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RESPIRATION DURING CHRONIC HYPOXIA AND HYPEROXIA IN LARVAL AND ADULT BULLFROGS (*RANA CATESBEIANA*)

II. CHANGES IN RESPIRATORY PROPERTIES OF WHOLE BLOOD

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SUMMARY

Rana catesbeiana Shaw tadpoles and adults were maintained at 20–23 °C under aerial and aquatic normoxia (P_{O_2} 150 mmHg), hyperoxia (P_{O_2} 275 mmHg) and hypoxia (P_{O_2} 75 mmHg) for 4 weeks, after which the following blood measurements were made: haematocrit, red blood cell count, haemoglobin concentration, mean corpuscular haemoglobin concentration, O_2 capacity, O_2 equilibrium curve, Bohr shift, Hill's coefficient and intraerythrocytic concentration of nucleotide triphosphates (ATP+GTP) and 2,3-DPG.

Normoxic tadpoles had much higher blood O_2 affinity (P_{50} 9–10 mmHg) than adults (P_{50} 35 mmHg) but a lower haemoglobin concentration, haematocrit and O_2 capacity. The concentration of intraerythrocytic phosphates was higher in normoxic tadpoles than in adults, indicating that the higher O_2 affinity of normoxic tadpole blood was due to the haemoglobins themselves, rather than affinity modulators.

Chronic hypoxia in tadpoles produced little change in whole blood P_{50}, and no significant change in any other blood variable. In adult bullfrogs, on the other hand, O_2 capacity doubled through polycythaemia, and the P_{50} decreased by 11 mmHg (35 %), though apparently not from any significant change in concentration of intraerythrocytic phosphates. Hyperoxia produced no haematological changes in either larvae or adults.

In adult bullfrogs exposed to chronic hypoxia, the morphology of the gas exchange organs does not change (Burggren & Mwalukomo, 1983), but instead profound adjustments occur in the blood, favouring O_2 transport under these conditions. The blood of the tadpole shows little or no response to chronic hypoxia, with morphological adjustments in skin, gills and lungs constituting the major response.

INTRODUCTION

Vertebrates experiencing environmental hypoxia generally respond initially by increasing convection of blood and air or water through the gas exchange organs, in order to maintain oxygen uptake. Since the energetic cost of increased cardiac output

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and ventilation is high, prolonged hypoxia may result in morphological changes of the gas exchange organs (McDonald & McMahon, 1977; Lechner & Banchero, 1980; Burggren & Mwalukomo, 1983) and/or changes in the respiratory properties of blood (see Wood, 1980 for review).

Thr morphological consequences of chronic hypoxia and hyperoxia in larval and adult bullfrogs are reported in the preceding paper (Burggren & Mwalukomo, 1983). In the tadpole, a marked branchial hypertrophy, an increase in capillary mesh density, and a decrease in the gas diffusion distance between blood and water in the skin, all accompany chronic hypoxia. However, adult bullfrogs exposed to the same hypoxic conditions showed no significant changes in the morphology of the skin or lungs. It was suggested that this striking difference in morphological response was related to the greater 'plasticity' of the larvae, which in any event undergo radical morphological change during metamorphosis.

No published study, however, has examined haematological responses to chronic hypoxia in larval compared with adult amphibians. The present study extends our observations on the different responses of tadpoles and adult bullfrogs to chronic hypoxia and hyperoxia by examining effects on the oxygen transport characteristics of the blood.

MATERIALS AND METHODS

Experiments were carried out during the summer of 1981 on a total of 26 adult bullfrogs (mean weight 95 ± 50 g) and on 33 tadpoles (mean weight 17 ± 5 g) of developmental stages XV–XX (after Taylor & Kollros, 1946). All animals were captured locally. Both adults and larvae were divided into three populations, which were held under hyperoxia (280–390 mmHg), normoxia (150 mmHg) or hypoxia (70–80 mmHg) at 20–23 °C for 25–28 days. Complete details of holding conditions are provided in the companion study (Burggren & Mwalukomo, 1983). After the acclimation period, the animal was killed, the heart was immediately exposed and a blood sample taken into a heparinized syringe. Haemoglobin concentration was measured spectrophotometrically with the cyanmethaemoglobin method. Whole blood NTP (GTP + ATP) and 2,3-DPG concentrations were measured with u.v. spectrophotometric assays provided by Sigma kit numbers 366-UV and 35-UV, respectively.

Whole blood O_2 content was determined using the method of Tucker (1967). Oxygen equilibrium curves at 23 °C were determined on a whole blood sample from each individual (i.e. blood samples were not pooled). Blood was tonometered with gases of known P_{O_2} and P_{CO_2} delivered by Wösthoff gas mixing pumps. The oxygen contents at constant P_{CO_2} (7 mmHg) and six specific values of P_{O_2}, corresponding to approximately 10, 30, 50, 70, 90 and 100 % oxygen saturation, were determined and used to construct the oxygen equilibrium curve. The pH of the blood at approximately 50 % saturation was measured with an IL 13 blood gas analyser. Oxygen equilibrium curves were then repeated at a P_{CO_2} of 14 mmHg to allow determination of the Bohr effect.

Statistical analysis

Treatment effects (i.e. three oxygen levels) among larval populations and adult

populations were assessed initially by analysis of variance (ANOVA). Where significant ($P < 0{\cdot}05$) treatment effects existed, differences between specific means were subsequently assessed with Student's t test for independent means.

RESULTS

Haematological changes during metamorphosis in normoxia

Haemoglobin concentration increased about 1·7 times during normoxic metamorphosis (Fig. 1), while mean corpuscular haemoglobin concentration (MCHC) increased 1·2 times (MCHC, in $g\,Hb/RBC \times 10^{-4} = 1{\cdot}70 \pm 1{\cdot}04$ for tadpoles, $1{\cdot}92 \pm 0{\cdot}51$ for adults).

The oxygen-carrying capacity increased approximately 40% during metamorphosis, as would be predicted from the increase in Hb concentration. The Hb-O_2

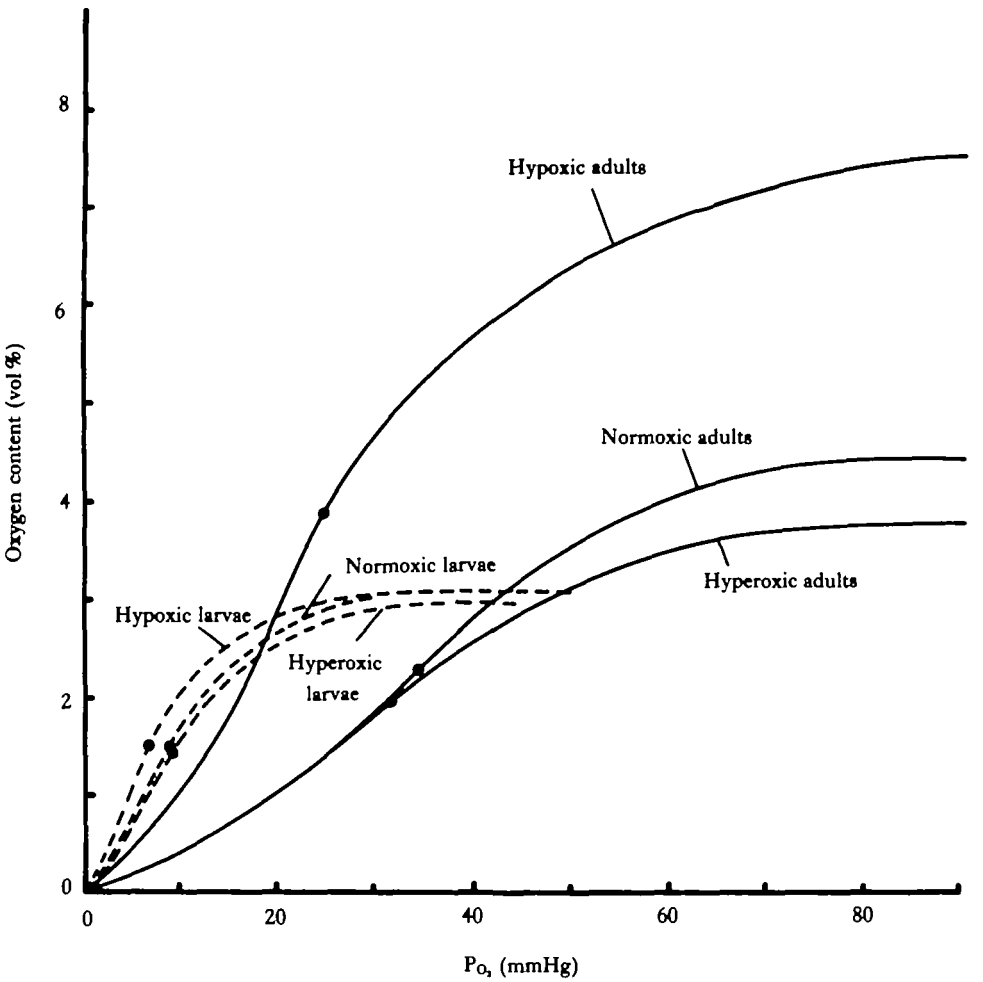

Fig. 1. Representative oxygen equilibrium curves of the whole blood of larval (dashed lines) and adult (solid lines) bullfrogs exposed to 4 weeks of normoxia, hypoxia or hyperoxia. These curves were determined at 23 °C and a P_{CO_2} of 7 mmHg. The black dot on each curve represents the P_{50} value.

affinity decreased greatly, with P_{50} rising from 9·4 mmHg in the tadpole to 33 mmHg in the frog (both values corrected to a plasma pH of 7·7). Metamorphosis had no effect on the cooperativity or Hill coefficient, n, which was $2{\cdot}5 \pm 0{\cdot}5$ ($N = 21$) in tadpoles *vs* $2{\cdot}4 \pm 0{\cdot}5$ ($N = 21$) in adults, nor on the Bohr effect, which was $-0{\cdot}54 \pm 0{\cdot}19$ ($N = 15$) in tadpoles and $0{\cdot}56 \pm 0{\cdot}37$ ($N = 19$) in adults.

The concentrations of intraerythrocytic organic phosphates decreased significantly during metamorphosis, due entirely to the decrease in NTP (chiefly ATP and GTP, Bartlett, 1976, 1980). Since haemoglobin concentration increased during metamorphosis, the molar ratio of phosphate to haemoglobin decreased from 3·6 in the tadpole to 1·3 in the adult. There was no correlation between organic phosphate levels and P_{50} in either tadpoles or frogs, nor was there any significant correlation between the concentration of NTP and DPG in individual samples ($P > 0{\cdot}1$ for correlation coefficient, r).

Effects of hypoxia and hyperoxia on larval and adult blood

Blood properties of adult and larval *R. catesbeiana* exposed to chronic hypoxia, normoxia and hyperoxia are indicated in Fig. 1. In the tadpole, red blood cell count, haematocrit, haemoglobin concentration, mean corpuscular haemoglobin concentration and oxygen-carrying capacity were not significantly affected by environmental P_{O_2}.

In sharp contrast to larval forms, however, adult *R. catesbeiana* showed major adjustments in response to 4 weeks of hypoxia. Highly significant increases occurred in haematocrit, red cell count and haemoglobin concentration, resulting in a near doubling of oxygen-carrying capacity (Figs 1, 2). Mean corpuscular haemoglobin concentration was not significantly affected, indicating that the major response was the production of more red blood cells with comparable haemoglobin concentrations.

Intraerythrocytic phosphate concentrations were significantly lower in hypoxic tadpoles, due to a lower concentration of NTP. There was no significant difference in 2,3-DPG. There were no differences in any measured phosphates between hyperoxic and normoxic tadpoles.

No significant changes in intraerythrocytic phosphate concentrations occurred with decreasing environmental oxygen in any of the frog populations (Fig. 1).

Hyperoxia produced no significant blood changes in tadpoles or adults.

Whole blood O_2 equilibrium curves

Oxygen equilibrium curves for whole blood under constant, physiological conditions of pH and P_{CO_2} from hypoxic, normoxic and hyperoxic tadpoles are indicated in Fig. 2. In tadpoles, whole blood P_{50} was slightly but significantly reduced from a mean of 9·2 mmHg in normoxic animals to 7·0 mmHg after 4 weeks of hypoxic exposure. The Bohr shift and Hill's coefficient of tadpole whole blood was not significantly affected by environmental P_{O_2} ($P > 0{\cdot}10$, ANOVA).

The Hb-O_2 affinity of the whole blood of adult frogs was unaffected by hyperoxic exposure, but hypoxic exposure produced a marked increase in affinity. The P_{50} of hypoxic frogs decreased about 35 % to 24 mmHg from 35 mmHg in normoxic animals (Fig. 2). As with tadpole blood, there were no significant changes in the Bohr shift or Hill's coefficient.

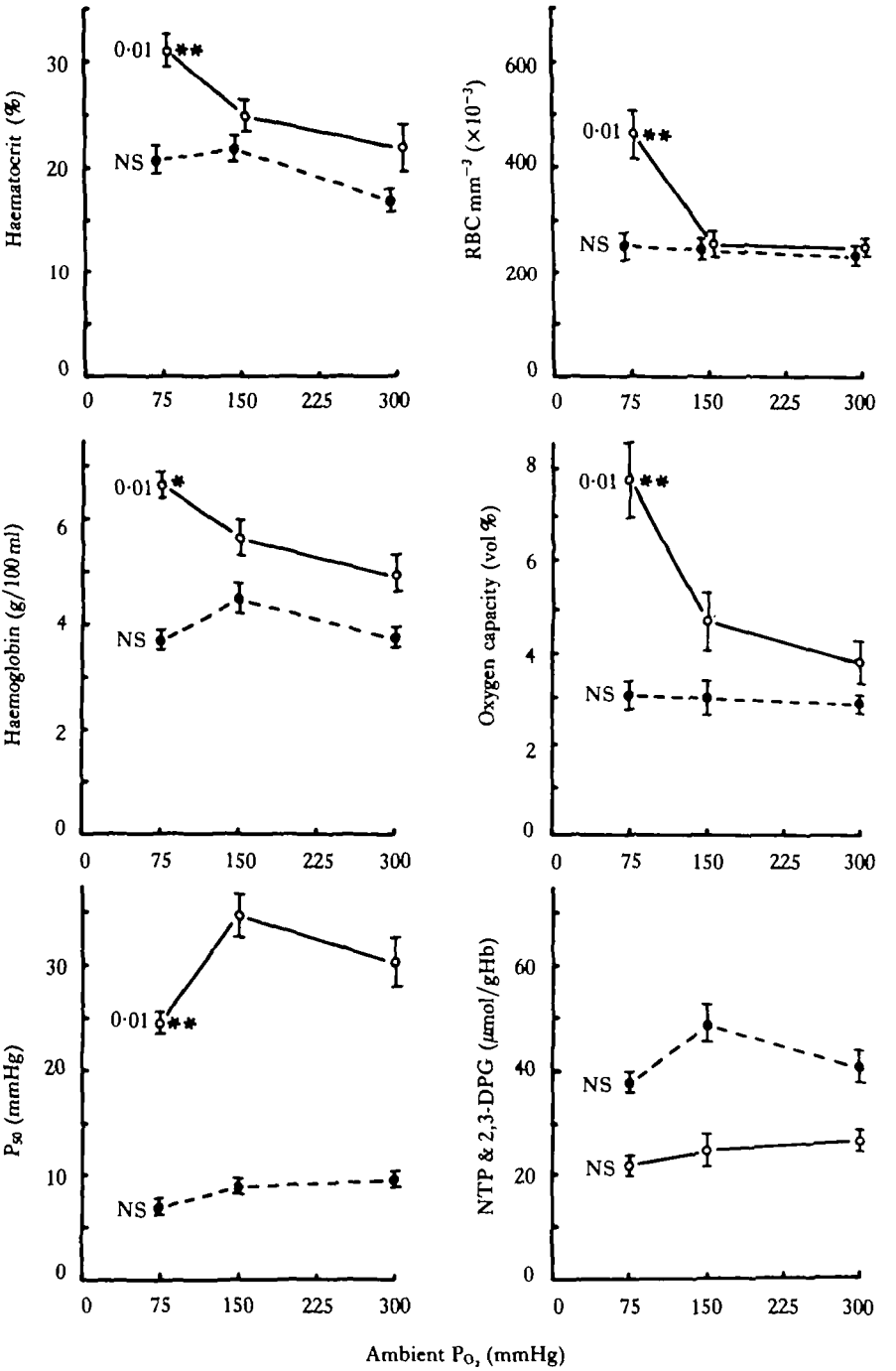

Fig. 2. Relationship between chronic ambient P_{O_2} and whole blood properties of larval (dashed lines) and adult (solid lines) bullfrogs, *Rana catesbeiana*. Mean values ±1S.E. are given. Number of adult frogs contributing to each point are as follows: normoxic, 7; hypoxic, 6; hyperoxic, 13. Number of larvae contributing to each mean are as follows: normoxic, 9; hypoxic, 17; hyperoxic, 7. The letters (NS = not significant) or numbers beside each set of lines refers to the value for *P*, i.e. significance level, for an ANOVA of data groups for the three oxygen levels. Means which are different from the control (normoxic) means are indicated by one ($P < 0{\cdot}05$) or two ($P < {\cdot}01$) asterisks.

Table 1. *Selected properties of whole blood of larval and adult bullfrogs (*Rana catesbeiana*). Appropriate references are indicated in parenthesis*

Variable	Tadpole	Adult
Haematocrit (%)	20·9 (1) 17·8 (6) 19·2 (8)	22·2 (1) 23·4 (7) 27·0 (3) 23·5 (2) 23·8 (6)
Haemoglobin (g/100 ml)	3·73 (1) 2·77 (6) 4·03 (8)	6·3 (1) 5·7 (7) 5·7 (2) 6·6 (6) 6·2 (8)
ATP or NTP (μmol/ml RBC)	7·56 (1) 4·2 (6) 6·02 (4) 5·98 (8) 7·9 (5)	3·56 (1) 3·38 (7) 1·80 (6) 11·0 (5)
2,3-DPG (μmol/ml RBC)	2·20 (1) 4·1 (5) 0·92–2·2 (6) 3·13 (4) 4·91 (8)	2·29 (1) 1·79 (7) 3·3 (5) 1·10 (6)
O_2 capacity (vol %)	2·91 (1)	5·84 (1) 7·15 (7) 9·2 (3) 8·02 (2)
Bohr shift ($\Delta \log P_{50}/\Delta pH$)	−0·60 (1) −0·18 (8)	−0·65 (1) −0·18 (3) −0·29 (2) −0·18 (8)
P_{50} (mmHg)	9·4 (1) 13 (6) 5·5 (8)	33· (1) 37· (6) 37· (7) 42· (3) 39· (2)
Hill's coefficient (n)	2·5 (1) 1·8 (8) 2·8 (9)	2·4 (1) 2·5 (3) 1·95 (8) 2·8–3·0 (9)

References and physiological conditions

1. Present study. (T = 23 °C, P_{CO_2}, 7 mmHg).
2. Lenfant & Johansen, 1967. (T = 22 °C, P_{CO_2}, 10 mmHg).
3. Tazawa, Mochizuki & Piiper, 1979. (T = 25 °C, pH 7·79).
4. Araki, Kazita & Shukuya, 1971. (T = 23 °C, pH 7·0).
5. Bartlett, 1976. (not stated).
6. Hazard & Hutchison, 1978. (T = 25 °C, $P_{CO_2} < 7$ mmHg).
7. Maginnis, Song & Reeves, 1980. (T = 25 °C, P_{CO_2}, 7·83 mmHg).
8. Johansen & Lenfant, 1972. (T = 20 °C, P_{CO_2}, 7·6 mmHg).
9. Riggs, 1951. (T = 20 °C, P_{CO_2}, 7·3–8·4 mmHg).

DISCUSSION

Haematological changes during metamorphosis in normoxia

Our measurements of blood variables in larval and adult bullfrogs under normoxia are generally closely comparable to those reported elsewhere, with the exception of the Bohr effect (Table 1). The Bohr effect measured in this study is the same for both frogs and tadpoles and is quite high compared to that recorded from most Amphibia (Lenfant & Johansen, 1967; Sullivan, 1974). Riggs (1951) and Aggarwal & Riggs (1969) report that tadpole haemoglobin does not exhibit a Bohr effect, but their studies were performed on blood haemolysates diluted in buffers, and Watt & Riggs (1975) report that the Bohr effect measured *in vitro* is dependent on the buffer used. Johansen & Lenfant (1972) report low but significant Bohr effects in the whole blood of both the tadpole and adult bullfrog.

A decrease in haemoglobin oxygen affinity associated with metamorphosis, as observed in this study, is common amongst amphibians (Wood, 1971; Hattingh & Bartels, 1973; Sullivan, 1974; Johansen & Lenfant, 1972; Toews & Macintyre, 1977), though not universal (Burggren & Wood, 1981). Organic phosphates, which are important effectors in changing Hb-O_2 affinity in mammals (Benesch & Benesch, 1967; Chanutin & Churnish, 1967), are also important modulators of amphibian haemoglobins (Aggarwal & Riggs, 1969; Araki, Kajita & Shukuya, 1971; Watt & Riggs, 1975; Wood, Hoyt & Burggren, 1982). However, they were not responsible for the decrease in O_2 affinity during metamorphosis under normoxic conditions in *R. catesbeiana*, since the large decrease in NTP levels during metamorphosis was in the wrong direction to produce the increase in P_{50}. Rather, the change in P_{50} is due to a change from high affinity larval haemoglobin to low affinity adult haemoglobin during metamorphosis in *Rana* (Riggs, 1951; Aggarwal & Riggs, 1969; Watt & Riggs, 1975; Just & Atkinson, 1972; Hazard & Hutchison, 1978). A decrease in organic phosphates with metamorphosis, as presently observed, is not universal amongst amphibians: an increase is observed in *Dicamptodon ensatus* (Wood, 1971) and *Typhlonectes compressicauda* (Garlick *et al.* 1979).

Effects on blood of chronic hypoxia and hyperoxia

Oxygen transfer in larval and adult *R. catesbeiana* maintained under chronic hypoxic conditions can potentially be enhanced by increases in the convective flow of blood and water/air through the gas exchange organs, morphological changes in the structure of the gas exchange organs, and/or adjustments in the oxygen-carrying properties of the blood. Hypoxic-induced increases in gill and lung ventilation and heart rate have been reported for *Rana* tadpoles (West & Burggren, 1982) and the adults of other anurans (see Boutilier & Toews, 1977 for references). However, these observations are during acute, not chronic, hypoxia and it is not known to what extent these increases in convective flow persist with time.

A companion study (Burggren & Mwalukomo, 1983) has shown that profound increases in surface area, lung volume, skin capillarization and decrease in the water/blood diffusion distance in the skin occur in response to chronic hypoxia in the tadpoles of *R. catesbeiana*, but that none of these morphological adjustments enhancing

gas exchange develop in the adult bullfrog. Similarly, the present study indicates major dichotomy between larvae and adults in the haematological responses to chronic hypoxia.

No significant changes in the oxygen-carrying capacity developed in tadpoles, and the left-shift in the O_2 equilibrium curve of whole blood was probably too small to have physiological significance.

The whole blood of adult bullfrogs, however, exhibits the 'classic' responses to chronic hypoxia – a greatly increased oxygen-carrying capacity and a major left-shift in the oxygen equilibrium curve. The left-shift of the oxygen equilibrium curve cannot be ascribed to a decrease in organic phosphates, since there was no significant change in their concentrations. It is possible that some other affinity modulator caused the left-shift, or that the proportions of the various haemoglobins changed toward a predominance of high affinity types. Induction of rapid haematopoeisis by haemorrhage (Meints & Forehand, 1977) results in a higher proportion of 'larval' red blood cells, presumably carrying higher affinity larval haemoglobins. Even in the absence of an increase in convective flow of blood through the lungs and skin of the adult, such adjustments in blood properties should considerably enhance oxygen transport to the tissues during environmental hypoxia.

Hyperoxia had no effect on the oxygen transport properties of either larval or adult blood. As indicated by a lack of morphological responses to hyperoxia (Burggren & Mwalukomo, 1983), a decrease in Hb-O_2 affinity or decrease in blood O_2 capacity in response to hyperoxia would leave an animal poorly acclimated for even transient hypoxia.

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