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OXYGEN TRANSPORT IN MARINE GREEN TURTLE (*CHELONIA MYDAS*) HATCHLINGS: BLOOD VISCOSITY AND CONTROL OF HEMOGLOBIN OXYGEN-AFFINITY

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Summary

Erythrocytes from green turtle hatchlings contain a single embryonic component, unlike those from other cleidoic eggs, in which adult hemoglobin (Hb) constitutes a significant fraction of total Hb at hatching. The functional properties of the isolated and purified green turtle hatchling Hb that distinguish it from adult Hb are a high affinity for oxygen and marked sensitivity to organic phosphate modulators. Hatchling erythrocytes also contain higher concentrations of ATP and 2,3-diphosphoglyceric acid, but their oxygen affinity is indistinguishable from that of adult erythrocytes. Hatchling erythrocyte mean cell volume is approximately half of the adult value, but hematocrit, blood hemoglobin concentration and blood viscosity of hatchlings and adults are similar. Oxygen-carrying capacity in green turtles, unlike that of other diving vertebrates, corresponds with a theoretically derived optimum. The possibility of allosteric control of Hb oxygen-binding in hatchlings may relate not to the challenge of exercise during the dispersal phase but to conditions in the late embryo in the nest.

Introduction

Many vertebrates enter the physical world of the adult incapable of sustained locomotory activity and have erythrocytes containing hemoglobins characteristic of both embryonic and mature phases of development (Brittain and Wells, 1983; Baumann, 1984; Grigg *et al.* 1993). The marine green turtle upon hatching, however, commences a period of immediate and intense physical activity. Preliminary studies addressing the partitioning of aerobic and anaerobic metabolism in this critical stage of development lead us to expect that hemoglobin in the hatchling functions under an acid–base and blood gas regime that differs from that of the adult sea-going turtle (Baldwin *et al.* 1989). The extraordinary ability of adult turtles to withstand prolonged periods of anoxia is well known (Lutz *et al.* 1985), and recent evidence points to both hematological and cardiovascular adjustments of turtle hatchlings to hypoxia (Kam, 1993). Post-hatching activity is supported by a brisk glycolysis resulting in the substantial accumulation of

Key words: green turtle, *Chelonia mydas*, blood, hemoglobin, oxygen affinity, viscosity, development.

plasma lactate, but little is known of the significance of the oxygen-transport properties of blood in meeting aerobic demand. Exercise is a major challenge to the respiratory system and it might also be expected that the blood oxygen-transport system is involved in supporting enhanced aerobic activity during hatchling dispersal.

Heron Island is a coral cay chelonery situated at 151˚55′E, 23˚26′S on the Great Barrier Reef, Australia. Nesting of green [*Chelonia mydas* (L.)] and loggerhead [*Caretta caretta* (L.)] turtles takes place between late October and late March, when eggs are deposited in chambers excavated high on the shore in the vegetation strand. Oxygen levels in the nest may fall to about half ambient levels, and hypercapnic conditions develop shortly before nocturnal hatching at about 9 weeks, when the young turtles dig out from the nest to migrate rapidly across the beach and shallow reef flats to the sea where they mature (Ackerman, 1977). The post-hatching phase is a critical time in the development of marine turtles and only two or three individuals per thousand survive this ordeal (Witham, 1980).

Blood oxygen-affinity in the green turtle is much higher than that of other marine turtles, such as the loggerhead and leatherback, although the reasons for this are unclear (Palomeque *et al.* 1977; Isaacks *et al.* 1978; Lapennas and Lutz, 1982; Lutz and Bentley, 1985; Lutcavage *et al.* 1990). While adult green turtle blood has been viewed in relation to diving activity (Maginniss *et al.* 1980, 1983; Lapennas and Lutz, 1982; Lutz and Lapennas, 1982; Lutz and Bentley, 1985; Lutcavage *et al.* 1990), the ontogeny of oxygen transport in these animals is poorly understood. Isaacks *et al.* (1978) were unable to distinguish between the oxygen binding of erythrocytes in hatchling and adult green turtles, despite the persistence of embryonic Hb at hatching. In the present study, we examine the oxygen-transport system in hatchling turtles and comment on the possible molecular basis for regulation of hemoglobin oxygen-binding by organic phosphates, which precedes the loss of significant allosteric modulation of adult hemoglobin (Bartlett, 1976; Lutz and Lapennas, 1982). In addition, hematological and viscometric properties of blood from adults and hatchlings are compared and discussed in relation to the respiratory demands of exercise.

Materials and methods

Blood sampling

Emerging hatchlings were gathered from nest sites on Heron Island over the 1991 and 1992 summer seasons. Blood samples of 0.15–0.25 ml were taken from the superficial cervical sinus into heparinized syringes fitted with fine (25 gauge) needles and placed on ice. This procedure could be completed in about 20 s with minimal handling and without injuring the hatchlings, which were returned on the next night tide. Adult females were intercepted during their seaward movement after completion of egg laying and nest building, and were also bled from the cervical sinus.

Several hatchlings were permitted to make their seaward dash (approximately 20 min of vigorous exercise on the beach) before blood samples were taken, in order that we might ascertain the possible effects of exercise on hematological variables and red cell

organic phosphate concentrations. These were compared with those of quiescent hatchlings which had been placed for 8 h in a darkened container.

Hematology and erythrocyte nucleotides

Blood was analyzed immediately after collection. Hematocrit values (Hct) were estimated by centrifugation, red cells were counted with an improved Neubauer hemocytometer (RBCC), and hemoglobin concentrations ([Hb]) were measured spectrophotometrically in Drabkin's solution (Dacie and Lewis, 1984). Mean cell hemoglobin concentration (MCHC) was calculated from [Hb]/fractional Hct, mean cell volume (MCV) from fractional Hct/RBCC, and mean cell hemoglobin (MCH) from [Hb]/RBCC. Plasma lactate was assayed in perchloric acid extracts using the Boehringer Mannheim enzymatic ultraviolet test kit. Adenosine triphosphate (ATP) and 2,3-diphosphoglyceric acid (DPG) were immediately assayed in deproteinized extracts of erythrocytes using Sigma enzymatic test kits. Frozen extracts from 100 μl of blood, deproteinized with 1.2 ml of 0.6 mol l^{-1} perchloric acid and neutralized with 5 mol l^{-1} K_2CO_3, were later assayed by reverse-phase HPLC using a 250 mm, 5 μm C-18 column (Lichrosorb RP18 Merck, Darmstadt) and 0.1 mol l^{-1} phosphate buffer (Ryder, 1985). Peaks were identified by reference to the column retention times of nucleotide standards and quantified by peak area integration.

Oxygen binding curves

The components of hemoglobin were characterized in hemolysates by isoelectric focusing under an ampholine gradient of pH 6–8 (Riggs, 1981). Hemolysates from hatchlings or adults were 'stripped' of organic phosphates by Sephadex G-25 gel filtration at 3 °C (Jelkmann and Bauer, 1976) and concentrated by membrane filtration under nitrogen (PM-10, Amicon Corp. Lexington, MA), and then used immediately without freezing. The Hb solutions thus obtained were spectrophotometrically scanned for methemoglobin formation (Benesch *et al.* 1973). Oxygen affinity studies of the resulting hemoglobin solutions were recorded in a Hemox analyzer (TCS Medical Products, Huntingdon Valley, PA) at 25 °C from a final concentration of 1.5 g Hb l^{-1} (equivalent to 0.023 mmol l^{-1} tetramer) in 0.01 mol l^{-1} Bis–Tris or Tris buffers made up to constant 100 mmol l^{-1} [Cl^-]. DPG (pentacyclohexylammonium salt) and inositol hexaphosphate (IHP) modulators were added to Hb solutions at 20 times the molar Hb concentration to test for allosteric effects. Excess was required to compensate for the dissociating conditions of the Hb/organic phosphate complex in dilute solution and resulted in saturation of the effector binding sites. The CO_2 effect was studied using either air or nitrogen containing 5 % CO_2 (P_{CO_2}=38 mmHg; 1 mmHg=0.1333 kPa) in the equilibrium mixture, and pH was measured in a Radiometer BMS2 apparatus.

Oxygen affinity of buffered erythrocyte suspensions was determined using a Benesch-type tonometer (Wells and Weber, 1989) and ionic compositions recommended for the green turtle by Lutz and Lapennas (1982). This iso-pH method was chosen to facilitate comparisons of affinity and the fixed-acid Bohr effect at constant intracellular pH.

Blood viscosity

Viscosity was measured using a cone-plate viscometer with a cone angle of 8° (model LVTD CP/11, Brookfield Engineering Laboratories, USA). Hatchling blood was pooled to obtain the required 0.5 ml samples needed for analysis. Measurements were made at 25°C over a range of shear rates. Further determinations of viscosity were made at a constant shear rate of $90\,s^{-1}$ on centrifugally separated erythrocytes reconstituted in autologous plasma to a range of hematocrit values.

Results

Hematology and erythrocyte organic phosphates

Hemoglobin concentration, hematocrit, and therefore blood oxygen-carrying capacity and MCHC, were similar for both adult and hatchling turtles (Table 1). Erythrocytes, however, are more numerous and therefore smaller in hatchlings. Both DPG and ATP concentrations in hatchling erythrocytes were higher than in adults. When expressed in molar ratios to Hb (assuming a relative molecular mass of 65 000), concentrations of the potential modulators of Hb function correspond to $0.5\,mol\,l^{-1}$ DPG and $1.2\,mol\,l^{-1}$ ATP per mole Hb in hatchlings, decreasing to one-fifth and half, respectively ($0.1\,mol\,l^{-1}$ DPG and $0.6\,mol\,l^{-1}$ ATP), in adult erythrocytes.

Turtle hatchlings show a dramatic increase in oxygen uptake upon handling (Hamsa *et al.* 1986). Nevertheless, the possibility that hematological values and erythrocyte nucleotides might show some lability during intense exercise was dismissed (see Table 2). As expected, the plasma lactate values increased threefold with exercise, but other hematological values and nucleotides remained unaltered ($P>0.05$).

ATP was the main nucleotide component measured by HPLC and the data corresponded well with those from non-specific enzymatic measurements. Small quantities of guanosine triphosphates were present, but neither AMP nor ADP was present in appreciable quantities. The degradation compound of nucleotide metabolism, inosine monophosphate, was present in similar concentrations in both hatchlings and adults (Table 3).

Oxygen equilibria of isolated hemoglobin components

Isoelectric focusing of adult and hatchling lysates revealed single sharply resolved components with isoelectric points, pH_I, of 7.00 and 6.85 respectively. Therefore, beyond gel filtration to remove Hb-bound organic phosphates, additional purification steps were not required for the separation of hemoglobin components. The presence of a single homogeneous band in the newly emerged hatchling suggests its origin in the embryonic phase, and we have therefore designated this component HbE, and that of the adult HbA.

The oxygen affinity coefficient P_{50} and Hill's cooperativity coefficient n at P_{50} are expressed as a function of pH in Fig. 1. 'Stripped' HbA has a low oxygen affinity, with a Bohr factor ($\Delta\log P_{50}/\Delta pH$) of -0.30; there was no significant effect of the cofactors DPG and IHP on affinity. CO_2 had a small effect in decreasing affinity and increasing the cooperativity coefficient n (Fig. 1A). In marked contrast, HbE had an intrinsically higher

Table 1. *Summary of hematological data and blood chemistry in green sea turtles*

	Hct (%)	Hb ($g\,l^{-1}$)	10^6×number of red blood cells per μl	10^{15}×MCV (l)	10^{12}×MCH (g)	MCHC ($g\,l^{-1}$)	[DPG] ($\mu mol\,g^{-1}$ Hb)	[ATP] ($\mu mol\,g^{-1}$ Hb)
Hatchlings	28.1±6.29	76.5±14.9	0.81±0.20	344±46	96±16	275±36	6.82±1.79	17.29±1.82
N	30	28	24	23	23	28	12	16
Adults	26.5±3.7	86.1±9.0	0.36±0.04	746±97	245±20	327±22	1.76±0.73	8.77±0.93
N	3	3	3	3	3	3	3	3

Data are means ± S.D.; *N*, number of turtles.
MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; DPG, 2,3-disphosphoglyceric acid; Hb, hemoglobin.

Table 2. *Hematology and blood chemistry in turtle hatchlings following 20 min of exercise*

	Hct (%)	Hb ($g\,l^{-1}$)	MCHC ($g\,l^{-1}$)	Lactate ($\mu mol\,ml^{-1}$)	ATP ($\mu mol\,g^{-1}$ Hb)
Resting (*N*=6)	29.42±3.74	84.7±10.8	289.9±37.2	2.78±0.93	15.93±1.75
Exercised (*N*=6)	27.08±11.12	69.8±16.1	273.7±49.1	8.25±2.75	17.76±1.34

Data are means ± S.D.; *N*, number of turtles.

Table 3. *HPLC analysis of erythrocyte nucleotides*

	ATP (μmol g^{-1} Hb)	GTP (μmol g^{-1} Hb)	IMP (μmol g^{-1} Hb)
Hatchlings (*N*=5)	19.28±4.95	2.15±0.80	0.69±0.31
Adults (*N*=3)	7.12±0.88	0.43±0.11	0.62±0.48

Data are means ± S.D.; *N*, number of turtles.

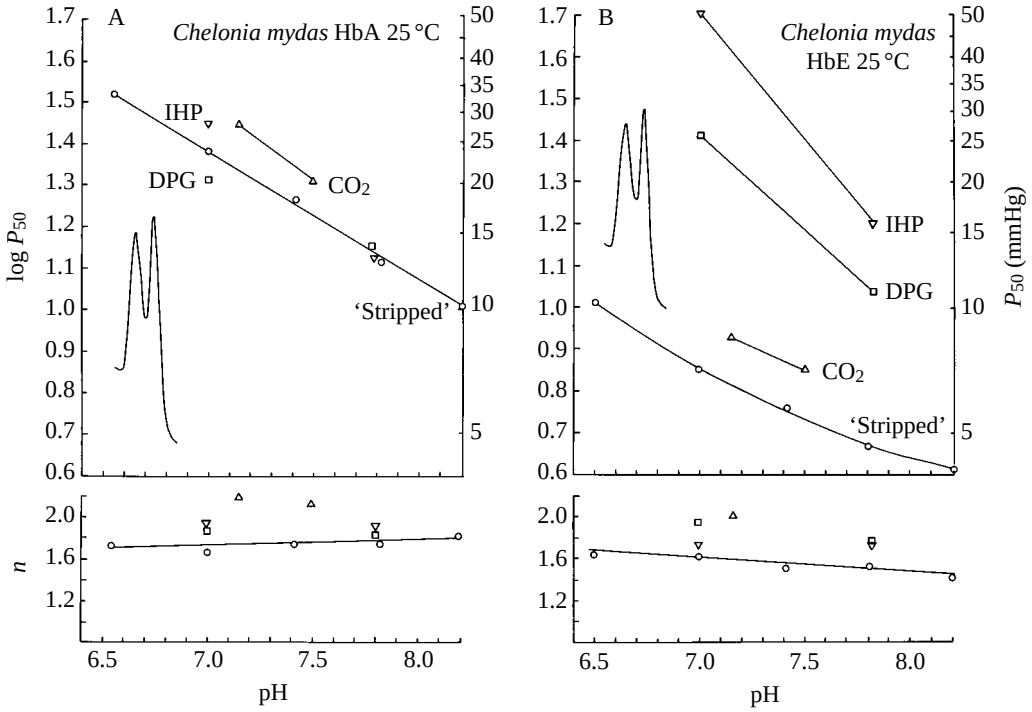

Fig. 1. Effect of pH on the oxygen equilibrium constants P_{50} and n for adult (A) and embryonic (B) turtle hemoglobin 'stripped' of organic phosphates (○) and in the presence of 2,3-diphosphoglyceric acid (□), inositol hexaphosphate (▽) and CO_2 (△). The insets are spectrophotometric scans confirming the absence of oxidized hemoprotein.

affinity for oxygen that was strongly influenced by DPG and IHP. The Bohr factor for HbE was −0.24 between pH 7 and 8, and the cooperativity coefficient n increased at low pH and in the presence of cofactors (Fig. 1B). The affinity of HbE with phosphate binding sites occupied was similar to that of HbA. Both hemoglobins were stable, as indicated by the absence of metHb in spectral scans, with no tendency to auto-oxidize during experiments.

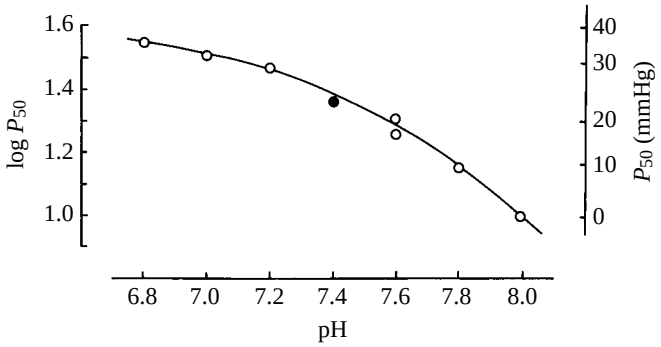

Fig. 2. P_{50} *versus* pH for hatchling erythrocytes at 25 °C (○) compared with a value for adult erythrocytes (●) from Wood *et al.* (1984).

Oxygen affinity of intact erythrocytes

The effect of pH on the oxygen affinity of intact erythrocytes is given in the Bohr plot of Fig. 2, where pH sensitivity is greatest at alkaline pH. The value for Φ, fixed acid, corresponds to −0.55 between pH 7.4 and 7.8. These affinity measurements accord with the data from HbE with cofactors present (Fig. 1B) and with affinity data for adult erythrocytes from Wood *et al.* (1984). (Adult turtles did not come ashore during the time we made hatchling erythrocyte affinity studies, and thus we were unable to repeat previously published experiments.)

Viscosity of whole blood

The dependence of viscosity on shear rate was most marked at low rates (comparable to blood flow) in hatchling blood relative to adult blood, and this may be related to the smaller size of the hatchling cells (Fig. 3.) At constant shear rate, the viscosity of both adult and hatchling bloods reconstituted to known hematocrits showed a rapid rise above approximately 30 % Hct (Fig. 4). Assuming that oxygen transport is proportional to viscosity, then the potential transport capacity may be calculated by the ratio $1.34[\mathrm{Hb}]/\eta$, where η is viscosity at a shear rate of $90\,\mathrm{s}^{-1}$ (Crowell and Smith, 1967). The potential oxygen-transport capacity for reconstituted hematocrits is shown in Fig. 5 and indicates optimal hematocrits close to those observed *in vivo*.

Discussion

Oxygen-transport capacity

The different behaviors of the newly hatched and adult turtles are not reflected in the blood variables measured. The increased size of erythrocytes during post-hatching development has little effect on the oxygen-transport potential of blood, apart from a modest decrease in viscosity at low shear rate and, presumably, reflects a shift in erythropoietic site from embryo to adult as occurs in other vertebrates (Brittain and Wells, 1983; Nikinmaa, 1990).

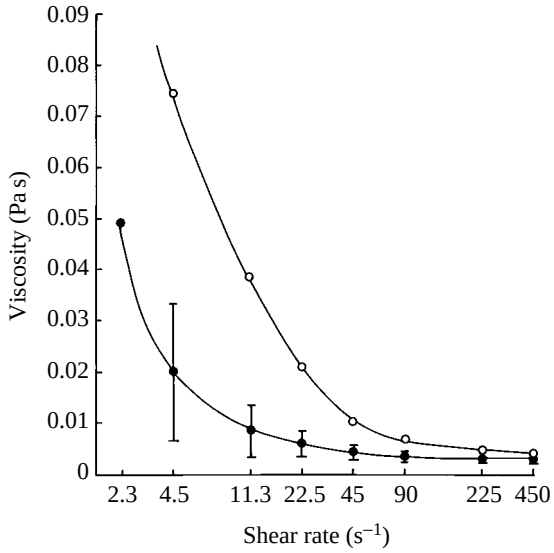

Fig. 3. Viscosity *versus* shear rate for adult (○) and pooled hatchling (●, values are mean ± S.E.M.) blood at 25 °C.

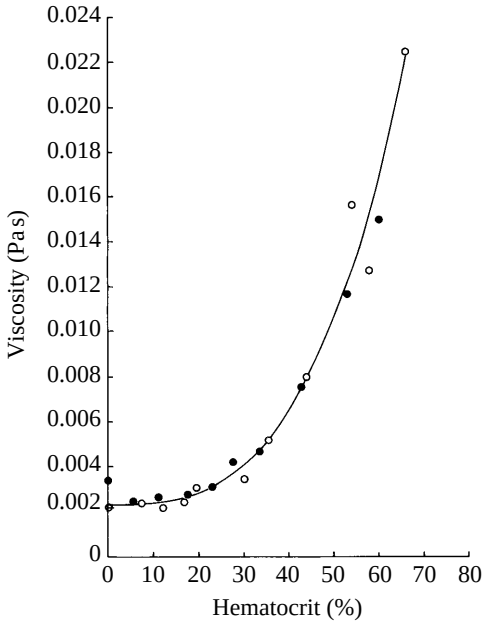

Fig. 4. Viscosity *versus* hematocrit for adult (○) and hatchling (●) erythrocytes at a constant shear rate of 90 s^{-1} and 25 °C.

Lutz and Bentley (1985) questioned whether higher hematocrit in diving turtles would confer an advantage because of the predicted increase in viscosity, and we now confirm that viscosity, and hence effective oxygen transport, are indeed compromised at

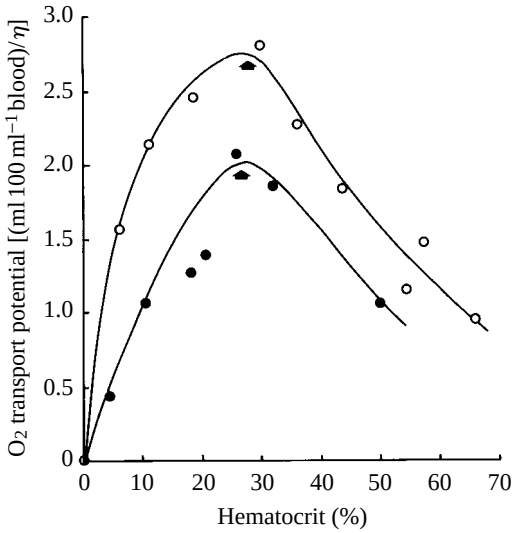

Fig. 5. Optimal hematocrit curves for adult (○) and hatchling (●) erythrocytes with normal resting hematocrits indicated by the arrows.

hematocrits higher than those recorded in green turtles. Other diving vertebrates have high hematocrits and viscosity (Hedrick *et al.* 1986; Wickham *et al.* 1989). Perhaps the peculiar anatomical arrangement, with lungs and ribs anchored to the carapace, and the constraint for the lungs to be operational in buoyancy control are energetically demanding (Milsom, 1989) and may thus favor efficient flow characteristics over high oxygen-carrying capacity. The differing behavioral repertoires of hatchlings and adults are not, therefore, supported by adaptations to transport capacity and rheology of the blood system.

The adult hemoglobin system

Neither DPG nor IHP had a significant allosteric effect on HbA, confirming the work of Isaacks *et al.* (1978) and Giardina *et al.* (1992). Lutz and Lapennas (1982) noted a small allosteric effect at low pH, although this might not be expressed *in vivo* owing to intracellular complexing with magnesium (Bunn *et al.* 1971; Weber and Lykkeboe, 1978). Biphasic *n* values were not seen in our results but are characteristic of other turtle hemoglobins (Reischl *et al.* 1984; Lutz and Bentley, 1985; Brittain, 1991).

Control of oxygen affinity during development

Other reptiles studied include snakes, lizards and crocodiles, in which oxygen-affinity changes during development may be ascribed either to Hb switching (Pough, 1977; Birchard *et al.* 1984; Ragsdale and Ingermann, 1991; Grigg *et al.* 1993) or to changes in red cell organic phosphate concentrations (Grigg and Harlow, 1981). These species emerge with some maternal protection until the locomotory performance of the juvenile

is enhanced. Hb switching also occurs in the late embryonic development of precocial birds (Borgese and Nagel, 1977; Baumann *et al.* 1982).

The value of the fixed acid Bohr coefficient for adult green turtles (Lapennas and Lutz, 1982; Wood *et al.* 1984) is similar to our value for hatchlings. Since an operational Bohr effect requires an arterio-venous pH difference, a high fixed acid content in either hatchling or adult would not enhance oxygen transport during exercise.

The evolution of ontogeny

Haeckel's so-called 'biogenetic law', whereby control features of hemoglobin oxygen-transport in mammals appear to be 'recapitulated' in embryos of primitive forms, has no molecular basis, but a more helpful explanation is that embryos generally live in more constant environments than adults and the same evolutionary driving forces do not come into play in early development. Embryonic crocodiles also contain erythrocyte DPG, but here the modulator appears to play no regulatory role either in reaction with embryonic Hb or when challenged by hypoxia (Grigg *et al.* 1993). Allosteric regulation of turtle embryonic hemoglobin in order to achieve the same oxygen-transport potential as that of the adult seems to be an unnecessary complication. A more economic explanation is the potential for hypoxia in the nest rather than during dispersal. A study of the *in ovum* environment and embryonic erythrocyte properties and information about the time at which adult Hb is synthesized would seem warranted.

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