Allosteric modulation of haemoglobin function in response to changes in oxygen supply and demand: insights from aquatic ectotherms*

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ABSTRACT

Phosphate compounds in the red blood cells of vertebrates play an important role in modulating haemoglobin function whereby tissue oxygenation is maintained despite changes in both environmental oxygen supply and metabolic demand. Three examples from Gordon Grigg's work on aquatic ectotherms illustrate how our thinking has developed in terms of adaptive responses to seasonal temperature changes, environmental hypoxia, and ontogeny.

Key words: Haemoglobin, oxygen transport, fish, crocodile.

*This paper is a tribute to Gordon Grigg's scientific curiosity and kindness which I have enjoyed for 25 years

.Introduction

The circulating red blood cell appears to be a vertebrate innovation and occurs in all classes from Agnatha to Mammalia. It is essentially a package of concentrated haemoglobin in solution whose role it is to transfer oxygen from a gas exchange surface to tissues working at a lower partial pressure of oxygen (PO_2). Here, oxygen may be exchanged for carbon dioxide where it is returned for release back at the exchange surface. What makes haemoglobin attractive to researchers is the diversity of animals and their variable demands for oxygen, coupled with the often uncertain supply of oxygen in many habitats. This leads to numerous questions about matching oxygen supply with demand and how animals adapt to different habitats. With our growing understanding of the ecological significance of physiological diversity, such questions are the basis of ecological physiology.

In the mid-20th century however, fundamental questions engaged researchers of protein structure and function. An explanation of how the haemoglobin molecule might work as a "molecular lung" was proposed by Bragg and Perutz (1952) and Perutz *et al.* (1960), and explained how stereochemical shifts in molecular structure accompanied oxygen binding to the protein, and thus why arterial blood is red and venous blood blue. These, and related studies, led to Perutz's award of the Nobel Prize in Chemistry for providing the first structural model of a protein that allowed functional interpretation.

But these advances in molecular biology did not shed light on the question: "Why have red blood cells?" After all, the invertebrates seemed to manage oxygen transport with haemoglobin dissolved in the plasma. Polymerisation of the haemoglobin protein's subunits in polychaete worms for instance, was thought to be a mechanism for preventing the excretion from the circulation through the nephridia of an otherwise small molecule. Red blood cells might therefore be a device to retain haemoglobin in the vertebrate circulation. Another popular idea at the time was that haemoglobin was packaged into cells to reduce the viscosity of blood. This idea arose from the colligative behaviour of an ideal Newtonian fluid: more particles meant higher viscosity. Blood however, is not a Newtonian fluid and the rheological properties of highly deformable red blood cells did not support the speculation (Schmidt-Nielsen and Taylor 1968).

Questions about seasonal compensation of oxygen transport in a catfish

Around this time, a young Australian researching his doctoral thesis at the University of Oregon, U.S.A., pondered a similar problem from a different perspective. Gordon Grigg acclimated brown bullhead catfishes, *Ictalurus nebulosus*, for at least three weeks under simulated summer (24-25 ºC) and winter (9-10 ºC) conditions in well-aerated water. The summer acclimated fish had consistently lower blood oxygen affinity compared to winter acclimated fish when measured at the same temperature (Grigg 1969). The affinity of blood for oxygen decreases with increasing temperature and allows for greater unloading of oxygen to tissues in response to increased metabolic demand, but at the expense of efficient loading at the gas exchange surface. The adjustments in catfish served to reduce the effect of seasonal temperature changes on blood oxygen transport (Fig. 1). This was the

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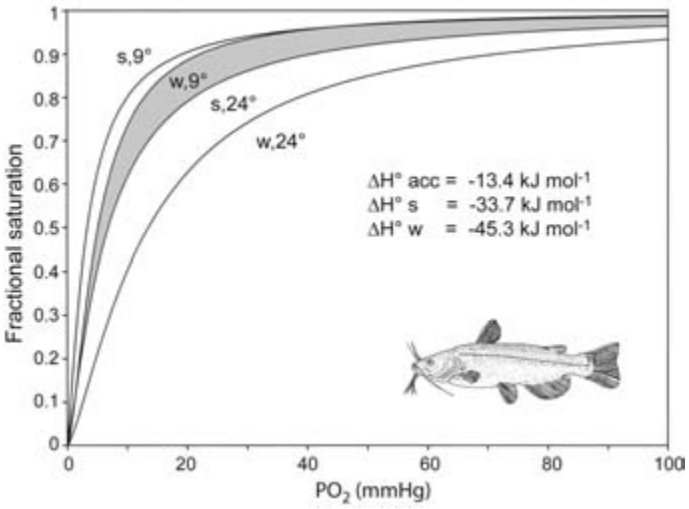

Figure 1. Whole blood oxygen equilibria reconstructed from P50 and *n* values for *I. nebulosus* in Grigg (1969) using: $Y = (K*(X^n))/(1 + (K*(X^n)))$, where $K = 1/P50^n$, P50 is the half-saturation value, and *n* is Hill's coefficient for co-operative oxygen binding derived from the slope of log *X* versus log (*Y*/(1-*Y*)) and *Y* = 0.5, where *X* = PO2 (1 mmHg = 133.3 Pa) and *Y* = fractional saturation. The effect of acclimation temperature on blood-oxygen affinity was quantified by the van't Hoff relationship:

$\Delta H° = -0.0192 ((T1T2)/(T2-T1)).\log(P50^1/P50^2)$ kJ mol^{-1}, where ΔH° = apparent heat of oxygenation, T1,T2 = low, high, measurement temperatures in K, $P50^1$, $P50^2$ = affinity constants at respective temperatures. ΔH° acc = seasonally acclimated; ΔH° s = summer acclimated to 24 °C; ΔH° w = winter acclimated to 9 °C. The shaded area represents the reduction of ΔH° due to acclimation.

first demonstration of seasonal acclimation of oxygen transport in a fish. Grigg further observed that this functional difference disappeared in dilute haemoglobin solutions when the cells were ruptured. In attempting to explain the mechanism behind these observations, he excluded changes in haemoglobin isoforms or acid-base balance, and concluded:

> "…it seems probable that the change in oxygen affinity is effected by a factor in the erythrocyte… the change… is not the result of a change in the nature of the hemoglobin itself. It must therefore result from a change in the level of interaction between the hemoglobin and some other factor…"(Grigg 1969, p. 1221).

He presented his findings in a seminar at the Portland Medical Center, Oregon where he learned that the same conundrum was being considered by medical researchers working with mammalian systems: could oxygen affinity be modified in the red blood cell? Although Grigg had correctly deduced that an erythrocytic factor must be regulating oxygen transport in his acclimated fishes, both the nature of the factor and its mechanism of allosteric binding to haemoglobin were to be the focus of future investigations.

It had been known for some time (and confirmed by Grigg for fish) that lysed red blood cells bound oxygen much more tightly than whole blood. It may have been thought that the haemoglobin became partly degraded when released from its cellular environment. It was also known that human red blood cells contained large quantities of an organic phosphate of unknown function. The phosphate, 2,3-diphosphoglyceric acid (DPG), is a metabolite that is formed "off piste" from the central glycolytic pathway and is present in mammalian red blood cells at approximately equimolar concentration with haemoglobin. When added back to purified haemoglobin solution, it lowered the oxygen affinity of haemoglobin and thus facilitates oxygen unloading (Fig. 2) because it preferentially binds to deoxygenated haemoglobin (Benesch and Benesch 1969). A range of other organic phosphates have subsequently been shown to have analogous effects in non-mammalian animals. The principal compounds in fish are adenosine triphosphate (ATP) and guanosine triphosphate (GTP), and are formed by oxidative phosphorylations within the erythrocyte (Bartlett 1980; Wilhelm *et al.* 1992; Val 2000).

With this breakthrough, the red cell became seen as a micro-environment providing for regulated oxygen transport through changes to the phosphate:haemoglobin ratio, and assumes great significance with respect to opportunities for animals to compensate for environmental hypoxia, and to explain the adaptations of species living in diverse environments. The roles of ATP and GTP in fish have been the focus of numerous studies and have led to the view that they are responsible for adaptive shifts in blood oxygen affinity in response to low ambient oxygen tensions and high environmental temperatures (Wells 1999). Examples are hypoxic eels, *Anguilla anguilla* (Wood and Johansen 1972) and tench, *Tinca tinca,* (Jensen and Weber 1982), and thermally challenged eels, *Anguilla anguilla* (Laurensen *et al.* 1985) and blackfish, *Gadopsis marmoratus* (Dobson and Baldwin 1982).

In the next case study, the idea that acclimatory changes to phosphate:haemoglobin ratios are truly adaptive, comes under close scrutiny.

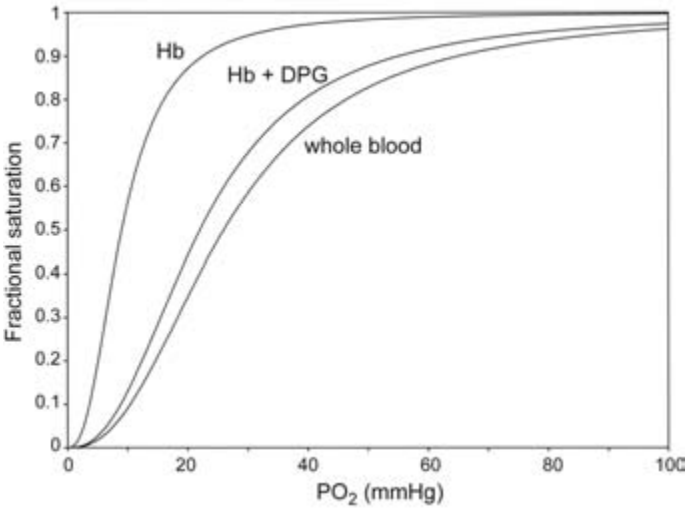

Figure 2. The oxygen equilibrium of purified haemoglobin (Hb) lies to the left of that of whole blood, signifying its higher affinity for oxygen. When DPG is added to purified Hb in saturating amounts (Hb + DPG), the equilibrium is nearly restored to an oxygen affinity close to that of whole blood at 30 °C, pH 7.4.

Questions about hypoxic acclimatory abilities in an Antarctic fish

The shallow, near-freezing coastal waters of the Antarctic continent have been geographically isolated for 20 million years and are now dominated by an unusual endemic fish fauna that evolved by radiation from benthic ancestors into a remarkably diverse species assemblage (Eastman 1993). Among the notothenioid fishes, members of the Channichthyidae are the only vertebrates without haemoglobin or myoglobin, and their hearts circulate a clear, colourless fluid (Fig. 3). Other Antarctic fishes have haemoglobin, although at lower concentration than in temperate species, and without the multiple isoforms typical of warm-adapted species (Wells 2005). These traits appear adaptive in the context of the constant low water temperature of -1.86 ºC in equilibrium with the atmosphere resulting in high concentrations of dissolved oxygen, and yet the metabolic demand for oxygen at this temperature is very low. Icefishes are not very active swimmers and have no need of haemoglobin and can presumably save energy by reducing protein turnover and circulating a low viscosity fluid (Wells *et al*. 1990). It is most unlikely that Antarctic notothenioid fish have been exposed to environmental hypoxia during their speciation.

There are numerous examples of fish and other animals that are regularly exposed to low levels of oxygen and show apparently adaptive responses by reducing the phosphate:hemoglobin ratios to secure oxygen uptake under tighter gradients from gill to tissue (Wells 1999). Grigg and Wells considered these arguments to be somewhat tautologous and proposed to study the responses of Antarctic fishes to environmental hypoxia- a condition that does not occur naturally. While this might seem pointless in an ecological context, the hypothesis that phosphate-haemoglobin interactions are adaptive in warm hypoxic aquatic habitats can be strengthened by testing the falsified hypothesis in Antarctic fishes (see Popper 1972): that these fishes would not show comparable acclimatory responses to hypoxia.

With this approach in mind, Grigg and Wells, accompanied by Lyn Beard undertook a series of experiments on the sea ice in Antarctica during the summer of 1986. A portable laboratory was erected over a 600 mm diameter hole drilled through the 2 m thick sea ice (Fig. 4) about 1 mile south of Scott Base in McMurdo Sound (77º S, 166º E). The semi-pelagic nototheniid species *Pagothenia borchgrevinki* was captured through the ice hole (Fig. 5) and acclimated in aquaria under either normoxic (PO_2 > 150 mmHg) or hypoxic (PO_2 = 60 mmHg) for 11-14 days at -1.5 ºC. Their ability to make marked acclimatory adjustments to oxygen delivery in response to hypoxic exposure was surprising. An increase in oxygen carrying capacity was manifested through raised haemoglobin content, and a left-shifted oxygen binding curve (reduced P50) (Fig. 6A) mediated by reduced ATP:haemoglobin ratios (Fig. 6B), appeared to protect oxygen loading in the gills (Wells *et al*. 1989). We concluded that such adjustments might be a general response to hypoxia reflecting a phenotypic plasticity, rather than a specific adaptation to episodically low environmental oxygen.

Figure 3. The Antarctic icefish, *Chionodraco*, has colourless blood due to the absence of haemoglobin. The semi-pelagic species, *Pagothenia*, has red blood and both species are members of the Notothenioidei fishes endemic to Antarctica.

Figure 4. A portable laboratory with a 7.5 kVA diesel power generator was set up over a fishing hole drilled through the 2 m thick ice cover on the Ross Sea, Antarctica. The hut was heated to prevent the seawater from re-freezing and to allow the researchers to conduct physiological experiments.

Figure 5. Gordon Grigg (far right) looks on as research associate Lyn Beard captures a *Pagothenia* through the hole in the fish laboratory floor.

Questions about blood-oxygen transport during crocodile development

Crocodiles are fascinating not just because of their size and ferocity, but because of their interesting evolutionary history arising from the early Archosaurs, and linking them to ornithischian dinosaurs and birds. They are also interesting because of their reconstructed form and function equipping them for a secondarily aquatic life. Mechanisms of oxygen transport and the role of haemoglobin during breath-holding appear to be unique because of the very low levels of red-cell allosteric modulators and the unique role of bicarbonate in regulating haemoglobin-oxygen affinity (Grigg and Gruca 1979; Grigg and Cairncross 1980). Bicarbonate also increases cooperative oxygen binding in *Crocodylus porosus* (Brittain and Wells 1991). The authors interpreted these unusual effects in terms of a reduction in the fixed-acid Bohr effect that might offset an acidosis resulting from a long dive or prolonged activity.

The amino acid sequence that accounts for these unique properties has been engineered by site-directed mutagenesis into a novel human globin mutant, and proposed as a blood substitute (Frietsch *et al.* 1999). Dubbed "Hb Scuba", the novel protein has been filed under U.S. Patent No. 5942488 with the description: "Disclosed is a mutant human β-globin modified so as to exhibit, when present as part of a hemoglobin molecule, an altered bicarbonate effect compared to the unmodified β-globin."

The unusual mode of allosteric modulation of haemoglobin in the adult crocodile raises questions about haemoglobin function in early embryonic development in the amniotic egg (Fig. 7), not least because of potentially different environmental circumstances faced in the crocodile nest where moisture and biological oxygen demand might reduce ambient oxygen levels. Grigg *et*

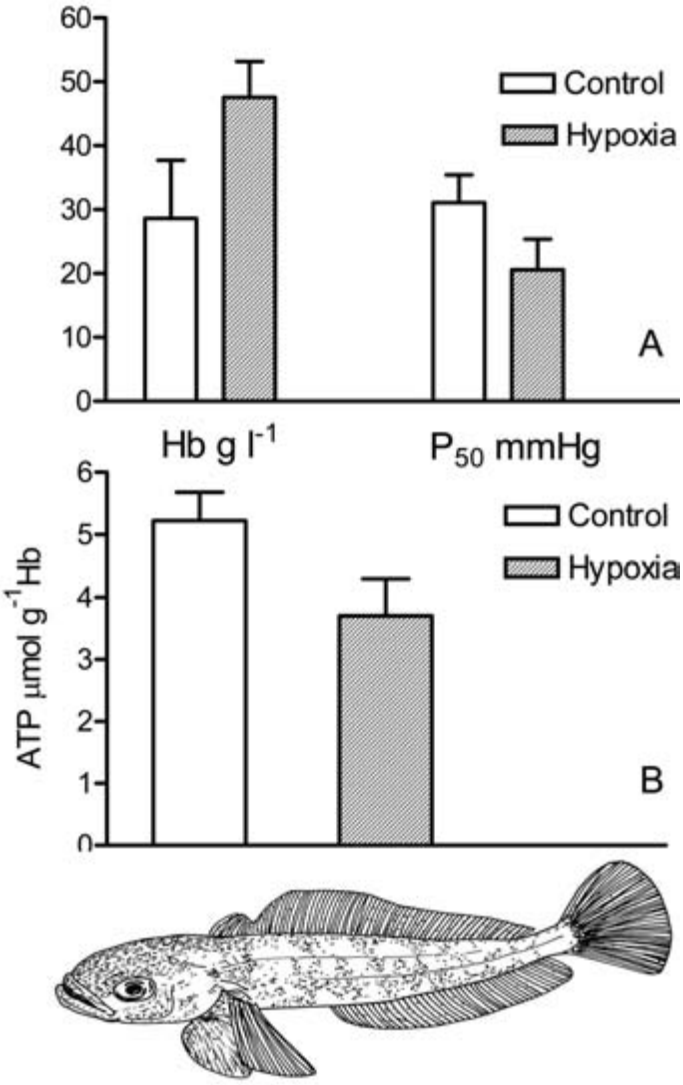

Figure 6. A. Haemoglobin concentration (Hb) and blood oxygen affinity increased (reduced P50) following acclimation of *Pagothenia borchgrevinki* to hypoxia at -1.5 °C. B. The increase in blood oxygen affinity is due to a decrease in erythrocyte ATP. Data are mean values ± S.D. for blood at pH 8.0 - 8.1 and -1.5 °C. (Based on data from Wells *et al.* 1989.)

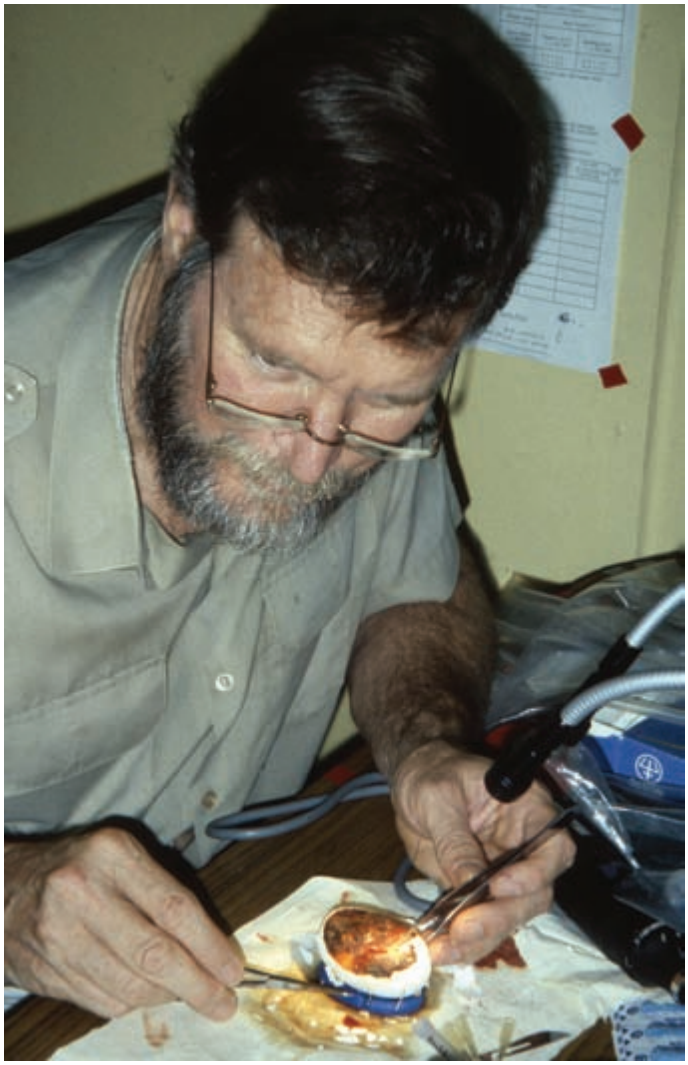

Figure 7. Gordon Grigg carefully dissects a crocodile egg (*Crocodylus porosus*) in order to obtain uncontaminated embryonic blood for physiological experiments.

al. (1993) traced the development of allosteric cofactors in the embryos and juveniles of the estuarine crocodile, *Crocodylus porosus*. A ten-fold decrease in red cell ATP during development of the egg was matched by an increase in blood-oxygen affinity (Fig. 8). These changes were paralleled by the substitution of an embryonic haemoglobin component for the adult haemoglobin (Fig. 9) which instead of binding phosphates, has a specific binding site for bicarbonate ions. The principal cofactors responsible for regulation of blood oxygen affinity in birds, inositol polyphosphates, were not present during crocodile development. Nonetheless, the investigators were surprised to find a pulse of DPG in late embryonic life that did not correlate with altered haemoglobin function. DPG is the principal regulator of oxygen affinity in mammals and is also found in the erythrocytes of avian eggs (Bartlett 1980). An interesting finding was the lack of responsiveness of affinity regulation when crocodile eggs were incubated at reduced ambient oxygen tension. Grigg *et al*. (1993) interpreted this observation as reflecting the orderly progress of development despite environmental perturbation.

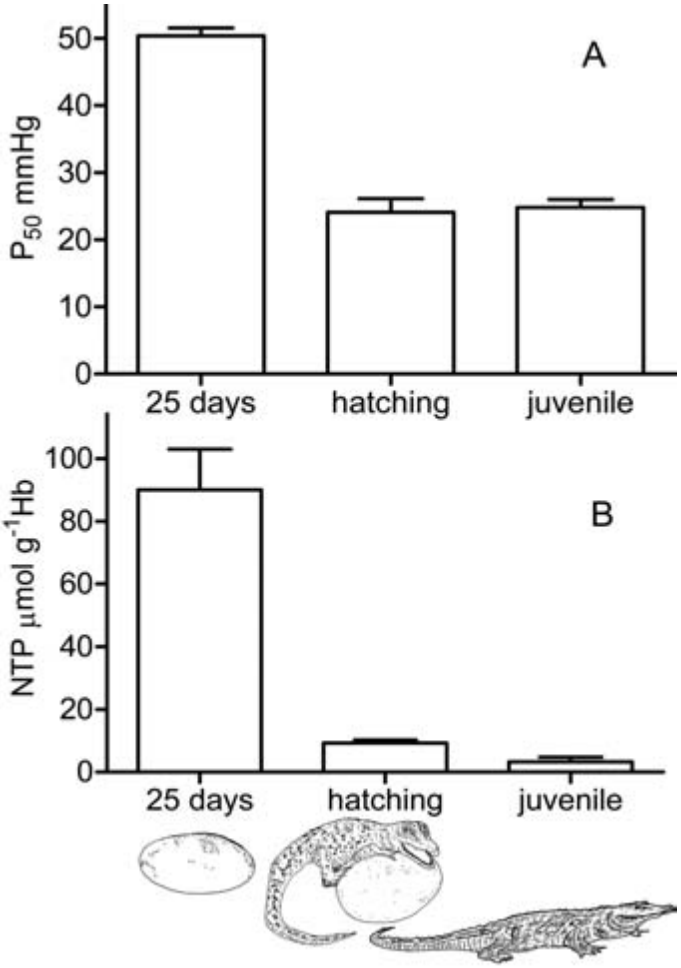

Figure 8. A. Blood oxygen affinity decreases during embryonic development and by the time of hatching at around 86 days, the whole blood oxygen affinity (P50) resembles that of juvenile crocodiles (PCO_2 = 16 mmHg, 30 °C). B. The increase in affinity corresponds with the loss of nucleoside triphosphates (NTP) from the developing erythrocytes. Data are mean values ± S.D. (Based on data from Grigg *et al*. 1993.)

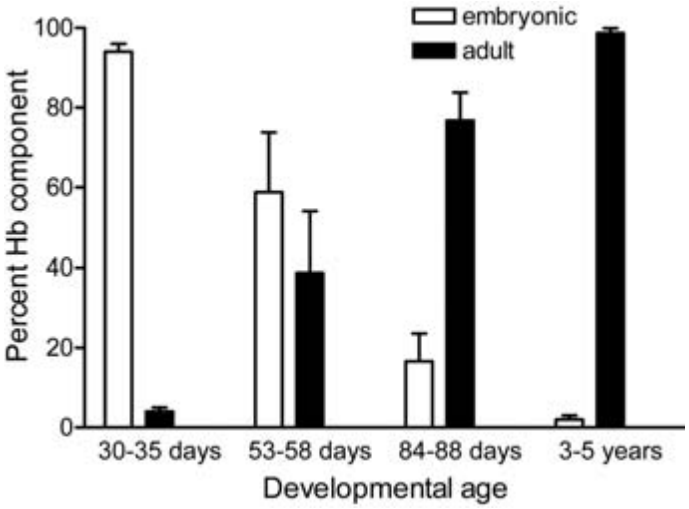

Figure 9. Embryonic haemoglobin is exchanged for adult haemoglobin during crocodile development so that by hatching (84-88) days the haemoglobin is nearly all in the adult form. Data are mean values ± S.D. (Based on data from Grigg *et al*. 1993.)

Concluding insights

The discovery of a range of red cell organic phosphate compounds and bicarbonate as regulators of haemoglobin oxygen transport allowed researchers the opportunity to explain adaptive shifts in oxygen transport efficiency in response to the altered environmental variables of oxygen and temperature. While the example of thermally acclimated catfish suggested an adaptive response directed towards energy conservation during seasonal temperature changes, the Antarctic fish were similarly able to demonstrate competent hypoxia acclimation in the absence of any need to do so. The complexity of the crocodilian blood oxygen transport system has shown the adaptability of a smooth transition from embryonic development to a specialised life as an aquatic breath-holder. Features of the blood in the amniotic egg phase however, did not seem to offer any acclimatory capability.

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