Biogeography and adaptation of Notothenioid fish: Hemoglobin function and globin–gene evolution

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Abstract

The recognition of the important role of the polar habitats in global climate changes has awakened great interest in the evolutionary biology of polar organisms. They are exposed to strong environmental constraints, and it is important to understand how they have adapted to cope with these challenges and to what extent adaptations may be upset by current climate changes.

We present an introductory overview of the evolution of the Antarctic fish fauna with emphasis on the dominant perciform sub-order Notothenioidei, as well as some specific comments on the biogeography of the three phyletically basal notothenioid families.

The wealth of information on the ecology and biodiversity of the species inhabiting high-Antarctic and sub-Antarctic regions provides a necessary framework for better understanding the origin, evolution and adaptation of this unique group of fish.

Notothenioidei offer opportunities for identification of the biochemical characters or the physiological traits responsible for thermal adaptation. The availability of phylogenetically related taxa in a wide range of latitudes has allowed to look into the molecular bases of environmentally driven phenotypic gain and loss of function.

In the process of cold adaptation, the evolutionary trend of notothenioids has produced unique specialisations, including modification of hematological characteristics, e.g. decreased amounts and multiplicity of hemoglobins. The Antarctic family Channichthyidae (the notothenioid crown group) is devoid of hemoglobin. This loss is related to a single deletional event removing all globin genes with the exception of the inactive 3′ end of adult α-globin. In reviewing hemoglobin structure, function and phylogeny, the evolution of the fish Root effect is analysed in detail. Adaptation of the oxygen-transport system in notothenioids seems to be based on evolutionary changes involving levels of biological organisation higher than the structure of hemoglobin.

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1. Biogeography of temperate, sub- and high-Antarctic notothenioid fish

1.1. Notothenioids dominate the modern Antarctic fish fauna

Perciform fishes of the sub-order Notothenioidei dominate the cold waters of the Southern Ocean surrounding Antarctica (Eastman, 1993). Perciforms are not monophyletic (Nelson, 2006) and the status of notothenioids as perciforms has recently become less certain given that some mitochondrial and nuclear sequence data have representative notothenioids nested within clades that include at least some traditionally recognised scorpaeniforms, an order that itself is not recoverable as monophyletic (Dettai and Lecointre, 2004; Smith and Wheeler, 2004; Stankovic et al., 2005). Notothenioids are, however, monophyletic (Balushkin, 2000; Chen et al., 2003; Near et al., 2004). There are eight notothenioid families encompassing 129 species (Fig. 1). With the exception of one bovichtid, the 101 Antarctic species are represented by the five families comprising the most phyletically derived clade of the sub-order.

Abbreviations: ACC, Antarctic Circumpolar Current; AFGP, Antifreeze glycoprotein; P50, Oxygen partial pressure required to achieve half-saturation.

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Antarctic notothenioids are distributed south of the Antarctic Polar Front. Non-Antarctic notothenioids, which comprise sub-Antarctic as well as temperate species, are found north of the Antarctic Polar Front 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 compose 77% of the fish diversity, 92% of abundance and 91% of biomass (Eastman, 2005). In the absence of competition from other fish groups they have filled most niches on the shelf and upper slope and may constitute a species flock (Eastman and McCune, 2000).

1.2. An Eocene fossil fauna is unlike the modern Antarctic fish fauna

The perciform stock that gave rise to notothenioids inhabited the shelf waters of the Weddellian Paleobiogeographic Province during the late Cretaceous and early Tertiary, prior to the final separation of Gondwana. This province extended along the Pacific margin of southern South America, Antarctica and southeastern Australia (Crame, 1999). The major Cenozoic fossil fish fauna from the Antarctic component of Gondwana is a Middle to Late Eocene (≈ 40 million years ago) fauna from the La Meseta Formation on Seymour Island, near the tip of the Antarctic Peninsula. It provides a glimpse of the high-latitude (≈ 60°S) shelf fauna at a time when waters were cooler temperate (≈ 10 °C). As biogeographic provinciality was less evident at high latitudes in the Eocene, the La Meseta fauna was cosmopolitan and taxonomically diverse rather than endemic and taxonomically restricted, as is the modern Antarctic fauna. Based on discoveries and identifications to date, the La Meseta fauna consists of 29 taxa with 21 chondrichthyan and eight actinopterygian including representatives of four perciform groups (Eastman, 2000, 2005).

Rajids (skates) and gadiforms (cods) are the only taxa unequivocally represented in both the Eocene and modern faunas. The presence of notothenioids in the La Meseta fauna is open to question because the partial dorsal cranium originally described as a gadiform (Eastman and Grande, 1991) has been reinterpreted as a notothenioid of the family Eleginopidae — Proeleginops grandesmanorum (Balushkin, 1994). Given the incompleteness of the fossil and the difficulty in distinguishing diagnostic osteological characters for notothenioids, the identification is not definitive. In summary, there has been a faunal replacement of the fishes in the shelf waters of Antarctica — the Eocene fauna is not ancestral to most of the modern fauna and most components of the modern fauna do not have a fossil history in Antarctica. This is unlike the situation in other Southern Hemisphere localities where there is continuity between Tertiary and modern faunas, with Australia as an example (Long, 1982).

1.3. Tectonic and oceanographic changes lead to isolation and cooling of Antarctic waters

Over the past 40 million years the Antarctic shelf has been subject to a series of tectonic and oceanographic events that began to alter the composition of the fish fauna and to initiate the process of faunal replacement. Key among these events was the opening of the Drake Passage between southern South America and the Antarctic Peninsula, dated at 23.5–32.5 million years ago (Thomson, 2004) and possibly even as early as 41 million years ago (Scher and Martin, 2006). This divided the notothenioid stock into Magellan (non-Antarctic) and Antarctic components. Opening of 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 the Antarctic waters. The Antarctic Polar Front, the northern boundary of the ACC, is a roughly circular oceanic feature between 50°S and 60°S. Just north of the Polar Front, the surface water temperature is ca. 3 °C warmer than the water to the south. Although the Antarctic Polar front is somewhat “leaky” (see below), its formation was likely a key vicariant event promoting the initial isolation and diversification of a cold-adapted notothenioid fauna to the south. As water temperatures decreased and ice appeared, the Antarctic notothenioids acquired antifreeze glycoproteins (AFGPs) (Fig. 1), a key innovation and a physiological necessity that allowed them to survive and diversify in ice-laden seawater that today reaches temperatures of nearly −2 °C (DeVries and Cheng, 2005). The phyletically basal bovichtids, pseudaphritids and eleginopids do not possess AFGP gene sequences in their genomes, indicating that they diverged before the tectonic isolation and associated cooling of Antarctica and the appearance of AFGPs (Cheng et al., 2003). The split between eleginopids and the Antarctic clade, the five families with AFGPs that inhabit the cold shelf waters of the continent, is variously estimated to have occurred at 5–14 million years ago (Chen et al., 1997), 27 million years ago (Bargelloni et al., 2000) and 40 million years ago (Nar, 2004). The 40 million year estimate is based on a fossil calibration of the contentious Proeleginops mentioned previously. Near (2004) discusses the reasons for the discrepancies among these dates.
The modern notothenioid fauna was probably shaped by a combination of these early vicariant events, later vicariant events involving advances of the ice sheet and, more recently and to a lesser extent, by dispersal including dispersal north across the Antarctic Polar Front in the case of some Antarctic notothenioids. The Antarctic Polar Front is now suspected to be “leaky” and to allow transport of planktonic invertebrates north in mesoscale eddies with cold cores (Clarke et al., 2005). The larvae of most notothenioids are planktonic and the recent origin of the non-Antarctic channichthyid, Champscephalus esox (a species also found at South Georgia in the Antarctic Region), may be an example of this phenomenon (Stankovic et al., 2002). As Antarctica became colder in the Miocene and Pliocene, expansion of the ice sheet led to destruction and disturbance of inshore habitat by ice, with repeated groundings of parts of the ice sheet as far as the shelf break (Anderson, 1999). Loss of habitat and changes in the trophic structure of the ecosystem probably led to the extinction of most of the persisting Eocene components of the fish fauna. Thus the diversity of the fauna was reduced and new niches became available to other groups that were diversifying in situ with notothenioids as the prime example. With little competition from a sparse non-notothenioid fauna, they underwent phyletic as well as morphological and ecological diversification (Eastman, 1993, 2000).

1.4. Biogeography of the phylogenetically basal non-Antarctic (or sub-Antarctic) notothenioids

Because research stations and logistic operations are centered near the Antarctic continent, the Antarctic notothenioid species are more readily available for study and a great deal known about their hemoglobins (Hbs). The three phylogenetically basal non-Antarctic families have been difficult to obtain and less frequent subjects of research. The recent ICEFISH cruise (www.icefish. neu.edu) in the periphery of the Southern Ocean allowed us to collect and study members of these families. The following sections summarise their systematics and biogeography.

Maps with species distributions and additional information on notothenioid biogeography are available in books by Kock (1992), Eastman (1993) and Miller (1993).

1.5. Bovichtidae — thornfishes

Within the Notothenioidei most cladistic analyses identify the Bovichtidae as the phylogenetically basal family (Fig. 1). Only Balushkin’s (1992, 2000) analyses involving morphological characters distinguish the Pseudaphritidae as phylogenetically basal. This placement has not been supported by molecular sequence data (Lecointre et al., 1997; Bargelloni et al., 2000; Near et al., 2004), other morphological data (Voskoboinikova, 2004; Eastman, 2006) or karyological data (Mazzei et al., 2006), which confirm that bovichtids are the phylogenetically basal notothenioid family, a hypothesis that we follow here.

There are 11 species of Bovichtidae with nine in the genus Bovichtus and two other monotypic genera, Cottoperca gobio and Halaphritisplatycephala. Cottoperca is confined to the waters of the Magellanic Province (southern South America, Burdwood Bank and Falkland Islands); Halaphritis is endemic to the inshore waters of Tasmania. Bovichtus, however, is wide ranging. With the exception of Bovichtus elongatus from the waters of the northern Antarctic Peninsula, the eight other species of Bovichtus are found outside the Antarctic Region. Their range include southern South America, southeastern Australia and Tasmania, New Zealand and associated southern islands, as well as some cold temperate peri-Antarctic islands in the Pacific, Indian and Atlantic oceans including St. Paul, Amsterdam, Gough and Tristan da Cunha. Bovichtus diacanthus from Tristan da Cunha (37°S) live near the northern limit for notothenioids (Eastman, 1993). Here they experience mean monthly temperatures of 13–19 °C but can tolerate summer tide pool temperatures up to 27.4 °C (Andrew et al., 1995). Cottoperca is subject to mean monthly temperatures of 5–10 °C on the Patagonian and Falklands shelves (Hart, 1946).

The young of Bovichtus occur as silvery, pelagic juveniles found in large shoals (Hardy, 1988; Andrew et al., 1995) and this aspect of their life history may have allowed the dispersal of this genus to these remote localities at the periphery of the Southern Ocean via the Antarctic Circumpolar Current or, in the case of the most northerly species, in mesoscale (tens to hundreds of kilometers) eddies spun off from this current sometime after its formation 23.5–32.5 million years ago (Thomson, 2004; Clarke et al., 2005).

1.6. Pseudaphritidae — congolli or tupong

This monotypic family contains Pseudaphritis urvillii from coastal waters, estuaries and rivers of Tasmania, Victoria, New South Wales and South Australia. Pseudaphritis is one of only two notothenioids inhabiting freshwater, ascending streams as far as 120 km (Andrews, 1980). Although nothing is known about the reproductive habits of this species, it is assumed that they spawn at sea. However based on the possibility that the spawning site is estuarine, McDowall (1988) suggested that Pseudaphritis might more correctly be considered marginally catadromous. Pseudaphritis was able to become established in an area of the Southern Hemisphere with a low-diversity freshwater fauna. They may be either a Gondwanan form that became associated with and drifted northward in the freshwater of the Australian plate, or a derivative of a marine dispersing bovichtid ancestor (McDowall, 1981).

1.7. Eleginopidae — mullet or róbalo

The monotypic family Eleginopidae is represented by Eleginops maclovinus. The status of Eleginops as the sister group of the Antarctic notothenioids (Fig. 1) is supported by phylogenetic analyses employing both morphological (Balushkin, 2000) and molecular data, including partial (Bargelloni et al., 2000) and complete (Near et al., 2004) mtDNA gene sequences. E. maclovinus has a non-Antarctic distribution that may reflect the historic pattern on the South American component of the Gondwanan shelf. Unlike the Antarctic clade of notothenioids, eleginopids did not become associated with the margins of the Antarctic plate and their
subsequent evolution was little influenced by large-scale tectonic movements or by the cooling of the Southern Ocean (Eastman, 1993). *Eleginops* became established in cool temperate, low-diversity inshore marine and freshwater habitats — the Falkland Islands, for example, have only two native species of freshwater fishes (McDowall, 2002). It is one of only two euryhaline notothenioid species. *Eleginops* inhabits coastal waters, sounds and tidal creeks in the Falkland Islands (Falkland Islands Government, 2003; Brickle et al., 2005a,b) and coastal waters, estuaries and rivers in southern South America from the Beagle Channel ($\approx 54^\circ$S) to approximately the Golfo San Matías, Argentina ($\approx 40^\circ$S) on the Atlantic coast (Gosztonyi, 1979) and Valparaiso, Chile ($\approx 32–33^\circ$S) on the Pacific coast (Gosztonyi, 1979; Pequeño, 1989; Ojeda et al., 2000).

Several aspects of the biology of *Eleginops* distinguish it from the sedentary, cold-adapted Antarctic notothenioids. For example, they have a relatively larger mass of red pectoral musculature and a greater capacity for sustained labriform swimming than other notothenioids (Fernández et al., 1999). The rapidity of their escape response is similar to eurythermal temperate non-notothenioids rather than to Antarctic notothenioids (Fernández et al., 2002) and their resting rates of oxygen consumption place them in an active category in comparison with sympatric non-Antarctic notothenioids (Vanella and Calvo, 2005). In the Falkland Islands *Eleginops* are subject to annual temperatures of $\approx 0–15^\circ$C (Falkland Islands Government, 2003).

2. The hemoglobins of Notothenioidei

2.1. Structure, function and adaptations

The recognition of the important role of the polar habitats in global climate changes has awakened great interest in the evolutionary biology of the organisms that live there. These organisms are exposed to strong environmental constraints, and it is important to understand how they have adapted to cope with these challenges and to what extent adaptations may be upset by current climate changes. Adaptations of the dominant group of Antarctic fish, the sub-order Notothenioidei, have been thoroughly investigated by several teams.

The Southern Ocean offers a uniquely stable thermal environment where cold adaptation of fishes may be expected to have occurred, obviating the need to retain the functional plasticity required in more variable ecosystems (Somero, 1995). Much of our knowledge of the effect of the environment on vertebrate physiology and evolution has come from the study of fishes. The study of fish adapted to the extreme conditions of the polar seas will allow us to take a renewed look at the development, impact and consequences of climate and anthropogenic challenges and the role played by temperature in establishing species distribution (di Prisco and Verde, 2006). Antarctic fish have evolved in an environment with the lowest temperature variations, and have limited abilities to withstand an increase in temperature. These species are highly susceptible to global warming events and may be one of the most vulnerable groups to climate change. The ability to cope with change depends on the phenotype and differs from individual to species level (Peck, 2005). Antarctic fishes (in particular the dominant sub-order Notothenioidei) including high- and sub-Antarctic species offer many opportunities for comparative approaches in order to identify the biochemical characters or the physiological traits responsible for thermal adaptation. From this evolutionary perspective, the availability of phylogenetically related notothenioid taxa living in a wide range of latitudes has led many comparative physiologists and biochemists to look into the molecular bases of environmentally driven phenotypic gain and, conversely, loss of function.

Although dissolved oxygen can be transported free in solution, most animals carry oxygen to the respiring tissues by one or more respiratory proteins. In fish, as in other vertebrates, the molecule that transports oxygen to the cell is Hb. In jawed vertebrate erythrocytes, Hb is a tetramer and its physiological function to bind or release oxygen depends on the partial pressure of oxygen. Hbs are highly sensitive to temperature and their structural and functional properties may in part mirror the environmental conditions encountered by species during their evolutionary histories. Consequently, the comparison between cold and non-cold-adapted fish Hbs may be informative for many biological questions. For example, many structural and functional features typical of most vertebrate Hbs share similarity with allosteric enzymes. These multi-subunit regulatory enzymes and Hbs are characterised by specific ligand-binding affinity and generally exhibit cooperativity in ligand binding. These properties (affinity and cooperativity) may be adaptively modified in different Hbs to provide adequate oxygen supply under a wide variety of environmental and physiological conditions. Many of the functional differences among vertebrate Hbs may be interpreted in terms of amino-acid substitutions although many others are due to changes in the composition of the milieu in which the protein works. The globin–heme complex can be adapted to various biological functions through the design of the heme pocket. Lastly, change in the concentration of Hbs in the erythrocyte provides another adaptive strategy to extreme environments.

Compared to temperate and tropical species, high-Antarctic notothenioids have evolved reduced erythrocyte number, Hb concentration and multiplicity (Eversion and Ralph, 1968; Bureau and Tomo, 1977; Wells et al., 1980). One notothenioid family has abolished Hb as oxygen carrier (Ruud, 1954). The colourless blood of the “icefishes” of the family Channichthyidae lacks Hb and erythrocytes. These are the only known adult vertebrates showing such an astonishing adaptation (see below).

Most Antarctic notothenioids are bottom dwellers. The red-blooded families generally have a single Hb (Hb 1) accounting for 95–99% of the total (except in Bovichtidae sensu stricto, Cottoperca and Bovichtus), and often a functionally similar, minor component Hb 2 (Verde et al., 2007). High-Antarctic notothenioids also have traces of another minor component, Hb C (di Prisco et al., 1991). Although a report in the literature (Sidell and O’Brien, 2006) does not support the ensuing hypothesis, we think that the reduction in Hb content/
multiplicity and erythrocyte number in the blood of Antarctic notothenioids counterbalances 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 (Wells et al., 1990). Multiplicity of Hbs in fish is usually linked to the need to respond to variable environmental conditions specifically associated with a given habitat (Jensen et al., 1998; Weber, 1990). Furthermore, the availability of several globin genes for α and β-globins may be interpreted as adaptation to cope with deleterious mutations in individual globin genes (Jensen et al., 1998; Weber, 1990). In high-Antarctic benthic notothenioids, a single Hb present in low amount is regarded as the consequence of its reduced role as oxygen carrier, possibly in relation to the fish life-style and metabolism, and to the peculiarity of the environmental conditions [high stability and constancy of physico-chemical features, higher oxygen content in Antarctic seawater (Verde et al., 2006)]. In other words, the lack of multiple globin genes is not lethal in thermostable environments. The oxygen affinity of Hbs (controlling oxygen binding at the exchange surface and subsequent release to tissues) of many high-Antarctic species is quite low (di Prisco et al., 1988), as indicated by the values of $p_{50}$ (the oxygen partial pressure required to achieve half-saturation). This feature is probably linked to the high oxygen concentration in the cold Antarctic waters. The Hbs of the most active species appear to have the lowest affinity (di Prisco et al., 1991).

According to the species-adaptation theory of Perutz (1983), the replacement of a few key amino-acid residues can lead to new functions. Active-site sequences are generally highly conserved among homologous proteins due to structural constraints in ligand/substrate binding. For some proteins, e.g. enzymes, adaptive changes in the structure occur at sub-unit interfaces, distant from the active site (Johns and Somero, 2004). These alterations in the strength of sub-unit–sub-unit interactions may affect thermal stability and the energy changes associated with conformational transitions due to ligand binding. Enhanced flexibility has been associated with a number of structural modifications. Common trends include a (i) reduced number of ion pairs, hydrogen bonds and hydrophobic interactions, (ii) decreased inter-sub-unit interactions, (iii) increased interaction with the solvent, (iv) reduction of non-polar residues in the core of a protein, (v) higher accessibility to the active site, (vi) increased exposure of non-polar residues to the solvent, (vii) clustering of glycine residues, and (viii) lower Pro and Arg content (Somero, 1995; D’Amico et al., 2006). In polar Hbs, however, it has not been possible to ascribe thermal adaptation to specific residue substitutions. Indeed, the situation is much more complex, and is probably linked to the combination and interplay of a number of factors in the architecture of the globin tetramer. The crystal structure of two functionally distinct Antarctic Hbs, displaying 95% sequence identity (D’Avino et al., 1994; Ito et al., 1995; Mazzarella et al., 1999), have shown that fish Hbs often do not obey the classic structural dogmas. In fact, the possession of one or more unique residues does not necessarily result in specific adaptation and/or function.

To date, the crystallographic structures of cold-adapted Hbs (Ito et al., 1995; Mazzarella et al., 1999) have revealed both the classical conformation and high similarity with mesophilic homologues. Cold adaptation of the oxygen-transport system in high-Antarctic notothenioids seems to be based on evolutionary changes involving levels of biological organisation higher than the structure of Hb. These include changes in the rate of Hb synthesis or in regulation by allosteric effectors, which affect the amount of oxygen transported in blood. These factors are currently thought to be more important for short-term response to environmental challenges than previously believed.

Bovichtids are regarded as the most primitive family (Eastman, 1993) with species showing many morphological characters considered plesiomorphic for the sub-order. They do not possess any of the physiological and biochemical adaptations to the extreme Antarctic environmental conditions shared by most other notothenioids (Eastman, 1993). Unlike most Antarctic notothenioids but similar to many other acanthomorph teleosts, adult C. gobio, thriving in sub-Antarctic waters just north of the Polar Front, has two major Hbs sharing the β chain. In the two Hbs, oxygen binding is strongly modulated by heterotrophic effectors, with marked Bohr and Root effects, and high oxygen affinity compared to Antarctic notothenioid Hbs (Verde et al., unpublished). Higher multiplicity and oxygen affinity have also been observed in B. diacanthus, one of the most northern notothenioids (Verde et al., unpublished). Although the presence of multiple Hbs in the blood of non-Antarctic notothenioids may be considered a plesiomorphic condition for many percomorph fishes, the more complex oxygen-transport system in C. gobio and B. diacanthus may have been maintained by positive selection to deal with the large temperature changes in waters north of the Polar Front.

Similar to many high-Antarctic notothenioids, the hemolysate of P. urvillii (family Pseudaphritidae) (Verde et al., 2004) and E. maclovinus (family Eleginopidae) (Verde et al., unpublished) have a single major Hb (Hb 1) and a minor component (Hb 2). The low amount of Hb 2 can be considered a synapomorphic character linking P. urvillii and E. maclovinus to most of the other notothenioids. E. maclovinus is the sister-taxon of non-bovichtid and non-pseudaphritid notothenioids. This is consistent with the observation that Eleginops is a sub-Antarctic species devoid of AFGPs.

Oxygen affinity is very high in the Hbs of non-Antarctic notothenioids C. gobio, B. diacanthus, P. urvillii and E. maclovinus; $p_{50}$ values indicate that a decrease in oxygen affinity occurred along the lineage of the high-Antarctic notothenioids, with the exceptions of Pleuragramma antarcticum, Trematomus bernacchii and Artedidraco orianaee, whose Hbs have higher oxygen affinity than other Antarctic notothenioids (Verde et al., unpublished). The relationship between higher oxygen affinity of non-Antarctic notothenioid Hbs and habitat features remains an open question. Spectroscopic and modelling studies on P. urvillii Hb 1 have shown that all the non-conservative replacements in the primary structure of α and β chains leave the conformation and electrostatic field surrounding the heme pocket essentially unmodified (Verde et al., 2004) with respect to Hb 1 of the high-Antarctic T. bernacchii (Ito et al., 1995).
2.2. The Root effect

The decreased oxygen affinity of Hb at lower pH values in the physiological range is known as the alkaline Bohr effect, reviewed by Riggs (1988). In many Hbs from teleost fishes, when the pH is lowered, the oxygen affinity decreases to such an extent that Hbs cannot be fully saturated even at very high oxygen pressure. At low pH, cooperativity is lost and the oxygen capacity of blood undergoes reduction of 50% or more of the value measured at alkaline pH (Table 1). This feature is known as the Root effect, reviewed by Brittain (2005). Root-effect Hbs are so strongly pH dependent that they are able to unload a large amount of bound oxygen at low pH and against a pressure gradient. The Root effect dictates to what extent the oxygen tension can be raised in acid-producing tissues. The physiological significance of Root-effect Hbs has been linked to the presence of at least one of two vascular structures creating high oxygen pressure: the swimbladder rete mirabile supplying the gas gland that regulates buoyancy, and the choroid rete mirabile supplying oxygen to the poorly vascularised retina (Wittenberg and Haedrich, 1974). A recent study carried out on temperate fish (Berenbrink et al., 2005) has shown that the Root effect apparently evolved 100 million years before the appearance of the choroid rete, whereas the swimbladder evolved independently at least four times. According to these authors, the swimbladder rete, the choroid rete and the Root effect have been lost several times in some fish groups. It is likely that the choroid rete of the eye represents the most ancient anatomical structure associated with the presence of Root-effect Hbs (Wittenberg and Haedrich, 1974). Among high-Antarctic notothenioids, many species have lost the choroid rete, although several retain portions of the rete and/or small vestiges of the choriocapillaris (Table 2) (Eastman, 1988, 1993, 2006, unpublished).

Fig. 2A shows the pattern of occurrence of the choroid rete traced as a discrete ordered character on the tree showing the phylogeny of high-Antarctic and non-Antarctic notothenioids. The comparison of two phylogenetically related groups of notothenioids, one including the non-Antarctic and another the high-Antarctic species, shows that the choroid rete is present in the first group, whilst the high-Antarctic group displays a more variable scenario. In the family Nototheriidae, in fact, Aethotaxis mitopteryx, Pagotthenia borchgrevinki and Trematomus newnesi have lost the choroid rete. In contrast, T. bernacchii has it, whilst in Gobionotothen gibberifrons the rete is markedly reduced. The two Arctidraconidae A. arianea and Pogonophryne scotti, as well as the group of Bathydraconidae Gymnodraco acuticeps, Cygnodraco mawsoni and Bathydraco marri also lack the rete, whilst Racoitiza glacialis still retains a vestigial form of rete. Similar to other teleosts, a pseudobranch is present in both types of fish.

Fig. 2B shows the evolution of the Root effect in notothenioids, traced as continuous character on a phylogenetic tree including both non-Antarctic and high-Antarctic species. The Hbs of the sub-Antarctic notothenioids C. gobio and B. diacanthus are characterised by a very high Root effect. It must be noticed that the choroid rete is very well developed in this lineage, which is the most basal of the sub-order. A general reduction in the Root effect is noticed during the evolution of the Antarctic notothenioids; the reconstruction reported in Fig. 2B shows values at the ancestral nodes close to 40%, but in many cases the values at the terminal branches undergo striking variations in both directions. The Root effect of the lineage leading to Nototheria coriceps and N. angustata, for instance, jumps to about 55%. In contrast, it significantly decreases in A. mitopteryx, P. borchgrevinki and T. newnesi. The clade of Arctidraconidae + Bathydraconidae reveals some intriguing features. While the Root effect drops to low values (as expected) in the Arctidraconidae lineage, as well as in one Bathydraconidae (G. acuticeps), it is found at unexpectedly high levels in two species of the latter family, both displaying similar sluggish life style.

The question whether the maintenance of the Root effect in Hbs of high-Antarctic notothenioids is related or not to environmental conditions still remains to be answered. The fact that high-Antarctic notothenioids still have Hbs endowed with Root effect also when the choroid rete is absent suggests that this function undergoes neutral selection, not representing a disadvantage for the species. Moreover, a viable hypothesis is that, as in the early ray-finned fish, the deleterious effects of acidosis can be prevented by high Hb buffer values. On the
other hand, the weakening of the Root effect in Hbs of many high-Antarctic notothenioids indicates that the Root effect is not an all-or-nothing phenomenon, suggesting that it may not depend on a single factor or mechanism, but it may possibly be generated by a combination of several factors.

3. Hemoglobin evolution

To address some of the questions regarding the physiological and biochemical adaptations in Antarctic notothenioids, part of this review has also been focussed on the phylogeny of the Hbs of the sub-order. Investigating the evolutionary processes leading to some adaptations in the oxygen transport requires a well-resolved phylogenetic hypothesis. Published molecular and morphological hypotheses of notothenioid phylogeny are currently strongly congruent. In the molecular phylogenetic analysis using the maximum-likelihood method (Giordano et al., 2006) 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 obtained topology is in general agreement with the hypothesis of four groups of globins, namely “Embryonic Hb Group”, “Notothenioid Major Adult Hb Group”, “Anodic Adult Hb Group” and “Cathodic Adult Hb Group” (Maruyama et al., 2004).

Fig. 3A,B reports the trees inferred by a Bayesian method, using the software Mr BAYES with mixed model (Ronquist and Huelsenbeck, 2003). As a result of the isolation of Antarctica, the genotyp of Notothenioida diverged with respect to other fish groups in a way interpreted as typical of a species flock (Eastman and McCune, 2000). In the phylogenetic trees, the basal position of P. urvillii and E. maclovius 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). 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 *T. newnesi* (D’Avino et al., 1994), *P. borchgrevinki* (Riccio et al., 2000) and *P. antarcticum* (Tamburrini et al., 1996), “embryonic” globins
A - α globins

Fig. 3. Phylogenetic trees describing the evolution of α and β-globins in fish. The trees for α-(Fig. 3A) and β-globins (Fig. 3B) were inferred by a Bayesian method, using the software Mr Bayes with mixed model. The α- and β-chains analyses were stopped after 2,000,000 and 1,500,000 cycles, respectively. At the end of the analyses, the average standard deviation of split frequencies dropped to less than 0.01 and the scale reduction factor was 1 for all parameters. Antarctic Notothenioid major globins are in black, Antarctic Notothenioid minor globins are in dark grey, non-Antarctic notothenioid globins in bold italic, and temperate globins underscored.

L. chalumnae, Latimeria chalumnae; E. electricus, Electrophorus electricus; C. auratus, Carassius auratus; C. carpio, Cyprinus carpio; C. clarkii, Catostomus clarkii; O. mykiss, Oncorhynchus mykiss; Ch. auratus, Chrysophrys auratus; A. anguilla, Anguilla anguilla; S. salar, Salmo salar; H. littorale, Hoplosternum littorale; C. kumu, Chelidonichthys kumu; O. latipes, Oryzias latipes. For full notothenioid names, refer to Table 1.
are expressed at significant levels. In these three species, however, the majority of the β chains is included in “Notothenioid Major Adult Hb Group”. Thus, a complete “switch” to exclusive expression of the embryonic β-globin gene has occurred in adult C. gobio. A switch towards a constitutive expression of the embryonic globins in adult fish may have occurred exclusively along the lineage leading to C. gobio, because P. urvillii conforms to the Hb pattern found in most Antarctic notothenioids (Verde et al., 2004). The preservation of an embryonic character in the putative C. gobio ancestor can be interpreted as a case of neoteny (Fig. 4), possibly consequential to the maintenance of a high expression level of the embryonic globin genes at the adult stage followed by impaired expression of the “adult” β-globin gene (Giordano et al., 2006).

4. Loss of hemoglobin expression in the icefishes

Icefishes (family Channichthyidae) are the notothenioid crown group. Their blood is completely devoid of Hb (see Fig. 1); compensatory adaptations have been developed that reduce tissue oxygen demand and enhance oxygen transport (e.g., modest suppression of metabolic rates, enhanced gas exchange by large, well-perfused gills and through a scaleless skin, and large increases in cardiac output and blood volume). Oxygen delivery to tissues occurs by transport of the gas physically dissolved in the plasma. However, the development in icefishes of these compensatory physiological and circulatory adaptations argues that loss of Hb and erythrocytes was probably maladaptive under conditions of physiological stress. Channichthyids diverged from other Antarctic notothenioids ∼7–15 million years ago, but radiation of species within the icefish clade appears to have been confined to the last one million years (Bargelloni et al., 1994).

The physiological role of Hb in oxygen transport in temperate and tropical fish is undisputed; however, it is not clear whether Hb is essential for oxygen transport in the Antarctic red-blooded families, or is a redundant vestigial relict. The answer was found by reversibly “poisoning” Hb of the red-blooded nototheniid T. bernacchii with carbon monoxide (di Prisco, 2000). Carbon monoxide is lethal for most organisms (including temperate fish) whose life depends on oxygen, because it binds to Hb with much higher affinity than oxygen, thus replacing this ligand and preventing transport of the ultimate respiratory electron acceptor. The survival of T. bernacchii under conditions of Hb incapacitation by carbon monoxide suggests that, in the cold and stable environment of the Antarctic seas, Hb does not have an obligatory role in delivering oxygen to the tissues. In other words, similar to icefish, red-blooded Antarctic fish can rely on the oxygen physically dissolved in the blood.

Icefish genomes retain a small, inactive portion of the adult α-globin gene; the other part and the adult β-globin—gene has been deleted (Cocca et al., 1995; Zhao et al., 1998; di Prisco et al., 2002). Southern blots of genomic DNA from red- and white-blooded (two species) notothenioids, probed with fragments of the genes flanking the ends of the embryonic/juvenile complex, indicated that icefishes have also lost embryonic/juvenile globin genes.

The evolution of icefishes to the Hb-less phenotype may have occurred by at least two mechanisms: (i) direct gene deletion, or (ii) a multi-step process involving transcriptional inactivation of the adult and the embryonic/juvenile globin complexes, followed by elimination of the non-functional genes.

![Fig. 4. Hypothesised patterns of globin–gene expression during evolution of Hbs of C. gobio and of Antarctic notothenioids. The expression levels of major (adult) and minor (embryonic) globin genes in the adult stage are indicated by different shades (black=high, grey=low, white=no expression). For the sake of simplicity, only one pair of adult genes and one pair of embryonic genes are shown.](image-url)
themselves (Cocca et al., 1995; Zhao et al., 1998). The current hypothesis is that inability to express Hb arose from a single, large-scale deletional event removing all globin genes with the exception of the 3′ end of adult α-globin. This transcriptionally inactive remnant, no longer under positive selection pressure for expression, subsequently experienced random mutational drift, without, as yet, complete loss of sequence information. These α-globin genetic remnants should indeed prove useful as tools for development of a molecular phylogeny of icefishes and for calibration of a vertebrate mutational clock free of selective constraints.

In red-blooded teleosts, juvenile and adult globin loci are typically composed of tightly linked pairs of α- and β-globin genes. In a recent study Near et al. (2006) demonstrated that the phylogenetically derived icefish species, Neopagetopsis ionah, possesses a complete, but non-functional, adult αβ-globin complex. Maximum-likelihood ancestral state reconstruction supports a scenario of icefish globin gene evolution involving a single loss of the transcriptionally active adult globin cluster prior to the diversification of the extant species in the clade. The inactive αβ-globin pseudogene complex of N. ionah may be considered an intermediate “genomic fossil” revealing key mechanisms on the pathway to loss of Hb expression by all icefish species (Near et al., 2006).

5. Concluding remarks

The two polar ecosystems offer numerous examples of the evolution of structure–function relationships among teleost fish. In the recent past, most reviews underlie how little is known about specific adaptations to a cold environment. However, polar research is becoming better focussed on the awareness of warming occurrence also in the polar regions. Over the last twenty years, important advances have been achieved in understanding the molecular mechanisms involved in evolutionary adaptation to temperature. In view of the importance of the Antarctic in determining the Earth’s climate, studies on the evolutionary adaptations of polar fish will help to feed and stimulate discussions on global climate changes, including those which are man-induced/enhanced.

The integration of phylogeny, structure/function relationships, molecular, functional and ecological data, is a necessary tool and will help us to understand the evolution of the oxygen-transport proteins and the effect of environmental conditions on phyllogenies. Over evolutionary time, the Hb phenotype is undergoing dynamic changes in response to cold adaptation. Antarctic notothenioids possess the physiological tools to adapt, having evolved a low-affinity oxygen-transport system which facilitates oxygen unloading. A future challenge is the study of their ability to develop repair mechanisms to changes induced by a wide variety of natural and anthropogenic processes, in the general framework of species and ecosystem responses, as well as the ways in which responses feed back to influence these processes. Along these lines, the oxygen-transport system of non-Antarctic notothenioids (as well as those of Arctic fish) provide precious comparative indications from environments endowed with wider changes in physico-chemical features.

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