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EFFECTS OF HYPOXIA AND HYPEROXIA ON OXYGEN-TRANSFER PROPERTIES OF THE BLOOD OF A VIVIPAROUS SNAKE

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Summary

Red cell oxygen affinity, red cell nucleoside triphosphate (NTP) levels and blood oxygen-carrying capacity were determined for male, nonpregnant and pregnant female, and fetal garter snakes *Thamnophis elegans* exposed to hypoxia (5 % oxygen) and hyperoxia (100 % oxygen). Male and nonpregnant female snakes were maintained under these conditions for up to 3 weeks and exhibited an apparent maximal change in oxygen affinity after 14 days of hypoxia and hyperoxia. Red cell NTP levels decreased and oxygen affinity increased with exposure to hypoxia, while exposure to hyperoxia promoted an increase in red cell NTP concentrations and a decrease in red cell oxygen affinity in the males. Hyperoxia-exposed nonpregnant females did not show a significant change in oxygen affinity. After 14 days of hypoxia, the pregnant females showed an increase in red cell oxygen affinity which was associated with a decrease in red cell NTP concentration and in the molar ratio of NTP/hemoglobin relative to normoxic controls. Fourteen days of hyperoxia did not result in a change in oxygen affinity of red cells from the pregnant female, but did promote a slight increase red cell NTP concentrations. The blood parameters of fetuses from females exposed to hypoxia or hyperoxia did not differ from those of normoxic control fetuses. The fetuses of females exposed to hypoxia suffered greater mortality, appeared less developed and had a lower average wet mass than the fetuses of normoxic- and hyperoxic-exposed females. Neither hypoxia nor hyperoxia altered the oxygen-carrying capacity of the blood in any group of snake.

Key words: reptile, *Thamnophis elegans*, fetus, oxygen affinity, garter snake, viviparity.

Introduction

Oxygen transfer from mother to fetus in the garter snake *Thamnophis elegans* appears to be facilitated by the blood of the fetus having a relatively high affinity for oxygen and by a pregnancy-associated reduction in the oxygen affinity of maternal blood (Berner and Ingermann, 1988; Ingermann *et al.* 1991*a*; Ragsdale *et al.* 1993). It is possible that the high oxygen affinity of fetal blood is a consequence of fetal hypoxia and that the pregnancy-associated change in maternal blood is a consequence of increased oxygen availability to the nucleated adult red cell. However, the influence of ambient oxygen levels on the blood oxygen affinity of a snake is not known.

The relatively high oxygen affinity of the fetal red cell in *T. elegans* is not due to a unique fetal hemoglobin; rather it appears to be due to relatively low red cell levels of nucleoside triphosphate (NTP, primarily ATP) (Berner and Ingermann, 1988). Levels of NTP rise and oxygen affinity decreases rapidly in neonatal red cells following birth (Ingermann *et al.* 1991*b*). Consistent with these observations is the possibility that the fetus is in a relatively hypoxic environment which limits red cell oxidative phosphorylation, thereby keeping NTP levels low and oxygen affinity high. A high affinity would facilitate the oxygen loading of fetal blood. Conceivably, upon birth, oxygen availability to these cells increases, oxidative phosphorylation and NTP levels increase, and oxygen affinity decreases. This decrease would enhance oxygen unloading to the tissues. That red cell NTP levels and oxygen affinities may be associated with oxygen availability in the snake is supported by the findings of Ogo *et al.* (1993), which show that the red cells of the viper *Bothrops alternatus* contain functional mitochondria and that uncoupling of oxidative phosphorylation in these cells results in lower ATP levels and a higher affinity for oxygen. An association between oxygen availability and red cell oxygen affinity is also supported by the findings that ambient oxygen levels influence red cell organic phosphate levels in the chick embryo (Ingermann *et al.* 1983) and in numerous fishes (e.g. Greaney and Powers, 1978; Nikinmaa and Soivio, 1982; Weber and Lykkeboe, 1978; Wood and Johansen, 1972; Wood *et al.* 1975).

One test of the putative correlation between the oxygen affinity of fetal blood and oxygen availability *via* oxidative phosphorylation is to expose the pregnant female to altered levels of ambient oxygen. In terms of the fetus, ambient

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hyperoxia might be expected to raise red cell NTP levels and decrease red cell oxygen affinity; hypoxia might produce opposite results, assuming that the fetus can survive subnormal oxygen levels. These expected responses are based, however, on the assumption that oxygen availability to the fetus is influenced by the gas that the mother breathes. At least in mammals, this has been shown in several acute studies. In humans, fetal heart rate slows and the fetus develops acidosis when the mother is acutely exposed to 10 % oxygen (Wood *et al.* 1971). In sheep, fetal arterial P_{O_2} decreases within a few minutes of exposure of the pregnant ewe to 12–13 % oxygen (Kitanaka *et al.* 1989).

The pregnancy-associated approximately 50 % rise in NTP concentrations and the concomitant decrease in oxygen affinity of red cells of the adult garter snake appear to be due, at least in part, to progesterone (Ragsdale *et al.* 1993). In mammals, pregnancy is associated with maternal hyperventilation due, in part, to changes in estrogen and progesterone levels (Bayliss *et al.* 1987; Hannhart *et al.* 1989; Regensteiner *et al.* 1989). Therefore, could the pregnancy-associated rise in red cell NTP levels in *T. elegans* be associated with increased oxygen availability to the maternal red cell? If so, hypoxia would be expected to cause a reduction in red cell NTP levels, perhaps to levels noted in nonpregnant animals or below these levels. Conversely, hyperoxia might prompt a rise in NTP levels in red cells of nonpregnant adults. We have tested these possibilities in the current study. This project examined the relationship between ambient oxygen and red cell organic phosphate levels and oxygen affinity by determining the effects of hypoxia and hyperoxia on the blood characteristics of pregnant and nonpregnant *T. elegans*.

To assess the time course of responses in red cell NTP levels and oxygen affinity to hypoxic and hyperoxic exposures, males and nonpregnant females were exposed to 5, 21 or 100 % oxygen for periods of up to 3 weeks. Further, since changes in ambient oxygen levels are correlated with changes in blood oxygen-carrying capacity in numerous animals (e.g. Pinder and Burggren, 1983; Soivio *et al.* 1980; Wells *et al.* 1989*a*; Wood and Johansen, 1972), this study also examined the oxygen-carrying capacities of blood from adult snakes and fetuses exposed to hypoxia and hyperoxia for 2 weeks.

Materials and methods

Adult garter snakes, *Thamnophis elegans* (Baird and Girard), were collected in Latah County, ID, USA. Sex was determined by probing the hemipenes; the males were housed separately from the females. Females were determined to be pregnant by abdominal palpation. The study used a total of 30 males (approximate mass 30–50 g), 28 nonpregnant females (50–85 g) and 17 pregnant females (80–130 g). The snakes were kept in 40 l terraria with access to an electric light/heat source on a 12 h:12 h L:D photoperiod. Food was given in the form of diced mice every 2 weeks (except during experiments) and water was given *ad libitum*. Snakes were maintained prior to and during experiments at 20 °C.

Snakes were housed for at least 2 weeks in the laboratory prior to testing to ensure that they were feeding. Male snakes were tested throughout the year and nonpregnant females were studied after February to ensure that they were in a nonreproductive condition. Pregnant snakes were all exposed to the different oxygen tensions over the same period in July; fetuses were therefore assumed to be at similar stages of development at the onset of the experiment. Each adult snake was tested only once, and snakes were divided into three experimental groups: hypoxic (5 % oxygen), normoxic (21 % oxygen) and hyperoxic (100 % oxygen). The hypoxic condition of 5 % was chosen on the basis of the findings of Pörtner *et al.* (1991) in which the toad *Bufo marinus* did not appear to be oxygen-stressed at levels above 2.6 % oxygen. Consequently, the present study focused on the effects of 5 % oxygen; an oxygen concentration presumably within the snake's capacity for aerobic respiration. The snakes were exposed to the differing oxygen tensions for various durations to characterize the length of time involved in acclimation to the ambient oxygen level, except for the pregnant females which were only exposed to the differing oxygen tensions for 14 days.

Initial hematological values were determined from approximately 0.4 ml of blood drawn from the hearts of ether-anesthetized male and nonpregnant female snakes, and hematocrit, hemoglobin (Hb) concentration and mean corpuscular hemoglobin concentration (MCHC) were measured. For the determination of hemoglobin concentration, 0.025 ml of the blood was mixed with 1.2 ml of Drabkins solution, frozen and thawed, then centrifuged at 10 000 ***g*** for 2 min to pellet the red cell ghosts. The hemoglobin concentration of the supernatant was determined spectrophotometrically (Ingermann *et al.* 1991*a*). All hemoglobin data refer to tetrameric hemoglobin. MCHC values were determined using the hematocrit values of the blood. For NTP and oxygen affinity determinations, red cells were washed three times by centrifugation (1000 ***g***, 5 min, 4 °C) in saline buffered to pH 7.4 (in mmol l^{-1}: 143 NaCl, 3 KCl, 1.5 $MgCl_2$, 1.5 $CaCl_2$, 20 Tris, pH adjusted with HCl). After the final wash, the cells were resuspended to give a hematocrit of approximately 12 %. Throughout the procedure, all cell suspensions were kept on ice. NTP (=GTP+ATP) concentrations were determined using Sigma Diagnostic Procedure no. 366-UV (Sigma Chemical Co., St Louis, MO, USA). Red cell NTP concentration (in mmol l^{-1}) was calculated as suspension NTP concentration divided by suspension hematocrit.

P_{50} values of the washed red cell suspension at pH 7.4 were determined using the method of Tucker (1967), with a TC500 Tucker cell and model 781 oxygen meter from Strathkelvin Instruments (Glasgow, Scotland). Cells were incubated for 10 min in a waterbath set at 20 °C with partial pressures of oxygen established by mixing nitrogen and compressed air. P_{50} values were determined from Hill plots, each using 6–10 data points corresponding to cells that ranged from 20 to 80 % saturated with oxygen (Hill plots appeared linear over this range).

Animals were placed in the testing chambers 24 h after the initial blood sampling. The snakes were housed two per chamber; four snakes per experiment. These chambers consisted of 4 l glass containers containing water bowls. Two chambers were aligned in series and the gas mixture flowed through the chambers at a rate of approximately 80 ml min^{-1}. Three-way valves were placed prior to the first chamber and between the two chambers, allowing the gas mixture to be analyzed both before and between the chambers. The gas mixture was analyzed periodically with a Servomex 570A Oxygen Analyser (Sybron, Analytical Products Division, Boston, MA, USA) to ensure that the oxygen content of the gas mixture remained constant throughout the procedure. For normoxic and hypoxic runs, nitrogen and compressed air were mixed to the desired percentage oxygen with a FLO-BOX gas mixer (MG Industries, Valley Forge, PA, USA). For the hyperoxic animals, 100 % oxygen was directed first through a floating ball flowmeter set at 80 ml min^{-1}.

After the test period, blood was again drawn as previously described. Hematocrit, blood hemoglobin, MCHC and NTP concentrations, NTP/Hb ratios and P_{50} were determined for each animal.

Initial hematological values were not collected from pregnant snakes. The pregnant females snakes were placed in their respective oxygen environments for 14 days, after which they were stunned by a blow to the head, decapitated and blood collected into microcentrifuge tubes. The female's body was packed under ice slush until fetal removal approximately 20–30 min later. Whole-blood hematocrit and hemoglobin concentration were determined. The remaining maternal blood was centrifuged at 10 000 ***g*** for 1 min and plasma was collected and stored at −80 °C for lactate concentration analyses. The red cells were resuspended, washed and analyzed for red cell NTP concentration, NTP/Hb ratio and P_{50}. Lactate concentrations were determined using Sigma Diagnostics Procedure no. 735 (Sigma Chemical Co.).

Fetal snakes at Zehr stages 35–37 (Zehr, 1962) were surgically removed from the pregnant females used above. (Zehr stage 37 is the last prenatal stage.) The fetus and its membranes were transferred to an ice-cold saline bath to remove the maternal blood. The fetus was then removed from the fetal membranes and transferred to a second ice-cold saline bath. To determine whole-blood values, the fetus was removed from the second saline bath, blotted dry, a small portion of the tail was cut off, and a drop of blood collected onto a sheet of Parafilm dusted with heparin powder. Samples of whole blood were then immediately analyzed as described previously. The blood from the remaining fetuses was pooled by sectioning the fetuses into heparinized saline and the crude cell suspension filtered through glass wool to remove tissue fragments. The suspension was washed three times and the red cell parameters determined as previously described. A representative fetus from each pregnant female was preserved to determine the Zehr stage and fetal wet mass.

Where appropriate, data are presented as means ± 1 S.D. For the tabular data, a paired *t*-test was used to determine significant differences between initial and final values within a group, while a Student's *t*-test was used to compare final values between treatment groups. For data expressed as percentages of initial values, an analysis of variance (ANOVA) was used to determine significant differences between treatment groups and the Student–Newman–Keuls method was used when indicated by ANOVA. Since maximal responses appeared to occur at 14 days, these data were used for statistical analyses.

Results

Oxygen affinity

To obtain nonpregnant adult baseline data and to characterize acclimation time, 14 males snakes were exposed

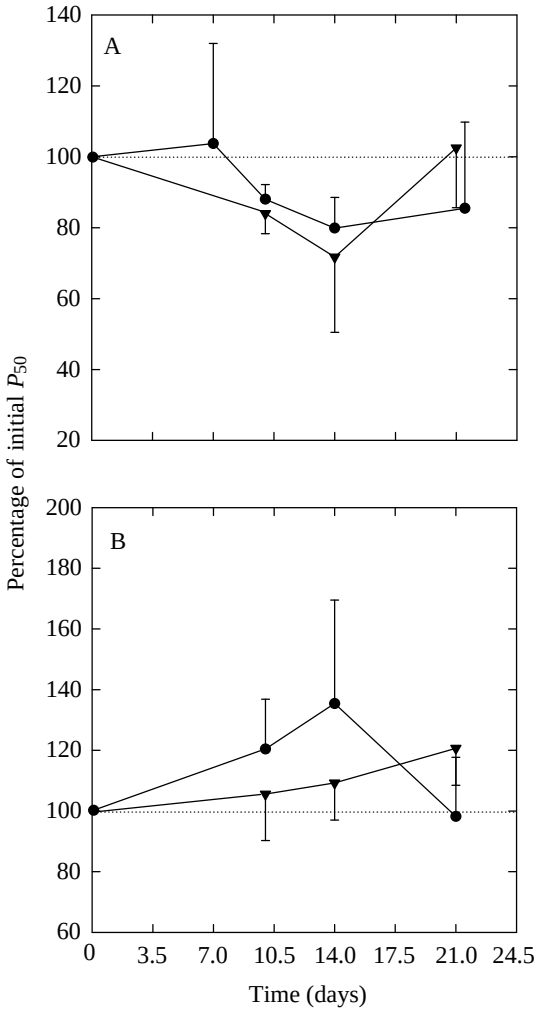

Fig. 1. (A) Percentage of initial red cell P_{50} for both male (●) and nonpregnant female (▼) garter snakes exposed to hypoxia for differing periods. Initial values for males and nonpregnant females were 3.99±0.67 and 5.40±1.31 kPa, respectively. (B) Percentage of initial red cell P_{50} for both male and nonpregnant female garter snakes exposed to hyperoxia for differing periods. Initial values for males and nonpregnant females were 4.22±0.53 and 4.51±0.49 kPa, respectively. N=3 for each group, except at day 14 where N=5. Values are means ± 1 S.D.

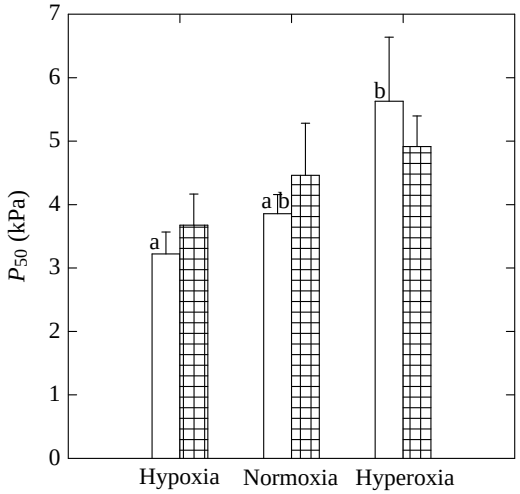

Fig. 2. Red cell P_{50} (in kPa) for both male (open columns) and nonpregnant female (crosshatched columns) garter snakes. Hypoxic, normoxic and hyperoxic groups are compared after 14 days of exposure (N=5 for each group). Like letters indicate a significant difference (P<0.05) between groups (e.g. red cell P_{50} values differ between males exposed for 14 days to hypoxia and normoxia). Values are means + 1 S.D.

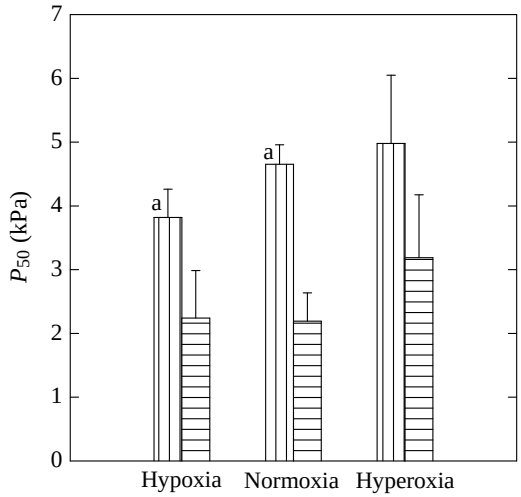

Fig. 3. Red cell P_{50} (in kPa) for both pregnant females (vertical stripes) and fetal (horizontal stripes) garter snakes following 14 days of exposure to hypoxia, normoxia and hyperoxia. Like letters indicate a significant difference (P<0.05) between groups. N=5 for normoxic and N=6 for hypoxic and hyperoxic groups. Values are means + 1 S.D.

to 5 % oxygen and 11 males were exposed to 100 % oxygen for differing periods (Fig. 1A,B). Although no time point had a final P_{50} that differed significantly from that of the respective other non-initial time points, the apparent maximal change in P_{50} appeared to be at day 14 for both groups of snakes. In the 14 day hypoxic group, the P_{50} had decreased significantly from the initial values (Fig. 1A). Although there was no statistically significant change in red cell oxygen affinity for the hyperoxic males when expressed relative to their initial values, there did appear to be an upward trend in red cell P_{50} with such exposure (Fig. 1B). Relative to normoxic controls at day 14, male snakes exposed to hypoxia had a significantly lower P_{50} value and those exposed to hyperoxia had a significantly increased P_{50} value (Fig. 2).

Twenty-two nonpregnant female snakes were also exposed to hypoxia or hyperoxia for various times (11 in each group) (Fig. 1A,B). There was no statistically significant change in the red cell P_{50} for any of the groups at any time point. Red cell P_{50} at 14 days for hypoxic females was not different from that of the normoxic females nor was the percentage change from the initial P_{50} value different (Figs 1A, 2). Nonetheless, trends in these data do suggest a response similar to that seen in the males. Males and females exposed to hypoxia did not differ in their P_{50} values at day 14 (Fig. 2). For hyperoxic nonpregnant females at day 14, the red cell P_{50} value did not differ from that of normoxic females nor did the percentage change from the initial P_{50} value differ between the two groups (Figs 1B, 2). The P_{50} values for males and nonpregnant females did not differ after 14 days of hyperoxic exposure (Fig. 2).

Red cells of pregnant snakes exposed to 5 % oxygen for 14 days had a lower P_{50} than did those of pregnant controls for this time; however, red cell P_{50} of pregnant females exposed to 100 % oxygen for 14 days did not differ from these controls (Fig. 3). Hypoxic exposure of pregnant females did not result in a change in fetal red cell P_{50} relative to normoxic controls (Fig. 3). Although there was also no statistically signficant difference in the red cell P_{50} value of fetuses in the hyperoxic *versus* normoxic groups, a P value of 0.068 suggests a trend towards significance (Fig. 3).

There were no apparent correlations between Hill coefficient and ambient oxygen level. Values of Hill coefficients for 14 days of hypoxia, normoxia and hyperoxia were as follows: male, 1.99±0.24, 2.10±0.23 and 2.06±0.41 (N=5,5,5); nonpregnant female, 3.17±1.15, 2.49±0.52 and 2.41±0.35 (N=5,6,5); pregnant female, 2.69±0.27, 2.53±0.21 and 2.32±0.47 (N=6,5,6); fetus, 1.96±0.78, 2.44±0.64 and 2.61±1.18 (N=5,5,6), respectively.

Red cell NTP concentration and NTP/Hb ratio

The lower red cell P_{50} of males exposed to 14 days of hypoxia relative to normoxic controls (Fig. 2) was associated with a significantly lower red cell NTP concentration (in mmol l^{-1}) and a nonsignificant downward trend in the change from initial concentration values (Fig. 4A,B). In contrast, there were no hypoxia-associated changes in the NTP/Hb ratio or in the change from initial molar ratios in these males (Fig. 4C,D). The significantly higher red cell P_{50} for males incubated for 14 days under hyperoxia relative to normoxic controls (Fig. 2) was associated with significant increases in red cell NTP concentration, percentage change from initial concentration values, final NTP/Hb values and percentage change from initial NTP/Hb values (Fig. 4A–D).

There were no statistically significant differences in red cell P_{50} between nonpregnant females exposed to hypoxia *versus* normoxia and hyperoxia *versus* normoxia for 14 days of

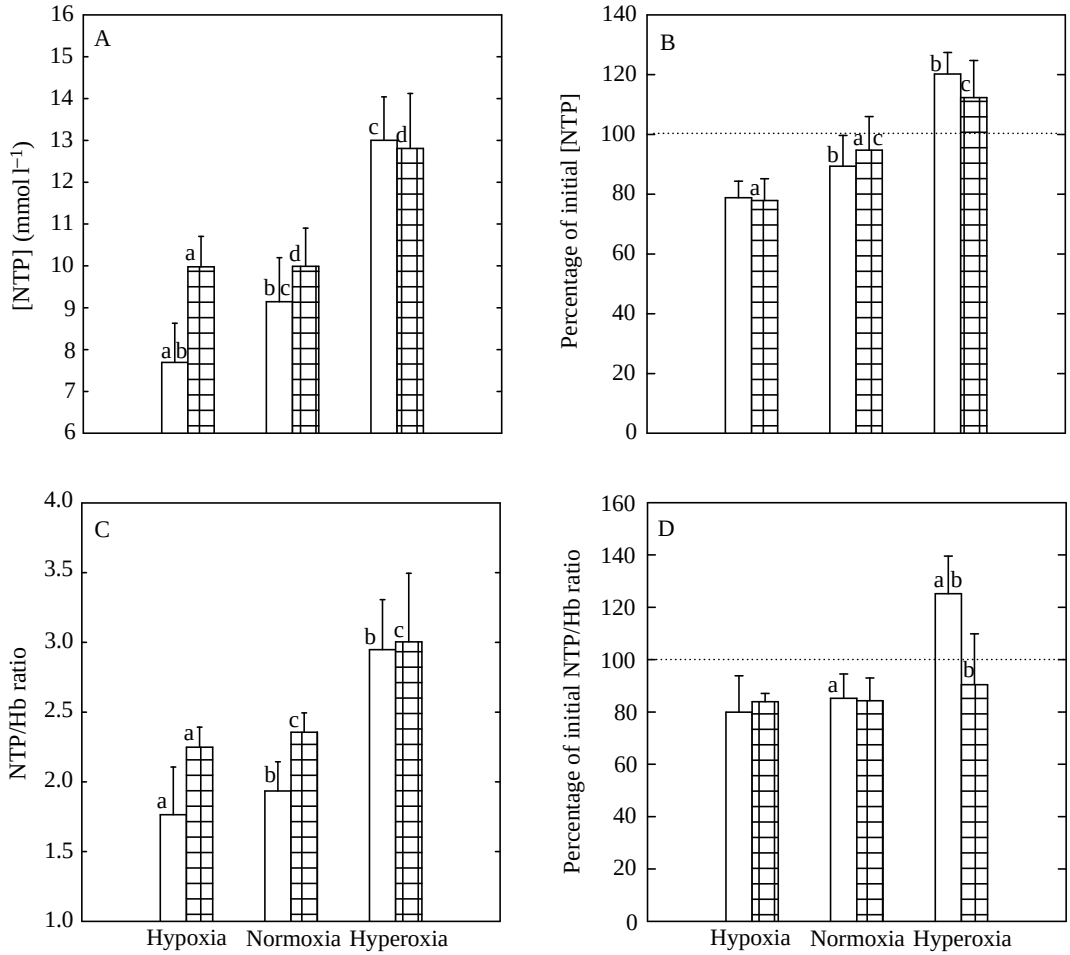

Fig. 4. NTP values after 14 days of exposure to hypoxia, hyperoxia or normoxia (N=5 for each group). (A) Red cell NTP concentrations (mmol l^{-1}) for both male (open columns) and nonpregnant female (crosshatched columns) snakes. (B) NTP concentration as a percentage of initial red cell NTP concentration for both male and nonpregnant female garter snakes. Initial values (mmol l^{-1}) for males exposed to hypoxia, normoxia and hyperoxia were 10.2±1.0, 10.2±1.0 and 10.8±0.6, respectively; initial values for the females were 12.8±0.8, 10.6±0.6 and 11.5±1.6 mmol l^{-1}, respectively. (C) Molar ratio of NTP/Hb for both male and nonpregnant female garter snakes. Hb represents tetrameric hemoglobin. (D) NTP/Hb ratio as a percentage of the initial value for male and nonpregnant female garter snakes. Initial molar ratio values for males exposed to hypoxia, normoxia and hyperoxia were 2.20±0.15, 2.27±0.16 and 2.35±0.16, respectively; initial values for the females were 2.68±0.17, 2.82±0.33 and 3.47±0.99, respectively. Like letters indicate a significant difference (P<0.05) between groups. Values are means + 1 S.D.

exposure. Nonetheless, there did appear to be a trend (similar to the significant change seen in the males) of increasing P_{50} with increasing ambient oxygen level (Fig. 2). This trend was associated with significant differences in the percentage change from initial NTP concentration in the hypoxia- and hyperoxia-exposed females relative to normoxic controls and a significant difference in NTP concentration and NTP/Hb ratio between normoxic and hyperoxic nonpregnant females (Fig. 4A–C). Red cells of male snakes exposed to hyperoxia showed a greater change in red cell NTP/Hb than did those of the females. Nonetheless, in both the males and nonpregnant females, the general trend appeared to be a rise in red cell NTP levels associated with a rise in P_{50} as ambient oxygen level increased.

Hypoxia resulted in a lower red cell P_{50} in pregnant females (Fig. 3). Associated with this change, hypoxia-exposed pregnant females showed lower red cell NTP levels and NTP/Hb ratios than did the normoxic pregnant females (Fig. 5A,B). Plasma lactate concentrations did not differ between these two groups of pregnant females (1.08±0.42 mmol l^{-1}, N=6, and 1.24±1.21 mmol l^{-1}, N=5, respectively). Hyperoxia did not appear to affect red cell P_{50} nor NTP/Hb ratio in the pregnant females (Figs 3, 5B). However, such exposure was associated with a greater red cell NTP concentration relative to the normoxic pregnant females (Fig. 5A). Plasma lactate concentrations did not differ between hyperoxic pregnant females (2.04±1.21 mmol l^{-1}, N=6) and normoxic controls.

The fetuses of pregnant females exposed to either hypoxia or hyperoxia did not differ from the fetuses of pregnant females exposed to normoxia in their red cell NTP concentrations or NTP/Hb values (Fig. 5). This corresponds to the lack of effect of ambient oxygen levels on fetal red cell P_{50} (Fig. 3).

Table 1. *Initial and final (14 days) values for mean corpuscular hemoglobin concentration and hematocrit*

	Hypoxia		Normoxia		Hyperoxia	
	Initial	Final	Initial	Final	Initial	Final
Male						
MCHC (mmol l^{-1})	4.28±0.42	4.31±0.37	4.28±0.41	5.45±1.20	4.28±0.46	4.57±0.39
Hct (%)	25.6±7.2*	21.6±4.5*	25.7±5.3	18.0±5.7	25.8±2.4*	19.2±2.5*
Nonpregnant female						
MCHC (mmol l^{-1})	4.81±0.36	4.43±0.27	3.52±0.78	4.06±0.86	3.47±0.80	4.12±0.49
Hct (%)	22.3±2.9	22.9±2.6	25.6±4.3	20.8±3.2	26.4±3.6**	23.1±2.9**
Pregnant female						
MCHC (mmol l^{-1})	–	5.23±0.94	–	5.57±0.21	–	5.52±0.48
Hct (%)	–	23.8±3.0	–	23.2±4.1	–	23.6±3.6
Fetus						
MCHC (mmol l^{-1})	–	3.83±0.28	–	3.44±0.36	–	4.25±0.30
Hct (%)	–	23.6±2.8	–	25.7±1.6	–	20.7±2.5

*P<0.05 (initial *versus* final); **P<0.01 (initial *versus* final).
MCHC, mean corpuscular hemoglobin concentration (hemoglobin expressed as the tetrameric form); Hct, hematocrit.
Values are means ± 1 S.D.; N=5, except for pregnant female and fetuses for hypoxia and hyperoxia where N=6.

However, the fetuses of hyperoxia-exposed females did have a greater red cell NTP concentration (but not NTP/Hb) than those of hypoxia-exposed females (Fig. 5).

Oxygen-carrying capacity

Exposure of snakes to 14 days of hypoxia or hyperoxia resulted in few significant changes in parameters related to oxygen-carrying capacity (Table 1; Fig. 6A,B). Relative to normoxic controls, there were no differences in blood hemoglobin concentration, MCHC or hematocrit, expressed as final values or as percentage changes from initial values, for males and nonpregnant females. Similarly, there were no differences in these final parameters between normoxic controls and hypoxia- or hyperoxia-exposed pregnant females or their fetuses.

Significant changes were observed in males exposed to hypoxia or hyperoxia for 14 days relative to their initial individual values. In each experiment, blood hemoglobin concentrations decreased significantly (from 1.09±0.33 to 0.94±0.25 mmol l^{-1}, N=5, for hypoxia; from 1.09±0.03 to 0.87±0.11 mmol l^{-1}, N=5, for hyperoxia), as did blood hematocrit (from 25.6±7.2 to 21.6±4.5 %, N=5, for hypoxia; from 25.8±2.4 to 19.2±2.5 %, N=5, for hyperoxia), but in neither experiment was there a significant difference between initial and final values of MCHC (Table 1).

Hyperoxia resulted in a decreased hematocrit (from 26.4±3.6 to 23.1±2.9 %, N=5) in the nonpregnant females relative to their initial individual values. This change was not, however, associated with changes in either blood hemoglobin concentrations (Fig. 6) or MCHC values (Table 1).

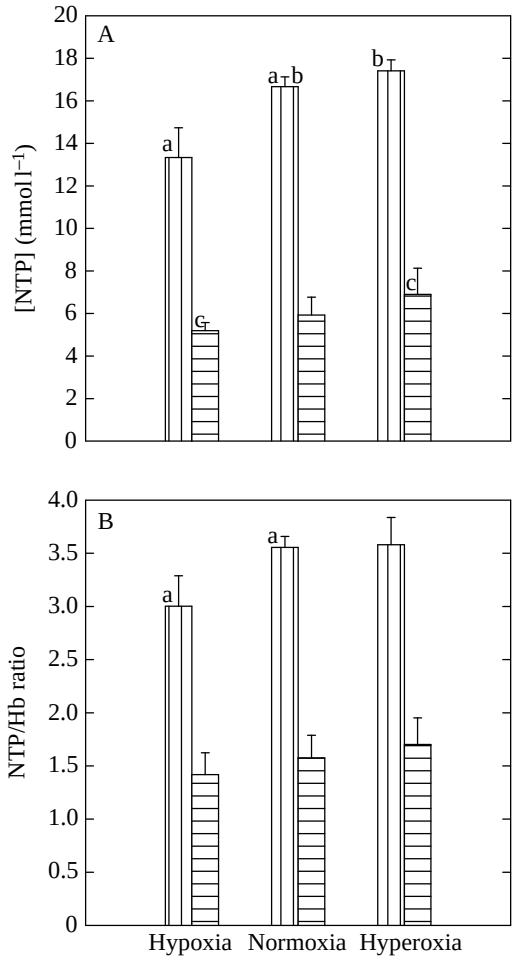

Fig. 5. NTP concentration and NTP/Hb ratio for pregnant females and fetuses after 14 days of exposure to hypoxia, normoxia and hyperoxia. (A) Red cell NTP concentrations (mmol l^{-1}) for pregnant females (vertical stripes) and fetuses (horizontal stripes). (B) NTP/Hb ratio for both pregnant females and fetuses. Like letters indicate a significant difference (P<0.05) between groups. Numbers of individuals are as indicated in Fig. 3. Values are means + 1 S.D.

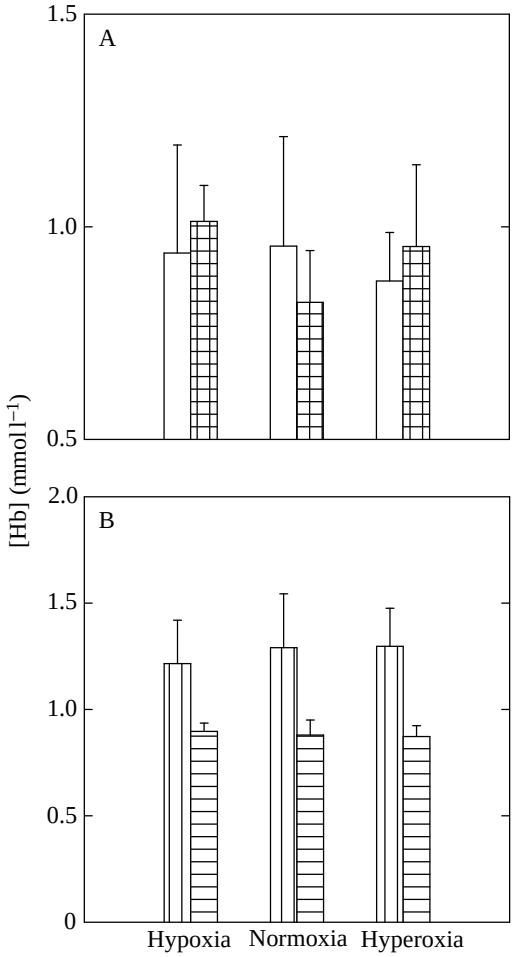

Fig. 6. Blood hemoglobin concentrations (in mmol l^{-1}) following 14 days of exposure to hypoxia, normoxia and hyperoxia. (A) Male (open columns) *versus* nonpregnant female (crosshatched columns) (N=5, each group). (B) Pregnant female (vertical stripes) *versus* fetal (horizontal stripes) snakes (N=5 for normoxic and N=6 for hypoxic and hyperoxic groups). There were no significant differences between the hypoxia *versus* normoxia *versus* hyperoxia groupings. Values are means + 1 S.D.

Mortality and development

During the time course of the study, no adult snakes died in the hypoxia or normoxia experiments; one adult died during hyperoxia-exposure after 2 days.

There was no obvious fetal mortality in pregnant females exposed to normoxia or hyperoxia. Of six pregnant females exposed to hypoxia, two had apparently normal litters. The remaining four pregnant females contained litters with 25–65 % dead fetuses. (Blood was only collected from live fetuses.)

The average wet mass of the live fetuses from hypoxia-exposed females was lower than the average mass of normoxic control fetuses (Fig. 7). The average wet mass of fetuses in the hyperoxic group did not differ from the average mass of the control fetuses but was, however, greater than that of the fetuses from females exposed to hypoxia. The fetuses from

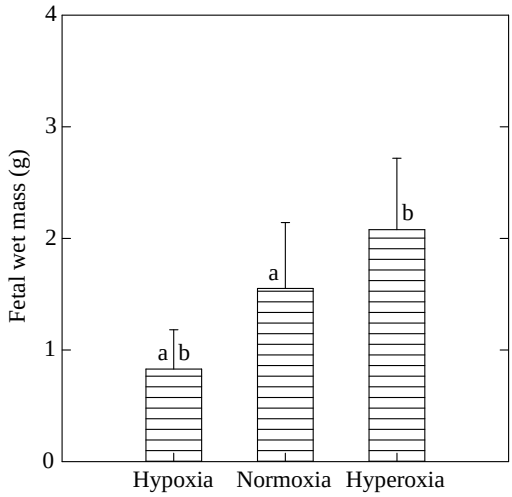

Fig. 7. Average fetal wet mass after 14 days of maternal exposure to hypoxia, normoxia or hyperoxia (N=5 for each group.) Like letters indicate a significant difference (P<0.05) between groups. Values are means + 1 S.D.

hypoxia-exposed females were primarily in Zehr stages 35–36, while the control fetuses ranged from stages 35 to 37 and the fetuses of the hyperoxia group were in stages 36–37.

Discussion

Oxidative phosphorylation is the primary energy source in the nucleated red cells of non-mammalian vertebrates (Bouverot, 1985). Thus, a decrease in oxygen availability may limit oxidative phosphorylation and lead to a decrease in red cell ATP concentrations, while an increase in oxygen availability may accomplish the opposite. Since nucleoside triphosphates are important allosteric regulators of hemoglobin function in the red cells of most ectotherms, oxygen availability may influence red cell oxygen affinity in these organisms. Data from fishes appear to support a link between oxygen availability and red cell function. Fish show a decreased red cell organic phosphate content and an increased red cell oxygen affinity with hypoxic acclimation (e.g. Greaney and Powers, 1978; Soivio *et al.* 1980; Wells *et al.* 1989*a*). Red cells incubated *in vitro* under metabolic inhibition show a similar change (Greaney and Powers, 1978; Tetens and Lykkeboe, 1981), while trout red cells acutely exposed *in vitro* to hyperoxia increase their NTP concentrations (Lane and Dai, 1992). However, not all organisms with nucleated red cells respond as do the fishes to altered oxygen availability. The oxygen affinity of red cells of amphibians does not appear to exhibit a clear pattern of response when animals are chronically exposed to low ambient oxygen levels (Pinder and Burggren, 1983; Wells *et al.* 1989*b*), while red cells of birds appear unresponsive under these conditions (McGrath, 1971; Pionetti and Bouverot, 1977). Whether the nucleated reptilian red cell is responsive to the oxygen availability of organism is not known. However, it may be expected on the basis of the findings of Ogo *et al.* (1993), which demonstrated correlations

between oxidative phosphorylation, intracellular ATP production and hemoglobin oxygen affinity from studies on isolated mitochondria and hemoglobin from *Bothrops alternatus* red cells. Consequently, this study examined the response of the garter snake *T. elegans* to changes in the ambient P_{O_2}.

Blood NTP concentrations of carp and trout respond to changes in water oxygen tension, and full acclimation of the blood requires approximately 1 week or more (Soivio *et al.* 1980; Weber and Lykkeboe, 1978). Therefore, the initial experiments of this study examined the temporal response of the nonpregnant adult snake to changes in ambient oxygen tension. Except for the nonpregnant female exposed to hyperoxia, the garter snake exhibited its apparent maximal change in red cell oxygen affinity after 2 weeks of exposure to the altered oxygen environment. Males and females tended to respond to hypoxia with an increase and to hyperoxia with a decrease in red cell oxygen affinity (Figs 1, 2). Generally, corresponding to the change in oxygen affinity was an apparent change in the red cell NTP concentration (rather than in the molar NTP/Hb ratio) (Fig. 4). Nonetheless, corresponding to what may be considered major changes in oxygen availability were relatively limited responses in organic phosphate levels and oxygen affinity: generally changes of 20 % or less. Therefore, while the snake appeared to respond to low and high ambient oxygen levels in predicted directions, the magnitude of the response was relatively modest. The magnitude of this response to hypoxia in the snake appeared to be intermediate between the slight changes in red cell oxygen affinity observed for the adult toad *Bufo marinus* (Wells *et al.* 1989*b*) or bullfrog *Rana catesbeiana* tadpoles (Pinder and Burggren, 1983) and the 35 % change in red cell oxygen affinity of adult *R. catesbeiana* (Pinder and Burggren, 1983). Further, the magnitude of this response in nonpregnant female *T. elegans* suggests that any hypothetical increase in oxygen availability (for example, due to hyperventilation and/or greater pulmonary perfusion) during pregnancy may contribute to, but cannot account for, the pregnancy-associated rise in red cell NTP levels and decrease in red cell oxygen affinity seen in this species (Ingermann *et al.* 1991*a*).

To test further the possibility that the pregnancy-associated rise in red cell NTP levels is related to changed oxygen availability during pregnancy, this study examined the effect of 14 days of hypoxia or hyperoxia exposure on pregnant *T. elegans*. Pregnant snakes exposed to hypoxia had a higher red cell oxygen affinity than pregnant snakes exposed to normoxia (Fig. 3). Corresponding to this increased oxygen affinity was a lowered red cell NTP concentration and NTP/Hb ratio (Fig. 5A,B). Hypoxic exposure did not reduce the red cell NTP concentration to nonpregnant values, again suggesting, as above, that oxygen availability cannot alone account for the pregnancy-associated rise in the NTP concentration of the adult red cell. Hyperoxia did not induce a significant change in the red cell oxygen affinity in the pregnant female. Nonetheless, the pregnant female after 14 days of hyperoxia did have a slightly elevated red cell NTP concentration relative to that of the normoxic controls, although this elevation was not reflected in an altered NTP/Hb ratio. Therefore, in terms of oxygen affinity and red cell NTP levels, pregnant females appeared to respond similarly to changes in oxygen availability as did nonpregnant adults, especially males. This response, however, does not appear to be of sufficient magnitude to account for the pregnancy-associated rise in red cell NTP levels.

Upon birth, red cell NTP concentrations of the neonate rise and oxygen affinity decreases to nonpregnant adult levels within about 6 h (Ingermann *et al.* 1991*b*). Further, *in vitro* incubation of neonatal red cells with metabolic inhibitors suggests that oxygen availability underlies these rapid red cell transitions at parturition (Ingermann *et al.* 1991*b*). These data further imply that the garter snake fetus is relatively hypoxic prior to birth. In mammals, the oxygen tension that the pregnant female breathes influences oxygen availability to the fetus (Kitanaka *et al.* 1989; Wood *et al.* 1971). If the snake fetus is also sensitive to the oxygen tension breathed by the mother, hyperoxic exposure may reduce putative fetal hypoxia (prompting a rise in red cell NTP level), while maternal hypoxia may exacerbate such hypoxia (perhaps prompting a decrease in NTP level). However, after 14 days of maternal exposure, neither red cell oxygen affinity nor NTP levels of fetal garter snakes had responded to changes in maternal oxygen availability relative to normoxic controls. Although not significant, there did appear to be an upward trend in the fetal P_{50} values with maternal hyperoxia, Fig. 3. The only significant response was between the extremes of 5 % and 100 % oxygen. Fetal garter snakes exposed to these conditions differed in their red cell NTP concentrations (Fig. 5A), although neither value differed from that of the normoxic control. That fetuses not only experienced the altered oxygen environment but were also affected by the lowered oxygen tension available to the mother is supported by the increase in fetal mortality and the decrease in fetal wet mass in the hypoxic group (Fig. 7). A reduction in fetal birth mass with maternal hypoxia has been noted in humans (e.g. Kruger and Arias-Stella, 1970; Lichty *et al.* 1957; Moore *et al.* 1982). These data indicate that the fetuses did experience changes in their oxygen availability, at least during maternal hypoxia, despite a lack of change in red cell oxygen affinity or NTP levels, changes that are associated with birth (Ingermann *et al.* 1991*b*). These observations suggest two principal possibilities. (1) Conceivably, the fetus of the hyperoxic females did not experience an increase in oxygen availability commensurate with that occurring upon birth (e.g. perhaps due to vascular shunting of blood from the fetus). (2) Alternatively, there is no direct link between oxygen availability and red cell NTP levels, i.e. the marked increase in red cell NTP concentrations which occur at birth are not due simply to a sudden increase in oxygen availability associated with the onset of air-breathing; some other or additional stimulus or interaction may be responsible for this change at birth. This latter possibility is supported by the observation that the effect of hypoxia on the red cell NTP levels of intact fish occurs at oxygen tensions well above those required to saturate cytochrome oxidase and can

be mimicked *in vitro* only by incubation of red cells under anoxia (Greaney and Powers, 1978; Tetens and Lykkeboe, 1981). Therefore, as suggested by Jensen *et al.* (1990) and Nikinmaa (1990), the influence of oxygen availability on the NTP concentrations of nucleated red cells may not be due to a direct effect on oxidative phosphorylation but rather involve or require additional input from the organism as a whole. As such, events associated with birth (other than the initiation of air breathing) may allow the red cells of the neonate to respond to increased oxygen levels with a change in red cell NTP level and oxygen affinity.

Hypoxic acclimation in fishes and mammals is associated with an increase in the oxygen-carrying capacity of the blood (e.g. Bouverot, 1985; Soivio *et al.* 1980; Wells *et al.* 1989*a*; Wood and Johansen, 1972). Exposure to hyperoxia in the trout results in a decrease in the blood hemoglobin concentration (Jewett *et al.* 1991; Lane *et al.* 1991). We predicted similar responses in these garter snakes, yet no snakes in the hypoxic, hyperoxic or control groups showed altered oxygen-carrying capacities when compared with initial values. That there were no differences among the test groups and the controls suggests that ambient oxygen levels do not alter hemoglobin/red cell production during the duration of these experiments. The unresponsiveness of the garter snake's oxygen-carrying capacity to low oxygen tension was similar to that reported for *Bufo marinus* (Wells *et al.* 1989*b*) but differed from the response of *Rana catesbeiana*, which increases its oxygen-carrying capacity (Pinder and Burggren, 1983). Like the blood of *R. catesbeiana*, the oxygen-carrying capacity of the blood of the garter snake appeared to be unresponsive to hyperoxia.

The results of the present study of the garter snake indicate that this terrestrial ectotherm generally shows modest changes in red cell oxygen affinity and NTP concentration but not in blood oxygen-carrying capacity in response to hypoxia or hyperoxia. The maximum response of oxygen affinity and NTP concentrations to oxygen availability appears to require several weeks to develop, and changes occur in directions consistent with those noted especially in fishes. Finally, oxygen availability does not appear to be able to account directly for the pregnancy-associated rise in red cell NTP levels, steady-state fetal red cell NTP levels or the rise in NTP levels in the red cells of the neonate upon birth.

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