REVIEW PAPER

Molecular adaptations in haemoglobins of notothenioid fishes

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Since haemoglobins of all animal species have the same haem group, differences in their properties, including oxygen affinity, electrophoretic mobility and pH sensitivity, must result from the interaction of the prosthetic group with specific amino-acid residues in the primary structure. For this reason, fish globins have been the subject of extensive studies in recent years, not only for their structural characteristics, but also because they offer the possibility to investigate the evolutionary history of these ancient molecules in marine and freshwater species living in a great variety of environmental conditions. This review summarizes the current knowledge on the structure, function and phylogeny of haemoglobins of notothenioid fishes. On the basis of crystallographic analysis, the evolution of the Root effect is analysed. Adaptation of the oxygen transport system in notothenioids seems to be based on evolutionary changes, involving levels of biological organization higher than the structure of haemoglobin. These include changes in the rate of haemoglobin synthesis or in regulation by allosteric effectors, which affect the amount of oxygen transported in blood. These factors are thought to be more important for short-term response to environmental challenges than previously believed.

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THE ENVIRONMENT AND NOTOTHENIOIDEI

The attention of physiologists and ecologists has long been attracted by environments that lie at the limits of the physical conditions capable of supporting life. In particular, the polar regions demand striking adaptations at the molecular, cellular or whole-organism level to allow organisms to survive, grow and reproduce (Clarke *et al.*, 2007*a*).

Antarctica, more than any other habitat on Earth, offers a unique natural laboratory for fundamental research on the evolutionary processes that shape biological diversity. The variety of adaptations underlying the ability of modern Antarctic fish to survive at the freezing temperatures of the environment represents the extreme of low temperature adaptations among vertebrates.

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Over the past million years, the Antarctic shelf has been subjected to tectonic and oceanographic events that began to alter the composition of the fish fauna and to initiate the process of faunal replacement (Clarke & Crame, 1992). Fragmentation of Gondwana into the modern southern continents and the displacement of the Antarctic continent to its current geographic location have been the most significant events responsible for these changes. The crucial opening of the Drake Passage between southern South America and the Antarctic Peninsula occurred 23·5–32·5 million (M) years before present (M B.P.) (Thomson, 2004) and possibly even as early as 41M B.P. (Scher & Martin, 2006). The Drake Passage lead to the development of the Antarctic Circumpolar Current (ACC) and this in turn was at least partially responsible for cooling of Antarctic waters from *c.* 20° C to the present extreme values near −1·8° C (Clarke, 1983).

The Antarctic Polar Front (APF), the northern boundary of the ACC, is a well-defined, roughly circular oceanic system, running between 50 and 60° S (Fig. 1). Along the APF, the surface layers of the north-moving Antarctic waters sink beneath the less cold and less dense sub-Antarctic waters, generating virtually permanent turbulence. Just north of the APF, the water temperature has an abrupt rise of *c.* 3° C, a critical factor for the isolation and adaptation of the ecosystem. The APF
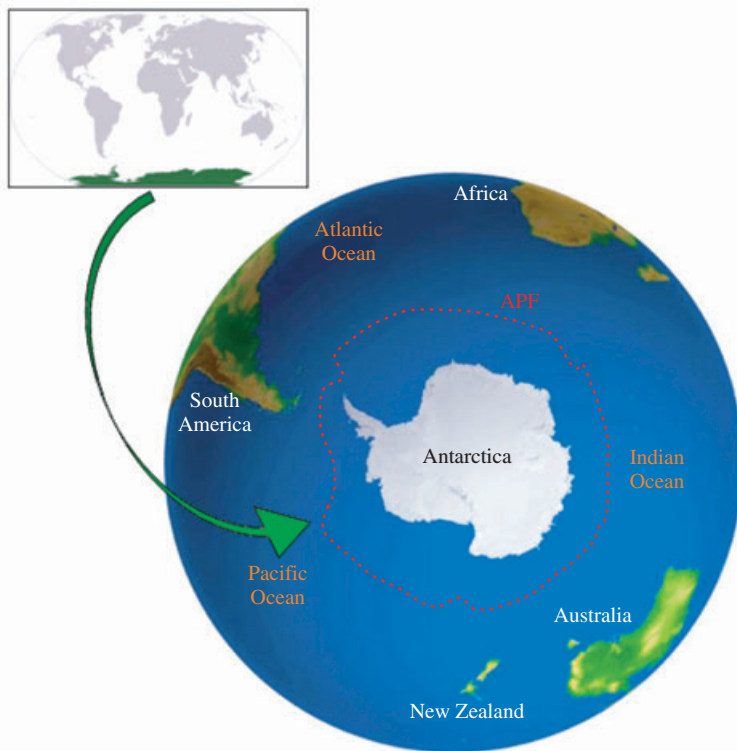

FIG. 1. Geographical location of Antarctica. The continent is surrounded by South America, Africa, Australia and New Zealand. The dashed lines denotes the Antarctic Polar Front, the northern boundary of the Antarctic Circumpolar Current, running between 50 and 60° S.

greatly limited the opportunities for migration, forcing the shallow-water fish fauna to either adapt to the changing climate or become extinct.

One group of teleost fish, the suborder Notothenioidei, became largely dominant as a consequence of success in adapting to the challenging environmental conditions, *e.g.* low temperature, sea ice, habitat reduction and seasonality of primary production (Clarke & Johnston, 1996). The ancestral notothenioid stock probably arose as a sluggish, bottom-dwelling teleost species that evolved some 40–60M B.P. in the shelf waters (temperate at that time) of the Antarctic continent and experienced extensive radiation, dating from the late Eocene, *c*. 24M B.P. (Near, 2004). Convincing arguments provided by Eastman & McCune (2000) have considered notothenioids as one of the very few examples of marine-species flock, due to the geographic, thermal and hydrologic isolation of the Antarctic shelf.

The perciform suborder Notothenioidei is the dominant component of the Southern Ocean fauna. High-Antarctic notothenioids are stenothermal (Eastman, 1993, 2005). They live at the freezing point of sea water (near $-1.9°$ C) and die at temperatures of $4-6°$ C. High-Antarctic notothenioids are distributed south of the APF. Non-Antarctic notothenioids, which comprise sub-Antarctic as well as temperate species, are found north of the APF and compose 22% (28 of 129 species) of notothenioid biodiversity (Eastman, 2005). Notothenioids exhibit considerable morphological and ecological diversity and on the high-latitude shelves they account for 77% of the fish diversity, 92% of abundance and 91% of biomass (Eastman, 2005). This level of dominance by a single taxonomic group is unique among piscine shelf fauna of the world.

Bovichtidae, Pseudaphritidae, Eleginopidae, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae and Channichthyidae are the families of the suborder (Eastman, 2005). All Bovichtidae (except one species), monotypic Pseudaphritidae and Eleginopidae and some species of Nototheniidae inhabit waters north of the APF (Fig. 2). Molecular phylogeny has recently begun to provide indications about the time of radiation in the Antarctic. Initial divergence took place *c*. 40M B.P. during the Eocene (Near, 2004). In fact, Bovichtidae, Pseudaphritidae and Eleginopidae essentially never experienced near-freezing water temperatures because they presumably diverged and became established in waters around areas corresponding to New Zealand, Australia and South America before Antarctica became isolated.
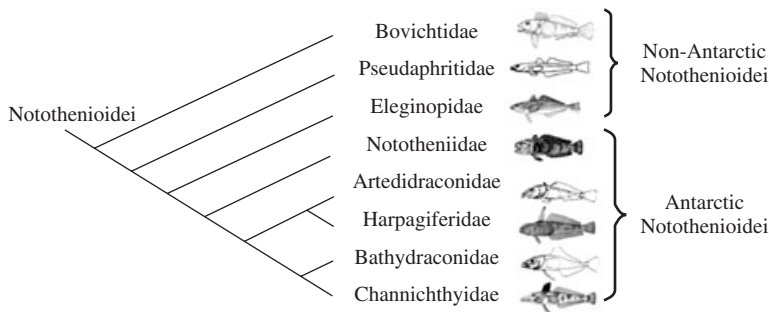

FIG. 2. Families of the suborder Notothenioidei; Bovichtidae (except one species), monotypic Pseudaphritidae and Eleginopidae are non-Antarctic.

The absence of any detectable antifreeze glycoprotein (AFGP) coding sequence, important for enabling survival as the ocean chilled to sub-zero temperatures, in some non-Antarctic species, *i.e. Bovichtus variegatus* Richardson, *Pseudaphritis urvillii* (Valenciennes) and *Eleginops maclovinus* (Cuvier), is consistent with this hypothesis (Cheng *et al.*, 2003). The finding of the genes but lack of AFGPs in another temperate notothenioid, the more recent nototheniid *Notothenia angustata* Hutton (Cheng *et al.*, 2003) supports the hypothesis that this species had developed cold adaptation before migration from the Antarctic continental shelf to temperate latitudes that occurred much later than other non-Antarctic species.

The availability of phylogenetically related notothenioid taxa living in a wide range of latitudes (in the Antarctic, sub-Antarctic and temperate regions) is a tool to potentially detect adaptive characters in response to climate change.

Climate change affects marine, terrestrial and limnetic polar systems. The great constancy of water temperature in the Southern Ocean has supported the evolution of stenothermal animal life, for the sake of an energy efficient mode of life in the cold (Pörtner, 2006). Stenothermal marine species appear particularly vulnerable to even small increases in temperature (Cook *et al.*, 2005; Clarke *et al.*, 2007*b*). Thus, the rate of current changes in relation to the capacity of extant species to acclimate or adapt is a crucial study area for the future management of polar ecosystems.

MOLECULAR ADAPTATIONS IN NOTOTHENIOIDS

In the process of cold adaptation, the evolutionary trend of Antarctic fish has led to unique specializations in many biological features in comparison with temperate and tropical species.

Examples include efficient microtubule assembly at temperatures as low as $-1.9°$ C (Detrich *et al.*, 1989, 2000; Redeker *et al.*, 2004), apparent loss of inducible heat-shock response (Hofmann *et al.*, 2000; Place *et al.*, 2004; Place & Hofmann, 2005), still possessed by some of non-Antarctic notothenioids (Hofmann *et al.*, 2005), enzyme-structural constraints (Fields & Somero, 1998; Russell, 2000; Hochachka & Somero, 2002; Collins *et al.*, 2003; D'Amico *et al.*, 2003; Feller & Gerday, 2003; Fields & Houseman, 2004; Johns & Somero, 2004), decreased membrane fluidity (Römisch *et al.*, 2003), constraints in aerobic energy supply, mitochondrial functioning and the capacity of anaerobic energy production (Johnston *et al.*, 1998; Pörtner, 2006) and higher levels of ubiquitin-conjugated proteins in tissues as evidence for cold denaturation of proteins *in vivo* (Todgham *et al.*, 2007). Recently, the complete loss of the nicotinamide adenine dinucleotide (NADH) 6 dehydrogenase mitochondrial gene was reported for Antarctic notothenioids (Papetti *et al.*, 2007), particularly surprising in the light of the fact that its absence was never reported in any other animal mitochondrial genome.

The biosynthesis of AFGPs is one of the most intriguing evolutionary adaptations discovered in the Antarctic fish. AFGPs allow to avoid freezing by binding water, thus preventing growth of ice crystals in the blood and other body fluids (DeVries, 1988; Cheng & DeVries, 1991). Produced by pancreatic tissue and the anterior portion of the stomach (Cheng *et al.*, 2006), AFGPs are a family of polymers composed of a glycotripeptide monomeric repeat, -Thr-Ala-Ala-, with each Thr linked to the disaccharide galactose-*N*-acetylgalactosamine (DeVries, 1988; Cheng & DeVries, 1991).

High-Antarctic notothenioids have ample gene families for the production of large amounts of AFGPs. In non-freezing environments, where the antifreeze function becomes nonessential, the AFGP function is reduced as observed in non-Antarctic notothenioid fishes. In *N. angustata* and *Notothenia microlepidota* Hutton, living in cool temperate waters, the AFGP system is reduced with very low blood AFGP concentration and only two to three genes showing some replacements in the functional repeat -(Thr-Ala-Ala)- (Cheng *et al.*, 2003). *Dissostichus eleginoides* Smitt, a non-Antarctic notothenioid of the family Nototheniidae, appears to have no functional AFGP sequences, consistent with its non-Antarctic distribution. The apparent absence of AFGP genes in *D. eleginoides*, however, is intriguing because the AFGP gene was thought to have evolved once, before the Antarctic notothenioid radiation, at the base of the family Nototheniidae. The hypothesis is that the species had the primordial AFGP genotype, lost or mutated following its migration to non-Antarctic habitats (Cheng *et al.*, 2003).

The study of freezing avoidance in Notothenioidei is now developing along new perspectives, linked to the recent discovery of AFGP-deficient, but freeze-resistant, notothenioids in early life stages (Cziko *et al.*, 2006). The absence of AFGP production in larvae suggests that suitable freezing resistance may temporarily be afforded by alternative mechanisms.

Specialized haematological features are striking adaptations developed by the Antarctic ichthyofauna during evolution at low temperature. In the seven red-blooded notothenioid families, the erythrocyte number is an order of magnitude lower than in temperate fish and is reduced by over three orders of magnitude in the 16 icefish species of Channichthyidae (Eastman, 1993), the most phyletically derived family, whose blood lacks haemoglobin (Hb) (Ruud, 1954). Icefish retain genomic DNA sequences closely related to the adult α-globin genes of its red-blooded notothenioid ancestors and contemporaries, whereas its ancestral β-globin-gene sequences have been deleted (Cocca *et al.*, 1995; Zhao *et al.*, 1998; di Prisco *et al.*, 2002). The discovery within the icefish family of two distinct genomic re-arrangements, both leading to functional inactivation of the locus, seems to point towards a multistep mutational process (Near *et al.*, 2006).

In channichthyids, no carrier has replaced Hb and the oxygen-carrying capacity of the blood is only 10% that of red-blooded fish. They cope with the lack of an oxygen carrier with increased blood volume and higher cardiac output (Egginton *et al.*, 2002); they have large gills and highly vascularized, scaleless skin, which favours cutaneous respiration. Recent studies highlight how the loss of Hb and their associated nitrogen monoxide–oxygenase activity may have favoured the evolution of these compensatory adjustments (Sidell & O'Brien, 2006). Although there is little doubt about the adaptive value of AFGPs, the evolutionary meaning of the deletion of the entire β-globin gene and parts of the α-globin gene observed in icefish is controversial (Sidell & O'Brien, 2006). It can be argued that such a unique feature is only possible in the oxygen-rich Antarctic waters, whereas it would be deleterious, if not lethal, in warmer waters. This view is in agreement with Near *et al.* (2006), who noted that Hb loss in icefish did not appear to be selectively neutral but is rather maladaptive, as indicated by the development of compensatory adaptations that enhance oxygen delivery, such as cutaneous uptake of oxygen and decreases in metabolic oxygen demand.

The loss of Hb in icefishes is paralleled by the loss of myoglobin (Mb) in six icefish species through at least four mutational events (Sidell *et al.,* 1997; Grove *et al.,* 2004; Sidell & O'Brien, 2006). Despite the costs associated with loss of these haemoproteins, the constantly cold and oxygen-saturated waters of the Southern Ocean provided an environment in which fish are able to survive even without oxygen-binding proteins.

Recent evidence suggests that at least three of the adaptations evolved by icefish, expansion of tissue capillarity density, enlargement of the heart and increases in mitochondrial densities in the heart, may be due to the homeostatic responses mediated by nitrogen monoxide. The loss of Hb, together with enhanced membrane-lipid densities (accompanied by high concentrations of mitochondria), becomes explicable by the exploitation of high oxygen solubility and low metabolic rates in the cold, where an enhanced fraction of oxygen supply occurs through diffusive flux.

Mb has also been lost in many notothenioids, at least in certain tissues. No notothenioid has Mb in its skeletal locomotory muscle, a loss of tissue-specific gene expression that must have occurred early in the notothenioid radiation some 7–15M B.P. (Sidell *et al.*, 1997). Unlike Hb, where a gene has disappeared from the genome, the gene encoding Mb is present in all icefish, even though in some cases the reading frame is disrupted, whereas in other cases the gene is transcribed but the message is not translated into protein (Grove *et al.*, 2004).

Recently, the genome-wide investigations of transcriptional and genomic changes associated with cold adaptation of Antarctic notothenioids have been reported (Chen *et al.*, 2008). Through comparative analysis of same-tissue transcriptome profiles of *Dissostichus mawsoni* Norman and temperate–tropical teleost fishes, Chen *et al.* (2008) identified 177 notothenioid protein families involved in mitigating stresses at freezing temperatures that were expressed many fold over temperate fishes, indicating cold-related upregulation. Further examination of the genomic and evolutionary bases for this upregulation demonstrated that evolution under constant cold has resulted in augmenting gene expression and gene functions contributing to the physiological fitness of Antarctic notothenioids in freezing polar conditions (Chen *et al.*, 2008).

THE HAEMATOLOGY IN NOTOTHENIOIDS

FISH HAEMOGLOBINS

Fish Hbs are particularly interesting because the respiratory function of fish differs from that of mammals. In fish, gills are in contact with a medium endowed with high oxygen tension and low carbon dioxide tension; in contrast, in the alveoli of mammalian lungs, the carbon dioxide tension is higher and the oxygen tension is lower than that in the atmosphere. The capacity of fish to colonize a large variety of habitats appears strictly related to the molecular and functional differences of their Hb system.

As in mammals, fish Hb is a hetero-tetramer having two identical pairs of α- and β-globins (Fig. 3).

The overall affinity of Hb for oxygen is expressed as the gas partial pressure required to achieve half-saturation (p_{50}). The cooperative ligand binding in the Hbs of higher vertebrates, expressed in the molecule because the binding of the
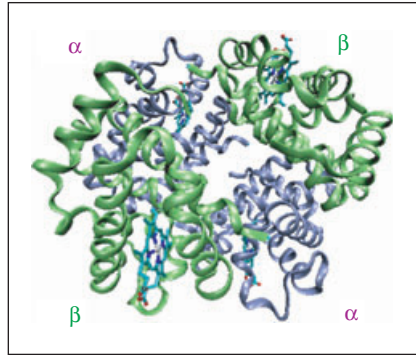
FIG. 3. Three-dimensional structure of emerald rockcod *Trematomus bernacchii* haemoglobin (pdb code 2h8D) (modified from Mazzarella *et al*., 2006*b*).

oxygen with one haem facilitates the binding of additional oxygen to the other haem sites, has important physiological consequences, because it allows oxygen to be efficiently released at relatively high oxygen partial pressure. During evolution, complex and sophisticated molecular mechanisms, such as effects of pH, carbon dioxide, organophosphates and temperature, have been developed to regulate oxygen transport by Hb in higher vertebrates.

Fish commonly exhibit pronounced Hb multiplicity with marked differences in the oxygen-binding properties and in their sensitivities to allosteric effectors, a differentiation that may serve to adapt oxygen transport to environmental variations and metabolic requirements (Weber, 1990; di Prisco & Tamburrini, 1992; Feuerlein & Weber, 1994; Weber *et al*., 2000; Fago *et al*., 2002). Hb multiplicity is usually interpreted as a sign of phylogenetic diversification and molecular adaptation, resulting from gene-related heterogeneity and gene-duplication events.

Red-blooded Antarctic notothenioids differ from temperate and tropical species in having fewer erythrocytes and reduced Hb concentration and multiplicity (none in channichthyids). The Hb content of erythrocytes is variable and in some species seems positively correlated with life style (Eastman, 1993). The vast majority of high-Antarctic notothenioid species have a single Hb (Hb 1), accompanied by minor Hbs (Hb C in trace amounts, and Hb 2, *c*. 5% of the total), having one of the globins in common with Hb 1 (di Prisco, 1998). High-Antarctic notothenioids have lost globin multiplicity, leading to the hypothesis that in the Antarctic thermostable environment the need for multiple Hbs may be reduced (Verde *et al*., 2006*a*, *b*). In comparison with high-Antarctic notothenioids, Hbs of many non-Antarctic notothenioids display higher multiplicity (di Prisco *et al*., 2007). It has been suggested that Hb multiplicity is more frequently found in fish that must cope with variable temperatures, whereas the presence of a single dominant Hb is usually associated with stable temperature conditions. This may explain why high-Antarctic notothenioids have a single major Hb, while sub-Antarctic and temperate notothenioids, such as *Cottoperca gobio* (Günther) and *Bovichtus diacanthus* (Carmichael) (family Bovichtidae) respectively, retained Hb multiplicity, presumably to cope with the small or large temperature changes in the respective habitats north of the APF (di Prisco *et al*., 2007).

Although a report (Sidell & O'Brien, 2006) challenges the ensuing hypothesis, the reduction in Hb content and multiplicity and erythrocyte number in the blood of high-Antarctic notothenioids is likely to counterbalance the potentially negative physiological effects (*i.e.* higher demand of energy needed for circulation) caused by the increase in blood viscosity produced by sub-zero seawater temperature.

The oxygen affinity of Hbs of many high-Antarctic species is quite low (di Prisco *et al.*, 2007), as indicated by the values of p_{50}. This feature is probably linked to the high oxygen concentration in the cold sea. In contrast, the affinity is higher in Hbs of the non-Antarctic notothenioids. The relationship between high affinity of non-Antarctic notothenioid Hbs and habitat features remains an open question as far as its structural basis is concerned. In fact, spectroscopic and modelling studies on Hb 1 of the temperate notothenioid *P. urvillii* have shown that all the non-conservative replacements in the primary structure of the α and β chains leave the conformation and electrostatic field surrounding the haem pocket essentially unmodified with respect to Hb 1 of the high-Antarctic *Trematomus bernacchii* Boulenger (Verde *et al.*, 2004*a*; Mazzarella *et al.*, 2006*b*).

THE ROOT EFFECT

The decreased oxygen affinity of Hb at lower pH values in the physiological range is known as alkaline Bohr effect (reviewed by Riggs, 1988), whose importance is to enable the animal to exchange oxygen and carbon dioxide at both lung or gill and tissue levels. In many Hbs of teleost fishes, the complete loss of cooperativity (indicated by a Hill coefficient equal to one), thus the inability to saturate the ligand sites at low pH even at high oxygen pressure, is a distinctive property with respect to the Bohr effect and is referred to as Root effect (reviewed by Brittain, 1987, 2005). The Root effect is responsible for a physiologically important response to lactic acidosis, which may induce complete oxygen unloading. The physiological significance of Root effect Hbs has been linked to the presence of at least one of two anatomical structures that require high oxygen pressure: the rete mirabile which supplies the gland that inflates the swimbladder with oxygen, and the choroid rete mirabile, a vascular structure that supplies oxygen to the poorly vascularized retina (Wittenberg & Wittenberg, 1974) (Fig. 4). Antarctic fish lack the swimbladder, and it is worth mentioning that only the few species possessing Hbs without a Root
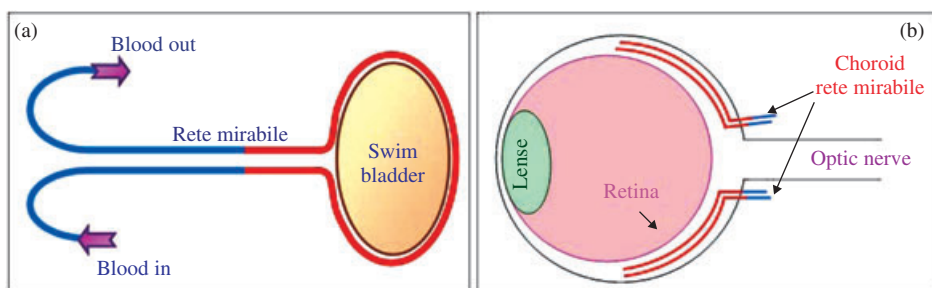

FIG. 4. Swimbladder (a) and choroid (b) retia mirabilia in fish. The retia are part of counter-current exchange systems. The acidic blood is in red, indicating oxygen release due to the Root effect.
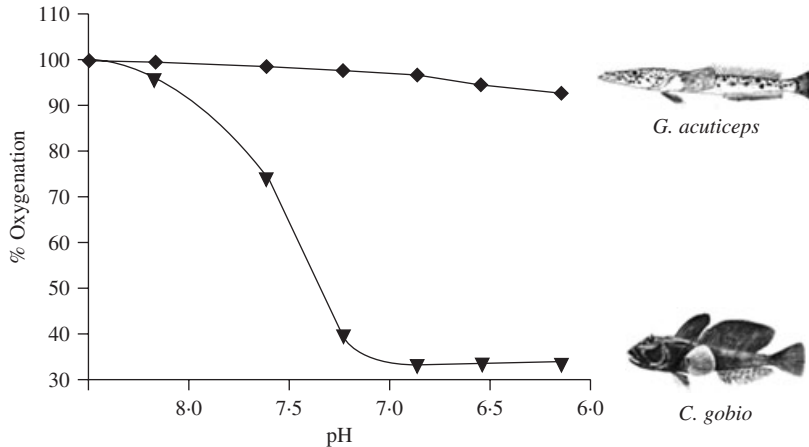
FIG. 5. Root effect of haemoglobins of the sub-Antarctic notothenioid *Cottoperca gobio* (Giordano *et al*., 2009), having the choroid rete, and the high-Antarctic notothenioid *Gymnodraco acuticeps* (Tamburrini *et al*., 1992), lacking the choroid rete.

effect, as well as those of the family Channichthyidae, are devoid of the choroid rete (Eastman, 1988). A general reduction in the Root effect is noticed during the evolution of the Antarctic notothenioids (di Prisco *et al*., 2007) (Fig. 5). Because high-Antarctic notothenioids still have Hbs endowed with Root effect also when the choroid rete is absent, this function may undergo neutral selection. Regarding neutrality, it has been argued that the possession of the Root effect may undergo neutral selection pressure in the simultaneous absence of retia mirabilia and presence of high Hb buffer capacity, as in some basal ray-finned fishes and in the ancestors of teleosts (Berenbrink *et al*., 2005; Berenbrink, 2007). This may generally also be the case in notothenioids with increased Hb surface–His content (Verde *et al*., 2008), because a role for some His residues as modulators of the Root effect has recently been postulated (Mazzarella *et al*., 2006*a*, see below). It remains to be shown to what extent each of these substitutions is the mechanistic cause of the reduced Root effect or the consequence of an altered selection pressure on Hb buffer properties once the Root effect was diminished (Verde *et al*., 2008).

Whatever the answer to the above questions may turn out to be, it seems that the multiple losses of the ocular oxygen-secretion mechanism in notothenioids are not necessarily associated with degenerate eyes or less visually oriented life styles, as an alternative oxygen supply route to the retina by a system of hyaloid capillaries is especially well developed in several notothenioid species that have lost the choroid *rete* (Eastman & Lannoo, 2004; Wujcik *et al*., 2007).

The study of the molecular bases of the Root effect has been tackled by many scientists over many years. Primary structures and analytical methods adopting an evolutionary perspective have provided useful indications on the physiology and evolution of the Root effect in fishes, but no unequivocal answer to the question of the structural implications. In recent years, X-ray crystallography succeeded in overtaking one of the classical views, which attempts to correlate all major changes in Hb function with a few residue substitutions, thus significantly contributing to
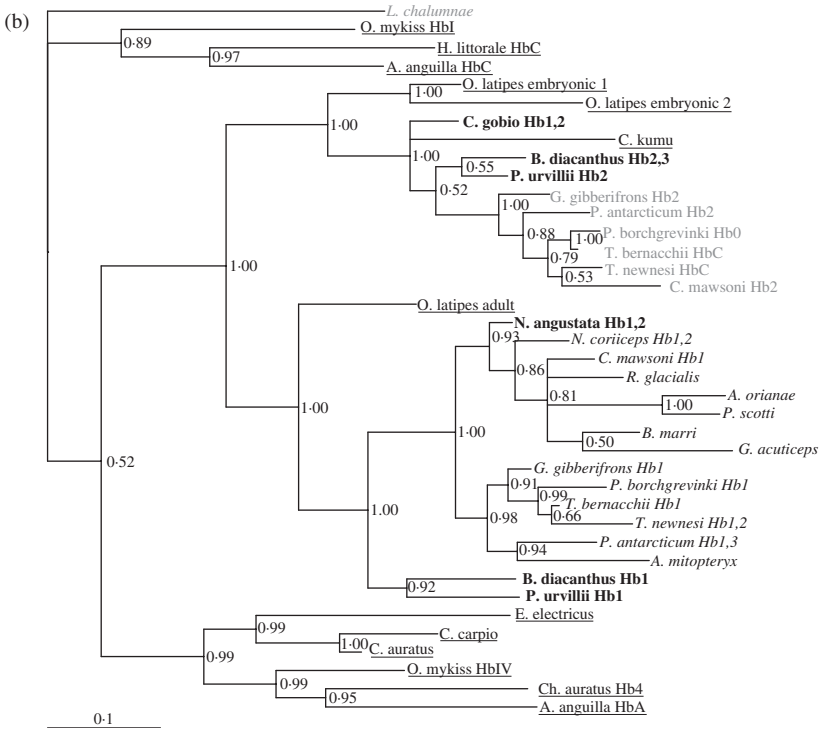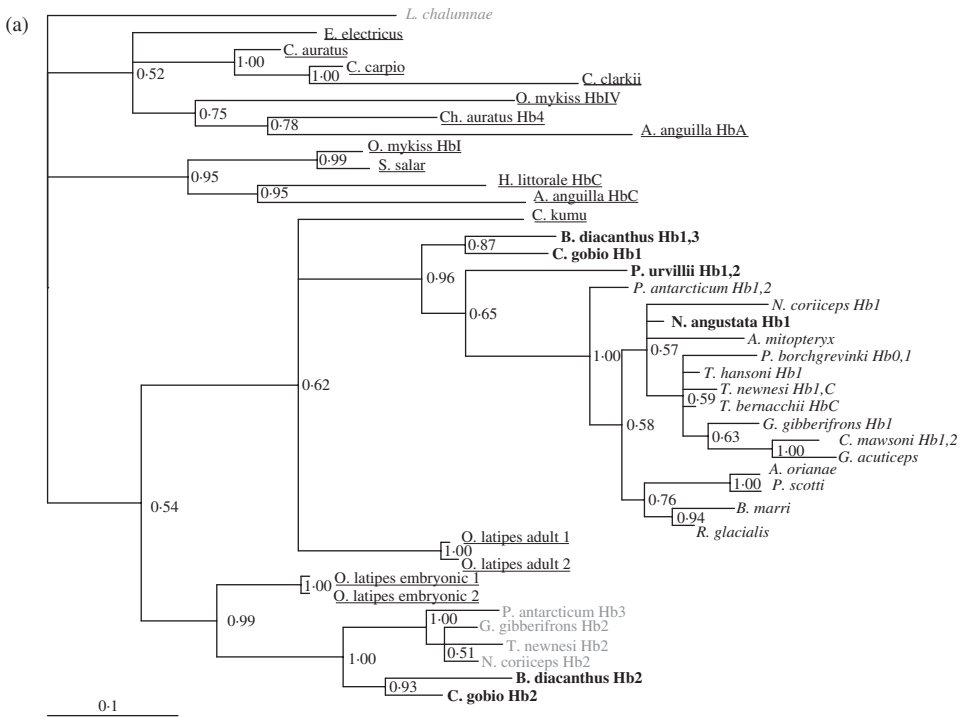
address the question in some instances. The extensive structural analysis of the Root effect by X-ray crystallography of Antarctic fish Hbs was stimulated by the good capacity to crystallize, together with the high sequence identity. These structural properties by themselves, however, are not sufficient to explain the presence of the Root effect. The current hypothesis is based on overstabilization of the T state, mainly induced by the inter-Asp hydrogen bond at the α_1/β_2 interface (Mazzarella *et al*., 2006*a*), possibly modulated by salt bridges involving histidyl residues (Mazzarella *et al*., 2006*b*).

Within the realm of tetrameric Hbs, there is one aspect in which Antarctic fish Hbs are exceptional. Although structurally and functionally analogous to mammalian Hbs, Antarctic fish Hbs follow a peculiar oxidation pathway when exposed to air or treated with chemical agents. The α and β chains of these proteins undergo distinct oxidation processes. Particularly unusual is the strong tendency of the β chains to form hexacoordinated bis-histidyl adducts in the ferric state (hemichrome) (Riccio *et al*., 2002; Vitagliano *et al*., 2004, 2008). Interestingly, the finding that Antarctic fish Hbs may assume states, such as hemichrome typically associated with Hbs with lower complexity, demonstrates that such states are also accessible to tetrameric Hbs. The bis-His co-ordination in the ferrous state, namely haemochrome (Rifkind *et al*., 1994), however, has never been observed. Thus, upon reduction, the hemichrome species is reversibly converted to the classical penta-co-ordinated deoxy form (Vitagliano *et al*., 2004). As yet, there is no clear understanding of the molecular constraints that prevent haemochrome formation in Antarctic fish Hbs. Current work by Mazzarella's team, based on a combined approach involving electron paramagnetic resonance (EPR) and crystallography, suggests a correlation between Root effect and hemichrome stability in Antarctic fish Hbs (Vergara *et al*., 2009).

MOLECULAR EVOLUTION AND PHYLOGENY

Four major α- and β-globin groups have been proposed in teleost fishes (Maruyama *et al*., 2004), *i.e.* Embryonic Hb Group (I), Notothenioid Major Adult Hb Group (II), Anodic Adult Hb Group (III) and Cathodic Adult Hb Group (IV) [Fig. 6(a), (b)] (Maruyama *et al*., 2004). Groups I and IV are still present in *Oryzias latipes* (Temminck & Schlegel) whereas Groups II and III were lost during the evolution of this species. Group I and IV are respectively located on chromosomes 8 and 19.

FIG. 6. Phylogenetic trees of fish α and β-globins (modified from di Prisco *et al*., 2007). The trees for α (a) and β-globins (b) were inferred by a Bayesian method, using the software Mr Bayes with mixed model. Antarctic notothenioid major globins are in italic, Antarctic notothenioid minor globins are in dark grey, non-Antarctic notothenioid globins in bold and temperate globins underlined. Full species names: *Latimeria chalumnae, Electrophorus electricus, Carassius auratus, Cyprinus carpio, Catostomus clarkii, Oncorhynchus mykiss, Chrysophrys auratus, Anguilla anguilla, Salmo salar, Hoplosternum littorale, Chelidonichthys kumu, Oryzias latipes, Notothenia coriiceps, Notothenia angustata, Pleuragramma antarcticum, Pagothenia borchgrevinki, Gobionotothen gibberifrons, Aethotaxis mitopteryx, Trematomus newnesi, Trematomus bernacchii, Cygnodraco mawsoni, Gymnodraco acuticeps, Racovitzia glacialis, Bathydraco marri, Pogonophryne scotti, Artedidraco orianae, Cottoperca gobio, Bovichtus diacanthus, Pseudaphritis urvillii, Eleginops maclovinus.*

According to previous results (Verde *et al.*, 2004*a*, *b*), globin paralogues (*e.g.* gene copies originated by duplication in a given genome) currently found in Antarctic fish diverged *c*. 250M B.P.; hence, unlike AFGP, whose appearance coincided with cooling of the Antarctic continent (Chen *et al.*, 1997), Hb diversification in major and minor groups appears less stringently correlated to changes in the environmental conditions. The time of the gene-duplication event that gave origin to the two paralogous groups of major and minor Hbs is similar, suggesting that they diverged long before the first stock of ancestral notothenioids.

The phylogenetic trees of the amino-acid sequences of *α* and *β* chains of Hbs of non-Antarctic notothenioids (temperate and sub-Antarctic), high-Antarctic notothenioids and temperate fish Hbs, obtained using a Bayesian method, are reported [Fig. 6(a), (b)] (di Prisco *et al.*, 2007). The globins of major and minor Antarctic fish Hbs cluster in two separate, strongly supported groups, with the anodic and cathodic globins of temperate fish Hbs forming the first divergence lineage. The globin-gene trees are in agreement with the species trees obtained by sequence studies on mitochondrial RNA (Ritchie *et al.*, 1996) and give strong support to the monophyly of Antarctic notothenioids, with non-Antarctic *C. gobio* as their sister taxon. The position of the *C. gobio* globins appears congruent with the phylogenetic evidence from nuclear and mitochondrial genes (Bargelloni *et al.*, 2000), suggesting that *C. gobio* is the sister taxon of *P. urvillii*, *E. maclovinus* and also of the Antarctic notothenioids.

The obtained topology is in general agreement with the maximum-likelihood method (Giordano *et al.*, 2006) and the hypothesis of four globin groups (Maruyama *et al.*, 2004). According to this hypothesis, *α*-globins that belong to notothenioid minor Hbs are included in the 'Embryonic Hb Group' and those of the major notothenioid Hbs are grouped into the 'Notothenioid Major Adult Hb Group'. All *β*-globin sequences from the major Hb 1 components (shared by Hb 1 and Hb 2 in most Antarctic notothenioids) belong to the 'Notothenioid Major Adult Hb Group', *β*-globins that belong to notothenioid minor Hbs are included in the 'Embryonic Hb Group', with good bootstrap support.

In the phylogenetic trees, the basal position of *P. urvillii* Hbs appears congruent with the postulated divergence before the appearance of AFGPs. The *α* chain of *P. urvillii*, shared by Hb 1 and Hb 2, branches off the clade of the major Antarctic Hbs, and the same applies to the *β* chain of Hb 1. The *β* chain of *P. urvillii* Hb 2 is in a basal position with respect to the clade of the Antarctic minor Hbs (Verde *et al.,* 2004*a*). The *α* chain of *C. gobio* Hb 1 also branches off the clade of the major Antarctic Hbs, whereas the *β* chain shared by Hb 1 and Hb 2 is included in the clade of the minor Antarctic Hbs (Giordano *et al.*, 2006).

In the majority of notothenioids, embryonic *α* and *β*-globins are expressed in trace or limited amounts in the adult stage, although in at least three species, namely *Trematomus newnesi* (D'Avino *et al.*, 1994), *Pagothenia borchgrevinki* (Boulenger) (Riccio *et al.*, 2000) and *Pleuragramma antarcticum* Boulenger (Tamburrini *et al.*, 1996), embryonic globins are expressed at significant levels (*c*. 25% of the total). In these three species, however, the largest fraction of *β* chains is included in the 'Notothenioid Major Adult Hb Group'. A complete switch to exclusive expression of the embryonic *β*-globin gene seems to be occurred in adult *C. gobio* (Giordano *et al.*, 2006).

CONCLUDING REMARKS

Gene expression patterns and, even more so, loss of genetic information, especially for Mb and Hb in notothenioid fishes, reflect the specialization of Antarctic organisms to a narrow range of low temperatures. These modifications become explicable by the exploitation of high oxygen solubility at low metabolic rates in the cold, where an enhanced fraction of oxygen supply occurs through diffusive oxygen flux (Pörtner *et al*., 2007). Conversely, limited oxygen supply to tissues upon warming is an early cause of functional limitation (Pörtner *et al*., 2007).

The evolutionary development of an alternative physiology based on Hb-free blood may adequately work in the cold for notothenioids in general. Clearly, the benefits due to Hb loss include reduced costs for protein synthesis. As pointed out by Pörtner *et al*. (2007), however, the shift from Hb-mediated oxygen transport to mechanisms based on diffusion may account for higher vulnerability of icefishes and of notothenioids in general, to warmer temperatures.

The southern polar environment is experiencing significant climatic change, as shown by sea-ice reductions on the western side of the Antarctic Peninsula (Clarke *et al*., 2007*b*). Species such as Antarctic notothenioid fishes that have had a long evolutionary history at constant temperatures may be uniquely vulnerable to Global Warming (Somero, 2005).

Recently, Chen *et al*. (2008) have reported genome-wide studies of transcriptional and genomic changes associated with cold adaptation in Antarctic notothenioid fish. Their results strongly suggest that evolution in the cold has produced dramatic genomic expansions and upregulations of specific protein gene families. Many of these upregulated genes are involved in the function of antioxidants, suggesting that augmented capacities in antioxidative defence are likely to be important components in evolutionary adaptations in cold and oxygen-rich environment.

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