, metamorphosed bullfrogs (Rana catesbeiana) will die if caught in submerged traps, while the PPs of tadpoles of Taylor-Kollros stages I-XIX (gills are lost at about stage XXII-XXIII, and stage XXV are newly metamorphosed) are <50 mmHg (Crowder et al., ’98). In Alabama, bullfrog tadpoles, which have functional lungs, are often found in water below their Ps in summer, in which case they are obligate air-breathers (Ultsch et al., ’99b), but they avoid such areas in winter, even in the absence of ice cover (Nie et al., ’99). We presume they have a similar wintertime behavior in more northern latitudes where ponds become frozen over. Bradford (’83) found the Ps of mountain yellow-legged frog (R. muscosa) tadpoles to be 15 mmHg at 4°C, compared to 30 mmHg for adults. He also found that in some winters most adults in high-altitude ponds (but not rivers) perish from hypoxia, while tadpoles survive. It should be noted, however, that Bradford found that tadpoles in laboratory studies died in a few hours if the PwO2 was <5 mmHg, and that while he found PwO2 in the overwintering environment to be as low as 6 mmHg, he did not observe anoxia, as is often stated. We suspect that tadpoles are more hypoxia-tolerant than adults, allowing them to function as a population reservoir during overwintering, but that neither form can tolerate anoxia for more than several days, since they lack the substantial whole-body buffering capacity such as is found in some anoxia-tolerant turtles (Stewart et al., ’03) or the alternative anaerobic pathways (e.g., ethanol) of a few fishes such as goldfish (Carassius auratus) and Crucian carp (C. carassius). Therefore ponds where all the water below the ice becomes anoxic for periods of a week or more will become depopulated of both adults and larvae, and new populations can arise only by immigration.

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LITERATURE CITED


Stewart ER, Reese SA, Ultsch GR. 2003. The physiology of hibernation in Canadian leopard frogs (Rana pipiens) and bullfrogs (R. catesbeiana). Physiol Biochem Zool (In press).