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Cardiovascular, respiratory and metabolic responses to temperature and hypoxia of the winter frog *Rana catesbeiana*

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Abstract

The objective of the present study was to determine the effects of hypoxia and temperature on the cardiovascular and respiratory systems and plasma glucose levels of the winter bullfrog *Rana catesbeiana*. Body temperature was maintained at 10, 15, 25 and 35ºC for measurements of breathing frequency, heart rate, arterial blood pressure, metabolic rate, plasma glucose levels, blood gases and acid-base status. Reducing body temperature from 35 to 10ºC decreased ($P<0.001$) heart rate (bpm) from 64.0 ± 3.1 (N = 5) to 12.5 ± 2.5 (N = 6) and blood pressure (mmHg) ($P<0.05$) from 41.9 ± 2.1 (N = 5) to 33.1 ± 2.1 (N = 6), whereas no significant changes were observed under hypoxia. Hypoxia-induced changes in breathing frequency and acid-base status were proportional to body temperature, being pronounced at 25ºC, less so at 15ºC, and absent at 10ºC. Hypoxia at 35ºC was lethal. Under normoxia, plasma glucose concentration (mg/dl) decreased ($P<0.01$) from 53.0 ± 3.4 (N = 6) to 35.9 ± 1.7 (N = 6) at body temperatures of 35 and 10ºC, respectively. Hypoxia had no significant effect on plasma glucose concentration at 10 and 15ºC, but at 25ºC there was a significant increase under conditions of 3% inspired O_2. The arterial PO_2 and pH values were similar to those reported in previous studies on non-estivating *Rana catesbeiana*, but P_aCO_2 (37.5 ± 1.9 mmHg, N = 5) was 3-fold higher, indicating increased plasma bicarbonate levels. The estivating bullfrog may be exposed not only to low temperatures but also to hypoxia. These animals show temperature-dependent responses that may be beneficial since during low body temperatures the sensitivity of most physiological systems to hypoxia is reduced.

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Key words
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- *Rana*
- Breathing frequency
- Blood pressure
- Heart rate
- Acid-base status
- Hyperglycemia

Introduction

Amphibians occupy environments that are severely hypoxic and/or are subject to extreme changes in temperature. Their cardiovascular and respiratory system demands are diverse and extreme in order to maintain an adequate P_aO_2 and acid-base status of the blood during changing oxygen availability and tissue demand (1). During seasonal periods of drought and/or low temperature some amphibians may experience hypoxia when they estivate (cf. 2). A few studies describe respiratory alterations in estivating amphibians. The most complete study documented ventilation and blood gases in

estivating *Bufo marinus*. During short-term estivation, hypoventilation combined with reduced CO_2 excretion yielded up to a 2-fold increase in blood P_aCO_2. The resulting respiratory acidosis was completely compensated for by elevation in plasma bicarbonate (3).

Hypoxia has been reported to induce a thermoregulatory response reducing body temperature in ectotherms, i.e., behavioral hypothermia (4). The interaction between body temperature and hypoxia has been shown to be advantageous in the groups studied (ranging phylogenetically from protozoans to mammals) because hypothermia decreases metabolic rate when O_2 supply is limited, thus facilitating survival (5). Some parameters have been measured in order to evaluate the effects of hypoxia at different temperatures such as: oxygen consumption (6), pulmonary ventilation (7,8), lactate production (9,10), blood gases, oxygen saturation and acid-base status (11-13) using animals during the active, non-estivating period. Other parameters such as heart rate, blood pressure and blood glucose levels have not been studied. On the basis of these considerations, we tested the hypothesis that hypoxia-induced alterations of arterial blood pressure, heart rate and plasma glucose levels may be temperature-dependent. We also measured oxygen consumption, breathing frequency, blood gases and acid-base status in the winter bullfrog *Rana catesbeiana*, assessing long-term changes of these parameters during estivation.

Materials and Methods

Adult bullfrogs (*Rana catesbeiana*) of either sex weighing 196.7 ± 10.3 g (mean ± SEM) were obtained from a commercial supplier. Experiments were performed from June to September (dry-winter season). Upon arrival, the animals were kept indoors in aquaria with free access to tap water and basking areas (temperature, 24-26°C).

Surgical procedure and anesthesia

For initial anesthesia, the toad was placed in a closed box saturated with ether vapor. The level of anesthesia was monitored by the hindlimb flexor reflex. Whenever necessary during surgery, ether was evaporated from cotton pads placed under the animal's belly. Arterial cannulation was performed using a PE-50 catheter filled with heparinized Ringer solution, occlusively inserted into the femoral artery. A second catheter (PE-100), inserted into the frog's buccal cavity via a tight-fitting hole made in the tympanic membrane, was used to measure breathing frequency. All animals recovered promptly from anesthesia. After surgery, the animals were left undisturbed for at least 24 h.

Analysis of blood gases

Arterial blood samples were analyzed for PO_2 (FAC Instruments, model 204A, São Carlos, SP, Brazil) and pH (Metrohm, model 654, Switzerland) immediately after withdrawal. The O_2 electrode (FAC Instruments) was calibrated with pure N_2 and atmospheric air. The pH electrode (Metrohm, Switzerland) was adjusted using Radiometer (Copenhagen, Denmark) precision buffer solutions (S1510 and S1500). Electrodes were kept at the temperature of the experimental animal using a constant temperature circulator (VWR Scientific, model 1160A, Niles, IL). Blood PCO_2 was estimated by the Astrup technique (14). Glucose concentration was determined quantitatively by enzymatic (hexokinase) determination (Sigma, St. Louis, MO).

Blood pressure, heart rate, breathing frequency, and O_2 consumption measurements

Arterial blood pressure was measured by connecting the arterial catheter to a Hewlett Packard pressure transducer (HP 1280, Colo-

rado Springs, CO, USA) kept at the level of the frog's heart. Heart rate was determined by counting pressure pulses. Breathing frequency was recorded using a differential air pressure transducer (Hewlett Packard, model 270) connected to the buccal catheter. Signals from transducers were recorded on paper (Hewlett Packard, model 7754A). Oxygen consumption was measured using a Krogh respirometer (15).

Experimental procedure

The experiment was performed on conscious unrestrained and undisturbed frogs. Five to seven animals were used in each group. During the experiments the frogs were housed in a 1-liter plastic chamber placed inside an environmental chamber (FANEM, B.O.D. 347 cd, São Paulo, Brazil) kept at the experimental temperature of 10, 15, 25 or 35°C. Transfer to the experimental temperature took place 24 h before the measurements. Cloacal temperature probes confirmed that there was no difference between animal temperature and environmental chamber temperature, as also reported for reptiles (16). The animal chamber was continuously flushed with humidified room air at the rate of 1.5 l/min. The humidifying flask was kept inside the environmental chamber to avoid temperature changes. At the end of the normoxic condition, buccal and arterial blood pressures were recorded for 20 min and arterial blood was sampled for analysis of blood gases, pH, and plasma glucose. Hypoxic gas mixtures (AGA, Sertãozinho, SP, Brazil) containing 3, 5, 7 or 10% oxygen were then applied in a random order for 60 min each. Buccal and arterial blood pressures were recorded and 1-ml arterial blood samples were withdrawn at the end of each experimental period. About 800 µl was reinfused into the animal's circulation after blood gas measurement. A small fraction (0.1 ml) of arterial blood was immediately centrifuged (Ravan microcentrifuge, model Ciclo I, São Paulo, Brazil) and plasma was frozen at -20°C until plasma glucose concentration was determined.

Calculations and statistics

Breathing frequency was obtained by counting the number of large amplitude buccal movements, distinguished from buccal oscillations (17). Breathing frequency, blood pressure and heart rate were calculated for 5-min periods. All values are reported as means ± SEM (N = 5-7 animals per group). The effects of hypoxia at each temperature and the effects of temperature under normoxia were evaluated by analysis of variance (ANOVA) and the difference between means was assessed by the Tukey test. Values of $P<0.05$ were considered to be significant.

Results

Breathing frequency (Figure 1, panel A) under normoxia was higher at higher temperatures ($P<0.01$ for 35°C compared to 10°C). The slope of the ventilatory response curve to inspired oxygen became markedly steeper at the higher temperatures. At low body temperature (10°C) there was no significant increase in breathing frequency, whereas at 15°C a significant change was measured in the presence of 3 and 5% inspired O_2. Even at 10% inspired O_2 significant differences were observed at 25 and 35°C. Seven percent inspired O_2 was lethal at 35°C.

Table 1 shows the effects of hypoxia on blood gases of frogs equilibrated at different temperatures. A significant increase of P_aO_2 and a decrease of arterial pH were measured with increasing temperatures. The hypoxia-induced tachypnea caused a respiratory alkalosis and a tendency to reduction of P_aCO_2 but the latter was not significant. There was a significant reduction of P_aO_2 under all the hypoxia levels tested at each one of the experimental temperatures. Figure 1 (panel

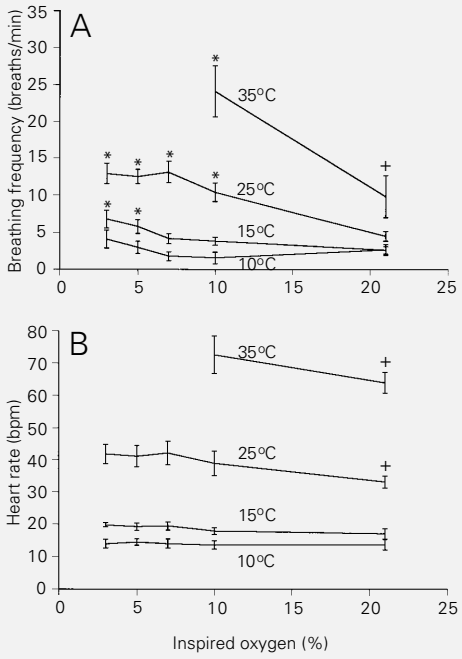

Figure 1 - Effects of hypoxia on breathing frequency (panel A) and heart rate (panel B) at different temperatures. Values are reported as mean ± SEM for N = 6 (10°C), 6 (15°C), 7 (25°C) and 5 (35°C). *P<0.05 compared to normoxic control (Tukey test) at the same temperature; +P<0.05 compared to 10°C (Tukey test).

Table 1 - Effect of hypoxia and body temperature on blood gases of *Rana catesbeiana*.

Data are reported as mean ± SEM for 5 animals in each group. *P<0.05 compared to normoxic conditions at the same temperature (Tukey test).

Temperature	Inspired O_2 (%)	P_aO_2 (mmHg)	pH_a	P_aCO_2 (mmHg)
15°C	21	36.0 ± 3.1	7.95 ± 0.006	-
	10	26.4 ± 4.0*	7.96 ± 0.006	-
	7	22.7 ± 2.9*	8.01 ± 0.005*	-
	5	22.0 ± 1.9*	8.03 ± 0.005*	-
	3	20.3 ± 1.0*	8.03 ± 0.005*	-
25°C	21	68.3 ± 5.4	7.87 ± 0.003	37.5 ± 1.9
	10	50.9 ± 3.6*	7.95 ± 0.003*	33.2 ± 2.6
	7	39.0 ± 3.9*	7.96 ± 0.004*	32.4 ± 2.1
	5	25.4 ± 2.6*	7.98 ± 0.005*	31.2 ± 2.7
	3	18.1 ± 2.3*	8.00 ± 0.005*	30.3 ± 3.1
35°C	21	83.7 ± 5.5	7.73 ± 0.11	-
	10	59.1 ± 4.9*	7.85 ± 0.11	-

B) shows the effects of hypoxia on heart rate at different temperatures. Under normoxia, heart rate increased significantly (P<0.001) at 25 and 35°C, taking heart rate at 10°C as reference. Hypoxia did not cause significant changes in heart rate at each experimental temperature. Blood pressure at 35°C was significantly higher than at 10°C (Figure 2) but no alteration in pressure was observed under hypoxia within the temperature range from 10 to 35°C (data not shown).

Figure 3 shows the effect of hypoxia on plasma glucose levels at different temperatures. Hypoxia caused no change in plasma glucose levels at 10 or 15°C, whereas at 25°C there was a significant increase at 3% inspired O_2 (P<0.05). At 35°C, 10% inspired O_2 failed to increase plasma glucose. Glucose levels increased significantly with rising temperatures for similar conditions of inspired O_2 (P<0.05).

Oxygen consumption increased with increasing temperatures (ml BTPS (body temperature pressure standard) min^{-1} kg^{-1}): 0.070 ± 0.020 at 10°C, 0.190 ± 0.038 at 15°C, 0.551 ± 0.086 at 25°C and 0.933 ± 0.094 at 35°C (P<0.001 for 25 and 35°C compared to 10°C).

Discussion

Pulmonary physiology of the winter bullfrog

The present study provides data on cardiorespiratory responses to hypoxia in the estivating bullfrog *Rana catesbeiana*, which had been equilibrated at different temperatures for 24 h. No data about the cardiopulmonary physiology of *Rana* during estivation have been reported thus far. Experiments were performed during the winter, when adult frogs stop eating and become more quiet, but are not torpid. In the field, frogs (*Rana catesbeiana*, *Rana clamitans*) were found estivating under 5 cm of leaf litter in Michigan (USA) from January to mid-April (winter season) when temperatures range from 0 to 3°C but none of them was torpid (cf. 18).

Most studies on the cardiopulmonary physiology of amphibians were performed during the active, non-estivating period of the species. The control of breathing of *Rana catesbeiana* was recently evaluated by

Kinkead and Milson (17) who measured blood gases and acid-base status of arterial blood. They reported P_aO_2 and pH values that are similar to those obtained in the present study but our frogs presented P_aCO_2 values 3 times higher than theirs. According to the Henderson-Hassebalch equation, increased P_aCO_2 causes a drop in pH unless a compensatory increase in bicarbonate concentration occurs. This is in agreement with a study on *Bufo marinus* (3). Under special laboratory conditions, *Bufo marinus* toads burrow and estivate and then hypoventilate, and reduce cutaneous CO_2 excretion, with a resulting 2-fold increase in P_aCO_2. During non-estivating periods, such a high P_aCO_2 value would induce hyperventilation (cf. 2) but, during estivation, ventilation is actually reduced. Instead, an increase in plasma bicarbonate completely compensates for the respiratory acidosis within the first three days of estivation (3). The source of bicarbonate is unknown but could result from ion exchange in the urinary bladder (cf. 18). Moreover, this is evidence that short-term (3) and long-term (present study) alterations during estivation might be similar among anuran amphibians.

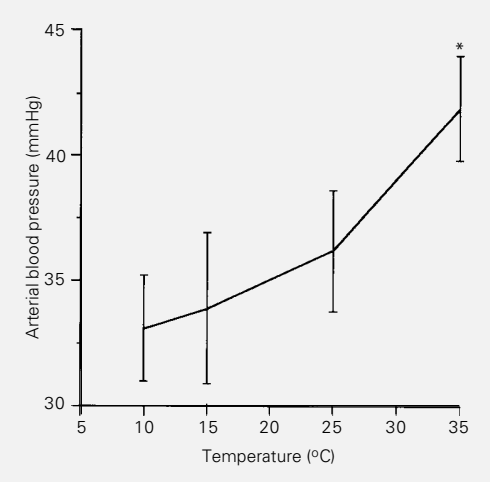

Figure 2 - Relationship between arterial blood pressure and temperature. Values are reported as mean ± SEM for N = 6 (10°C), 6 (15°C), 7 (25°C) and 5 (35°C). *P<0.05 compared to 10°C (Tukey test).

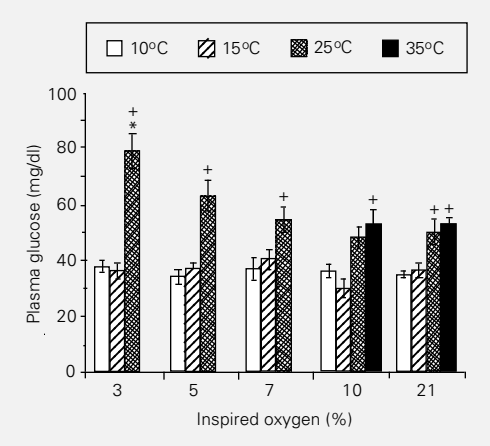

Figure 3 - Effect of hypoxia on glucose levels at different temperatures. At 25°C, hypoxia (3% inspired O_2) caused a significant increase in plasma glucose (*P<0.05 compared to the other conditions of inspired O_2; Tukey test). Under any degree of hypoxia, the highest temperatures were accompanied by an elevation of plasma glucose (+P<0.05, taking values at 10°C as reference; Tukey test). Values are reported as mean ± SEM for N = 6 in each group.

Effect of temperature on blood gases

The effect of temperature on P_aO_2 under normoxic conditions is consistent with previous reports on anuran amphibians (7,8,11-13,17). Arterial PO_2 was lower at reduced temperatures (Table 1). These results are consistent with the model proposed by Wood (19) for animals with intracardiac shunts.

As previously reported for ectotherms, arterial pH varied inversely with temperature (2,8-13,16,20-23). Changes in arterial pH with temperature may result from a relative bradypnea, i.e., oxygen consumption increased 2.9-fold from 15 to 25°C whereas breathing frequency increased approximately 1.7-fold. Pulmonary ventilation increased with rising temperatures but this increase was not large enough to maintain a constant ratio of ventilation to oxygen uptake (often called 'air convection requirement'). This seems to be a general trend among vertebrates (cf. 20).

Effect of temperature on pulmonary ventilation

In most amphibians and reptiles, pulmonary ventilation increases with increasing body temperature. The effect of temperature on ventilation was reported earlier for *Bufo.* Kruhøffer et al. (7) were the first to report that hypoxia-induced hyperventilation in *Bufo paracnemis* is augmented in response to increased body temperature. More recently, it was shown that the hypercapnic drive to

breathing is also increased at high temperatures in the same species (8). To our knowledge, no paper reported on the effects of temperature associated with hypoxia in frogs. Our measurements were performed at different temperatures, and permit us to conclude that hypoxia-induced alterations of ventilation are a temperature-dependent process in *Rana catesbeiana* (Figure 1A). Conversely, in studies of turtles (*Pseudemys scripta*) no clear relationship between temperature and ventilation was observed (22,23).

Effect of temperature on plasma glucose levels

In agreement with the literature (24,25), plasma glucose levels varied considerably among individuals. The effect of hypoxia on glucose levels was evaluated earlier in *Bufo paracnemis* (26) and *Bufo marinus* (27). Both studies reported a marked hypoxia-induced hyperglycemia at room temperature. The present study showed that at low temperatures (10 and 15°C) hypoxia failed to induce any increase in the glucose levels (Figure 3). In addition, a recent study (28) has shown that hypoglycemia elicits behavioral hypothermia in the toad *Bufo paracnemis*. Possibly, all of these conditions (hypothermia, hypoxia and hypoglycemia) occur simultaneously during estivation.

In conclusion, exposure to hypoxic environments elicits a regulated reduction in body temperature (behavioral hypothermia) in a variety of organisms ranging from protozoans to mammals (4,5). To evaluate the functional significance of hypoxia-induced hypothermia some physiological responses to hypoxia have been measured in frogs at different temperatures. It has been proposed that hypothermia is beneficial because it reduces oxygen consumption (6) according to the Q_{10} effect (ratio of oxygen uptake at temperature t + 10°C over oxygen uptake at temperature t), promotes a leftward shift of the oxyhemoglobin dissociation curve (increased affinity) and blunts the energetically costly responses to hypoxia, e.g., hyperventilation (7,8) when the oxygen supply is limited. The present study shows that a metabolic response to hypoxia (hypoxia-induced hyperglycemia) is temperature-dependent. Hypothermia may be beneficial in relation to hypoxia-induced hyperglycemia because reduction of body temperature dampens cellular oxidative demands during oxygen deprivation. On the other hand, cardiovascular parameters seem to be unaffected by hypoxia since heart rate and arterial blood pressure showed no significant changes under hypoxia at least at the temperature range from 10 to 25°C (Figure 1). Conversely, temperature under normoxia seems to be an important factor in all parameters evaluated in this study.

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