

The Ross Sea and its rich life: research on molecular adaptive evolution of stenothermal and eurythermal Antarctic organisms and the Italian contribution

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Abstract The official involvement of Italy in Antarctic research dates back to 1985, when Mario Zucchelli Station (the former Terra Nova Bay Station) was established in Terra Nova Bay. Italy joined the Antarctic Treaty in 1987. This article is an overview of the wide-ranging research in marine biology performed in the last three decades by the author's team in the Ross Sea. Fundamental questions have been addressed, related to cold adaptations—with special attention to the molecular bases—evolved by marine organisms along with progressive cooling in this geographic area, also analysed in comparison with other important areas, such as the Peninsula, the Weddell Sea, the sub-Antarctic and the Arctic. The basic stepping stone of this research was the integration of ecophysiology with molecular aspects, in the general framework of biodiversity, adaptation and evolution. Investigations have addressed a number of Ross Sea taxa, comprising fish, birds, urchins, whales,

seals and bacteria. Its significance has special meaning in view of the control that Antarctica exerts on the world climate and ocean circulation, which has awakened great interest in the evolutionary biology of the organisms that live there.

Keywords Ross Sea · Climate change · Molecular adaptive evolution · Hemoprotein · Enzyme · Metallothionein

Foreword

As the Guest Editors Schiaparelli and Fontaneto recalled in their Call for Papers, biologists have been working for well over a century in the Ross Sea. Advances in the knowledge of the ecosystem in this important area have steadily piled up, taking advantage of the well-equipped laboratories and aquaria of infrastructures such as McMurdo Station (USA), Scott Base (New Zealand), Mario Zucchelli Station (MZS, Italy), as well as of recent multi-year international programmes cruises, e.g. Climatic Long-term Interaction for the Mass balance in Antarctica (CLIMA), Ross Sea Marginal Ice Zone Ecology (ROSSMIZE), Latitudinal Gradient Project (LGP); see also Faranda et al. (2000) and references therein. A wealth of information on the Ross Sea and its ecosystem is to date available, which adds to that collected over many decades in other regions of primary importance, e.g. the Antarctic Peninsula and the Weddell Sea.

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Biology of the Ross Sea and Surrounding Areas in Antarctica

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Since the late '70s, the international biological community had been involved in programmes launched by the Scientific Committee on Antarctic Research (SCAR), such as Biological Investigations of Marine Systems and Stocks (BIOMASS) and Biological Investigations of Terrestrial Antarctic Systems (BIOTAS). Since the mid '80s, Italy had a strong role in launching new SCAR programmes, i.e. Ecology of the Antarctic Sea-Ice Zone (EASIZ), Evolutionary Biology of Antarctic Organisms (EVO-LANTA), Regional Sensitivity to Climate Change in Antarctic Terrestrial Ecosystems (RiSCC). They all envisaged the Ross Sea as a key area for research. Merging of EVOLANTA and RiSCC gave birth in 2006 to SCAR-IPY's Evolution and Biodiversity in the Antarctic—The Response of Life to Change (EBA). When EBA lapsed, its legacy took the shape of two new SCAR biology programmes (di Prisco & Convey, 2012; di Prisco et al., 2012a; Gutt et al., 2012): State of the Antarctic Ecosystem (AntEco) and Antarctic Thresholds-Ecosystem Resilience and Adaptation (AnT-ERA).

The inception of Italy's research in 1985, when MZS (formerly TNBS) was established in Terra Nova Bay, was a crucial factor, greatly favouring the development of marine biological research (mostly on the shelf of Terra Nova Bay, and in other areas of the Ross Sea), also in terms of international collaboration. Part of this research had begun some years earlier in Palmer Station (USA), in the Antarctic Peninsula.

Marine biological research in the Antarctic is bound to take the impacts of ongoing climate change in due account. Polar systems are undergoing exceptionally rapid warming and decrease in ice, especially in the Arctic and the Antarctic Peninsula, where the impacts on the ecosystem are already visible. In contrast, extent and duration of ice in the Ross Sea have significantly increased (Comiso & Nishio, 2008; Stammerjohn et al., 2012), and are responsible for the modest net increase in the Southern Ocean since 1979. The Ross Sea food web is as yet largely pristine (industrial fishing is impractical because of sea ice), however its chemistry is rapidly changing, mostly because of increasing CO₂ and decreased pH. Both features make the Ross Sea an excellent example of natural laboratory for testing impacts of climate change on ecological and biological processes. Bracegirdle et al. (2008) suggest that in a few decades the

Ross Sea will also experience reduction in summer sea ice, driven by warming and changes in winds. When such reversal occurs, some ecological changes observed in the sea around the Peninsula may take place in the Ross Sea, and will ultimately produce alterations to the food web, e.g. phytoplankton composition/production, krill availability and top-predator abundance (Smith et al., 2014). From this standpoint, although most of the marine organisms that prosper therein are not unique, the Ross Sea is a compendium/model of conditions at least partially experienced by most Antarctic marine habitats. Hence, in studying biodiversity, comparisons of adaptations in the Ross Sea and in other Antarctic marine regions have mutual advantages.

The crucial role of Antarctica in ongoing climate changes will be highlighted by comparisons with other areas, including the sub-Antarctic and the Arctic.

Due to isolation, also by virtue of the Polar Front (see below), the Antarctic ocean exhibits stable living conditions. The evolutionary processes of Antarctic organisms and the time scales in the context of geological and climatic changes have been extensively analysed (Peck, 2011). The variability of environmental temperature may be a major factor in dictating organismal responses to environmental change; physiological differences and variability may be correlated with variations at the level of gene expression (Peck et al., 2014, 2015).

Proteins are the main target of adaptation mechanisms. The structure and function of proteins are the basis for understanding the adaptive evolutionary forces operating at sub-zero temperature, and the knowledge gained at the molecular level is a crucial factor for predictions of the evolutionary consequences of global warming. At all analysed levels, functional adaptation to permanently low temperature appears to require maintenance of flexibility of molecules in order to adequately support cellular functioning.

Temperature is the prime driver that shaped the current structure and function of polar communities. Amongst temperature-influenced abiotic factors, O₂ and CO₂ (at several-fold higher concentrations than in temperate and tropical marine habitats) play an important role in life-sustaining processes. The temperature-dependent balance between O₂ demand/supply and the associated functional capacity of macromolecules shape the performance window in

polar species (Pörtner et al., 2007). The benefits of high O₂ are counterbalanced by kinetics of biological processes at low temperatures which decrease rates, and by increased production of reactive oxygen species (ROS). Oxygen is obviously necessary for aerobic metabolism, but it can become poisonous in triggering oxidative-stress bursts.

On the ground of these considerations, in all Antarctic organisms metabolism in general and biological processes involving O₂ (respiration, transport/release, scavenging, ROS, etc.) and other gases are bound to attract the interest of biologists.

This timely Special Issue has an important role. With some notable exceptions, e.g. Smith et al. (2014), there is paucity of comprehensive reviews centred on the Ross Sea, in particular on molecular adaptive evolution of stenothermal and eurythermal marine organisms. This article attempts to fill information gaps by providing an overview of a large part of the Italian studies carried out over several decades on mechanisms of adaptation developed by a number of Ross Sea marine taxa, and wishes to integrate ecophysiology with molecular aspects, in the general frame of biodiversity, adaptation and evolution. Due reference is made to important contributions of teams of other countries along the same lines.

Ichthyofauna

Historical background

Over the past 40 million years, the Antarctic shelf has undergone a series of tectonic and oceanographic events that began to alter the composition of the fish fauna. The opening of the Drake Passage between Tierra del Fuego and the Antarctic Peninsula, 23.5–32.5 million years ago (mya) (Thomson, 2004) and possibly even as early as 41 mya (Scher & Martin, 2006), was a key event. The Passage led to the development of the Antarctic Circumpolar Current (ACC), partially responsible for cooling of the Antarctic waters, and divided the fish stock into non-Antarctic and Antarctic components. The Antarctic Polar Front (APF), the northern boundary of the ACC, runs between 50 and 60°S. Just north of the APF, the surface water temperature is ca. 3°C warmer, promoting initial isolation of cold-adapted organisms (Clarke et al., 2005). Oxygen solubility increases with

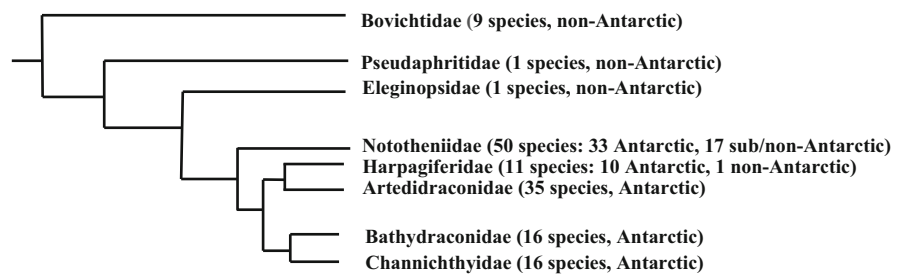
temperature decrease, thus the cold seas are an O₂-rich habitat. Most fish are stenothermal, hence their ability to cope with the ongoing temperature increase is likely to be limited. An important question is to what extent fish may adapt to environmental change.

The perciform suborder Notothenioidei, mostly confined within Antarctic and sub-Antarctic waters, dominates the modern Southern Ocean ichthyofauna. Notothenioids probably appeared in the early Tertiary and began to diversify on the Antarctic shelf in the middle Tertiary, adapting to progressive cooling; they account for 77% of the shelf-fish diversity and 91% of biomass (Eastman, 1993, 2005). Bovichtidae, Pseudaphritidae, Eliginopsidae, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae, Channichthyidae (the eight families of the suborder; see <http://www.oucom.ohiou.edu/dbms-eastman/>) encompass 139 species. Phylogenetic arrangement for families (Fig. 1) is based on Near et al. (2004) and Near & Cheng (2008). According to Eastman and Eakin (January 15th, 2015), “although recent work indicates that the Nototheniidae and Bathydraconidae are paraphyletic (Near et al., 2012; Dettai et al., 2012), we are provisionally retaining the traditional family names as relationships and taxonomy of notothenioids, and acanthomorphs in general, are entering a period of flux (Dettai et al., 2012; Betancur-R et al., 2013; Near et al., 2013)”. Figure 1 does not show the paraphyly.

Seven families have haemoglobin (Hb)-containing erythrocytes, whereas the species of Channichthyidae are devoid of Hb (Ruud, 1954). Bovichtidae, monotypic Pseudaphritidae and Eliginopsidae became established around New Zealand, Australia and high-latitude South America.

As ice appeared, Antarctic notothenioids acquired antifreeze glycoproteins (AFGPs), a key innovation and physiological adaptation that allows them to survive in ice-laden seawater at temperatures of nearly −2°C (DeVries & Cheng, 2005, and references therein). The AFGP gene evolved from a pancreatic trypsinogen-like serine protease gene: the ancestral gene provided the front and tail of the emerging AFGP gene (Chen et al., 1997). By binding ice crystals, AFGPs inhibit their growth. However, paradoxically, life-long accumulation of the AFGP-ice complex could be lethal for fish because AFGPs also prevent the melting in vivo, thus limiting the opportunities to eliminate ice from the body. Cziko et al. (2014) have shown that this crucial adaptive trait alone is

Fig. 1 Cladogram of the families of Notothenioidei (Near et al., 2004; Near & Cheng, 2008). Adapted from Verde et al. (2012d)



insufficient to permit survival in polar environments. There is need of both complementary protection by epithelia to reduce ice entry and evolution of mechanisms to sequester internalised ice crystals from circulation. These additional adaptations would erase the potentially antagonistic pleiotropic effects of melting inhibition due to AFGPs.

Bovichtids, pseudaphritids and eleginopsids have no AFGP genes, indicating that they diverged before cooling (Cheng et al., 2003).

North of the APF, non-Antarctic notothenioids (sub-Antarctic and temperate species) account for 23% (32 of 139 species) of biodiversity (see <http://www.oucom.ohiou.edu/dbms-eastman/>). *Bovichtus diacanthus* lives in Tristan da Cunha (37°S) in summer tide pools at up to 27°C, near the northern limit for notothenioids.

The pseudaphritid *Pseudaphritis urvillii*, a freshwater fish, is found in coastal waters, estuaries and rivers of southern Australia. The other monotypic family Eleginopsidae is the sister group of Antarctic notothenioids (Balushkin, 2000; Bargelloni et al., 2000; Near et al., 2004). *Eleginops maclovinus* inhabits coastal waters, sounds and tidal creeks in the Falkland Islands and coastal waters and estuaries in southern South America, at annual temperatures of 0–15°C.

The Antarctic (and polar) institutions of several countries have been traditionally implementing studies of the Ross Sea ichthyofauna for many decades, as well as in the Peninsula, the Weddell Sea, the sub-Antarctic.

The Italian contribution to research on the ichthyofauna of the Ross Sea (and polar fish in general) covers ecology, population dynamics, genetics, physiology, biochemistry; molecular aspects are a strong component; it began over 35 years ago in 1981 in Palmer Station (USA). Gathering contributions from

other countries, overviews have been published in eleven journal Special Issues (di Prisco et al., 1988; di Prisco, 1997b; Rodhouse et al., 2000; Ørbaek et al., 2002, 2003; Eastman et al., 2004; Moens et al., 2007; di Prisco et al., 2009; Fukuchi et al., 2010; Lewis et al., 2012; Verde et al., 2012a; and references therein) and nine books (di Prisco, 1991; di Prisco et al., 1991a, 1997, 1998, 2000b; Bolognesi et al., 2008; Turner et al., 2009a; di Prisco & Verde, 2012; Verde & di Prisco, 2012; and references therein).

Table 1 lists the investigated Antarctic fish species of the Ross Sea (accounting for the vast majority), the Peninsula, the Weddell Sea, the sub-Antarctic. The investigated Arctic species are listed in Table 2.

The Ross Sea

Biological research activity in the Ross Sea includes extensive studies performed in McMurdo Station, USA (e.g. Chen et al., 1997; DeVries & Cheng, 2005; Sidell & O'Brien, 2006; and references therein) and at Scott Base, New Zealand (e.g. Wells et al., 1980, and references therein). Italian teams are addressing ecology, biogeography, physiology, population genetics/dynamics, etc. (see also Cattaneo-Vietti et al., 2000; Vacchi et al., 2012; Tota et al., 2012; and references therein), as also shown by other articles of this Special Issue. Our research addresses molecular cold adaptation in the framework of evolution (di Prisco, 1997a, 2003; Verde et al., 2000, 2004a; Russo et al., 2010b).

Haemoglobin

Oxygen carriers are ideal for studying physiological adaptations and interrelationships between environment and molecular evolution. Considering the variety of species that possess Hb, this molecule (a most direct

Table 1 Species sampled in the Ross Sea (in other Antarctic areas, as indicated)

Suborder	Family	Species
Notothenioidei	Bovichtidae	<i>Cottoperca trigloides</i> (South Georgia)
		<i>Bovichtus diacanthus</i> (Tristan da Cunha)
	Pseudaphritidae	<i>Pseudaphritis urvillii</i> (Tasmania)
	Eleginopsidae	<i>Eleginops maclovinus</i> (Falkland Islands)
	Nototheniidae	<i>Dissostichus mawsoni</i>
		<i>D. eleginoides</i> (sub-Antarctic)
		<i>N. angustata</i> (New Zealand)
		<i>Notothenia coriiceps</i>
		<i>N. rossii</i> (Antarctic Peninsula)
		<i>N. larseni</i> (Antarctic Peninsula)
		<i>Gobionotothen gibberifrons</i> (Antarctic Peninsula)
		<i>Nototheniops nudifrons</i> (Antarctic Peninsula)
		<i>Pagothenia borchgrevinki</i>
		<i>Pleuragramma antarctica</i>
		<i>Trematomus bernacchii</i>
		<i>T. eulepidotus</i>
		<i>T. hansonii</i>
		<i>T. lepidorhinus</i>
		<i>T. loennbergii</i>
		<i>T. newnesi</i>
		<i>T. nicolai</i>
		<i>T. pennellii</i>
		<i>T. scotti</i>
		<i>Aethotaxis mitopteryx</i> (Weddell Sea)
	Harpagiferidae	<i>Harpagifer antarcticus</i> (Antarctic Peninsula)
	Artedidraconidae	<i>Artedidraco loennbergi</i>
		<i>A. orianae</i>
		<i>A. shackletoni</i>
		<i>A. skottsbergi</i>
		<i>Histiodraco velifer</i>
		<i>Pogonophryne scotti</i>
	Bathydraconidae	<i>Acanthodraco dewitti</i>
		<i>Cygnodraco mawsoni</i>
		<i>Gymnodraco acuticeps</i>
		<i>Prionodraco evansii</i>
		<i>Parachaenichthys charcoti</i> (Antarctic Peninsula)
		<i>Bathyrdraco marri</i> (Weddell Sea)
		<i>Racovitsia glacialis</i> (Weddell Sea)
	Channichthyidae	<i>Chaenodraco wilsoni</i>
		<i>Chionodraco hamatus</i>
		<i>C. myersi</i>
		<i>Cryodraco antarcticus</i>
		<i>C. atkinsoni</i>
		<i>Neopagetopsis ionah</i>
		<i>Pagetopsis macropterus</i>
		<i>Chaenocephalus aceratus</i> (Antarctic Peninsula)

Table 1 continued

Suborder	Family	Species
Zoarcoidei	Zoarcidae	<i>Champscephalus gunnari</i> (Antarctic Peninsula)
		<i>Lycodichthys dearborni</i>
		<i>Austrolycichthys brachycephalus</i>
Rajoidei	Rajidae	<i>Lichenchelys nigripalatium</i> (Antarctic Peninsula)
		<i>Raja georgiana</i>
		<i>Bathyrāja eatonii</i>

Table 2 Species sampled in the waters of North East Greenland

Suborder	Family	Species
Zoarcoidei	Anarhcadidae	<i>Anarhichas minor</i>
	Zoarcidae	<i>Lycodes reticulatus</i>
Gadoidei	Gadidae	<i>Arctogadus glacialis</i>
		<i>Boreogadus saida</i>
		<i>Gadus morhua</i>
Cottoidei	Liparidae	<i>Liparis tunicatus</i>
Rajoidei	Rajidae	<i>Raja hyperborea</i>

link between the exterior and body requirements) performs its primary function under extremely variable conditions, and may thus have experienced a major evolutionary pressure to adapt and modify its functional properties. Few proteins have been studied in such a wide array of organisms as Hb.

Hbs, found in bacteria, fungi, plants and animals, are ancient proteins that probably evolved from enzymes that protected against toxic O₂. The Earth's atmosphere was anoxic for about the first 2.7 billion years of its history, then reached the current O₂ levels about 540 mya. The Hb ancestor may then have adapted to scavenge excessive O₂ and/or to be involved in detoxification of NO (Holland, 2006).

Higher-vertebrate Hbs have developed a common molecular mechanism based on ligand-linked conformational change in a multi-subunit structure; generally, the molecule exhibits positive allosteric cooperativity between O₂-binding sites (homotropic interactions). Hb can exist in alternate quaternary molecular conformations (Perutz et al., 1987), with transition between low- and high-affinity states, tense (T) and relaxed (R) (Monod et al., 1965), involved in the modulation of O₂ affinity by physiological effectors (heterotropic interactions), which may preferentially bind to the T or R state, thereby lowering or enhancing the overall O₂ affinity.

Fish are sensitive sentinels of environmental challenge and responses to temperature adaptation. Elucidating molecular mechanisms of adaptation is one of the main goals in evolutionary biology. Evolutionary processes have allowed marine organisms to colonise habitats, yet conserving basic Hb features, e.g. the helical structure, high constancy in the number of amino-acid residues and subunit cooperativity, *vis-à-vis* of widely different selective pressures. The replacement of few key residues may lead to a different function (Perutz, 1983); in polar Hbs thermal adaptation does not seem to depend on specific substitutions, and is probably linked to the combination and interplay of many factors. Cold adaptation of O₂ transport in high-Antarctic notothenioids seems based on levels of biological organisation higher than the Hb structure, including changes in the rate of Hb synthesis and regulation of the amount of transported O₂. These factors are very important for short-term response to environmental challenges. In the same time span, changes in amino-acid sequence altering O₂ affinity would be long-term adaptations, occurring at a much slower rate. Fish may also modulate O₂ delivery by changing the concentrations of effectors, and by expression of multiple, functionally different Hbs.

One of our main study themes has been the O₂-transport system of fish, and also of other marine organisms (see below), notably the structure, function and evolutionary adaptations of haemoproteins (di Prisco et al., 1991a, 1998, 1999, 2007; di Prisco & Giardina, 1996; di Prisco, 2000, 2003; Giordano et al., 2010; Verde et al., 2000, 2011a, b).

We have collected blood samples of virtually all notothenioid species dwelling in the Ross Sea (Table 1). Some Antarctic fish developed unique specialisations, e.g. reduced erythrocyte number and Hb concentration/multiplicity (Everson & Ralph, 1968; Wells et al., 1980). This may be advantageous in coping with increased viscosity of body fluids at low

temperature (Wells et al., 1990), and finds partial compensation in the increased blood volume and higher cardiac output. One family has abolished Hb (Ruud, 1954): the colourless blood of Channichthyidae (icefishes) lacks Hb and functional erythrocytes. These are the only known adult vertebrates showing such an exceptional trait loss (see below).

Our investigations on the haematological features of polar fish showed that most red-blooded species of Notothenioidei (mostly bottom dwellers) have a single Hb (Hb 1) accounting for 95–99% of the total. Hb 1 is accompanied by the minor or “embryonic” components Hb C (in traces) and, in most species of Nototheniidae, by Hb 2, having one of the globins in common with Hb 1 (di Prisco et al., 1988, 1991a, b, 1997; D’Avino et al., 1989, 1992; Kunzman et al., 1992; Giangiacomo et al., 2001; Marinakis et al., 2003; Patarnello et al., 2011; Giordano et al., 2012b). In two active and cryopelagic nototheniids, *Trematomus newnesi* (D’Avino et al., 1994), *Pagothenia borchgrevinkii* (Riccio et al., 2000a), and in the pelagic, sluggish but migratory *Pleuragramma antarctica* (this synonym replaces *Pleuragramma antarcticum*; Eschmeyer, 2014) (Tamburrini et al., 1996), minor Hbs are expressed at higher levels (ca. 25% of the total). Lack of multiple globin genes is not detrimental in thermostable environments, as shown in the modern families (see below). On the other hand, multiple Hbs in active fish are linked to the need to respond to variable conditions associated with different habitats; also, the availability of several α and β genes may provide protection against deleterious mutations in an individual gene.

Species of modern Harpagiferidae, Artedidraconidae and Bathydraconidae, with the exception of *Cygnodraco mawsoni* (Caruso et al., 1991), lack minor Hbs (di Prisco et al., 1990; Kunzman et al., 1991; Caruso et al., 1992; Tamburrini et al., 1992). Intriguingly, Hbs of adult Artedidraconidae are endowed with weak pH and effector regulation and very low cooperativity of O₂ binding (Tamburrini et al., 1998), thus retaining functional properties typical of ancestral organisms, although being “modern”.

The O₂ affinity of Hbs of many high-Antarctic species is quite low, as indicated by high values of p_{50} (partial pressure of O₂ to achieve half-saturation), especially in active species (di Prisco et al., 1988, 1991a).

Unlike notothenioids, sluggish Antarctic zoarcids, have 4–5 electrophoretically distinct Hbs (di Prisco

et al., 1990). The reason for this multiplicity is still unknown; microheterogeneity cannot as yet be excluded.

Trematomus bernacchii Hb 1 was the first fish Hb obtained and described in crystalline form (Camardella et al., 1992), in collaboration with the Nobel Prize laureate Max Perutz. We have also crystallised Hb 1 and Hb C from *T. newnesi* (Mazzarella et al., 1999, 2006a); X-ray diffraction turned out to be an invaluable tool for molecular investigation of the relationship between structure, O₂ binding and R- to T-state transition, in combination with biophysical techniques, e.g. spectroscopy, EPR, light scattering (Riccio et al., 2002; Vergara et al. 2007; Vitagliano et al., 2008; Merlino et al., 2009, 2010; Coppola et al., 2012).

The family Channichthyidae and the reduced role of Hb

Channichthyidae (icefishes) are the notothenioid crown group. Radiation of species within the icefish clade is dated at about 7 mya (Near et al., 2012). Lack of Hb is balanced by high O₂ solubility and low metabolic rates. Icefish developed compensatory adaptations that reduce O₂ demand and enhance O₂ transport (e.g. decreased metabolic rates; enhanced gas exchange by large, well-perfused gills and through scaleless skin; large increases in cardiac output and blood volume). Delivery occurs by transport of O₂ dissolved in the plasma.

The icefish evolution to the Hb-less phenotype arose from large-scale deletional events, which removed all globin genes, including embryonic/juvenile, with the exception of the transcriptionally inactive end of the adult α -globin gene (Cocca et al., 1995, 2000; Zhao et al., 1998; di Prisco et al., 2002). *Neopagetopsis ionah* has a complete, but non-functional, adult $\alpha\beta$ -globin complex (Near et al., 2006). This pseudogene complex may be an intermediate “genomic fossil” revealing key mechanisms on the pathway to loss of expression by all icefish.

Only icefish have taken such a radical course, whereas the other Antarctic families have only partial Hb reduction. The benefits include reduced costs for protein synthesis, simplified metabolic pathways and lower amounts of oxygen radicals. However, the shift to O₂ transport based on diffusion may cause higher

vulnerability to warmer temperatures (Pörtner et al., 2007).

Does Hb remain absolutely vital for adequate O₂ transport in red-blooded notothenioids, or is it a vestigial relict which may be redundant under stress-free living conditions? We have shown that, following gradual reduction of the haematocrit in cannulated red-blooded fish, and reversibly “poisoning” Hb by carbon monoxide (lethal for organisms whose life depends on O₂), survival occurred without visible ill effects. Thus routine O₂ transport is possible without functional Hb, even during bouts of enforced exercise (di Prisco et al., 1992; di Prisco, 2000). Similar to icefish, red-blooded Antarctic fish can carry O₂ in the plasma, suggesting that, in the cold and stable Antarctic sea, Hb is not essential for this function. A single Hb in limited amount may be a consequence of its reduced role as O₂ carrier (Verde et al., 2006c); an additional physiological role might be that of an “O₂ store” under hypoxic or anoxic conditions. In temperate and tropical fish, the essential role of Hb in O₂ transport is undisputed.

Comparison with other areas

Comparison of adaptations of cold-adapted Antarctic notothenioids with sub-Antarctic and temperate notothenioids, and with Arctic fish, has been a powerful tool to understand whether (and to what extent) an extreme environment has required specific adaptations (Verde et al., 2004a, 2006b; Giordano et al., 2006; di Prisco et al., 2007; Coppola et al., 2012). We joined several US and Norwegian cruises, e.g. the 2004 International Collaborative Expedition to collect and study Fish Indigenous to Sub-Antarctic Habitats (ICEFISH; <http://www.icefish.neu.edu/>), at depths ranging from tide pools to the abyss (5,400 m), and the ongoing cruise series of the TUNU-Programme: Euro-Arctic Marine Fishes—Diversity and Adaptation (TEAM-Fish), covering the entire Euro-Arctic triangle.

The sub-Antarctic (and further North)

Bovichtidae, the basal notothenioid family (Eastman, 1993), did not have any of the physiological and biochemical adaptations to the extreme environmental conditions shared by most notothenioids. Unlike Antarctic notothenioids but similar to many other fish,

adult *Cottoperca trigloides* (this synonym replaces *Cottoperca gobio*; Eschmeyer, 2014), thriving in sub-Antarctic waters north of the Polar Front, has two major Hbs sharing the α chain (Giordano et al., 2006, 2009). We observed higher multiplicity also in *B. diacanthus*, one of the most northern notothenioids (Giordano et al., unpublished). This more complex O₂-transport system may have been maintained by positive selection to deal with the large temperature changes experienced by these species thriving north of the APF.

Similar to most high-Antarctic notothenioids, the pseudaphritid *P. urvillii* (Verde et al., 2004a) and the elegendinopsid *E. maclovinus* (Merlino et al., 2010; Coppola et al., 2012) have major Hb 1 and minor Hb 2, sharing the α chain; the latter species, similar to *T. newnesi*, also has Hb C. The low amount of Hb 2 can be a synapomorphy linking *P. urvillii* and *E. maclovinus* to the other notothenioids. *E. maclovinus*, lacking AFGP, is the sister taxon of non-bovichtid and non-pseudaphritid notothenioids. A switch to exclusive expression of the embryonic (minor) β -globin gene has occurred in adult *C. trigloides* (Giordano et al., 2006).

The O₂ affinity is very high in Hbs of non-Antarctic *C. trigloides*, *B. diacanthus*, *P. urvillii* and *E. maclovinus*. Values of p_{50} indicate that a decrease in affinity occurred along the lineage of the high-Antarctic notothenioids, with the exceptions of *P. antarctica*, *T. bernacchii* and *Artedidraco orianae*, whose Hbs have higher O₂ affinity than other Antarctic notothenioids. The relationship between higher O₂ affinity in non-Antarctic notothenioid Hbs and habitat features remains an open question. It is worth noting that 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 haem pocket essentially unmodified with respect to high-Antarctic Hb 1 (Verde et al., 2004b).

The relevance of studying Hbs of non-Antarctic species in the evolution of Notothenioidei is shown by the analysis of the features of the Hb system of another temperate notothenioid, the more recently evolved *Notothenia angustata* (family Nototheniidae), common near the coast of southern New Zealand. The sequence identity between Hbs of non-cold-adapted *N. angustata* and cold-adapted *Notothenia. coriiceps* is

the highest ever found among notothenioids (Fago et al., 1992). *N. angustata* has residual AFGP genes that produce minuscule AFGP quantities (Cheng et al., 2003), which can be slightly increased by cold acclimation. These findings support the hypothesis that, unlike *P. urvillii*, this species had developed cold adaptation before migration from the Antarctic shelf to temperate latitudes in a relatively recent geological time, much later than *P. urvillii*.

The Arctic

Biological comparison of species inhabiting the polar regions pinpoints the differences in evolutionary pressures between the two ecosystems.

Unlike the Southern Ocean, the sea above the Arctic Circle is surrounded by North America, Greenland, Europe and Asia, and is influenced by extensively populated terrestrial areas and industrial activities. Exchange of Atlantic and Arctic waters occurs through the passage between Greenland and the Svalbard Islands.

In the Arctic (and in the Antarctic Peninsula), changes are occurring much faster than in other regions, with dramatic effects on species that depend on sea ice for feeding and reproduction.

Arctic fish have higher biodiversity. Being exposed to seasonal temperature variations, they exhibit higher physiological plasticity than Antarctic fish, exposed to stable temperatures. While cold Arctic conditions have similarly driven evolution of AFGPs in some marine fishes (Chen et al., 1997), many physiological features do not show cold adaptation.

For comparative purposes, we have extended our investigation to Arctic fish species (Table 2).

Many Arctic fish species display Hb multiplicity. For instance, the spotted wolffish *Anarhichas minor*, a benthic, sedentary fish of the family Anarhichadidae (Zoarcoidei), has three functionally distinct major Hbs, whose amino-acid sequences and O₂-binding properties have been described (Verde et al., 2002, 2003a). Three functionally distinct major Hbs have also been observed in three Gadidae, namely the Arctic cod *Arctogadus glacialis*, the polar cod *Boreogadus saida* and the Atlantic cod *Gadus morhua* (Verde et al., 2006a). *A. glacialis*, found in the Greenland fjords and shelf, is sedentary. *B. saida* and *G. morhua* are pelagic and migratory, the former thriving in polar seas, whereas *G. morhua* also thrives

in northern temperate waters. Multiple Hbs provide a strategy for fine regulation of O₂ transport in response to variations in environment and/or metabolic demands (e.g. in physiological hypoxia), illustrating how adaptive modifications of physiological pathways may arise from opportunistic retention of plesiomorphic characters (Verde et al., 2012d).

The O₂-transport system of the liparid *Liparis tunicatus*, e.g. Hb structure/function and globin-gene evolution, resembles that of many species of Antarctic Notothenioidei, i.e. it has a major Hb 1 (the first Arctic Hb obtained in crystalline form) and a minor Hb 2, sharing the α chain, both with low O₂ affinity (Giordano et al., 2007b).

Unlike Antarctic Zoarcidae, the Arctic zoarcid *Lycodes reticulatus* (Riccio et al., 2011) has a single major Hb 1. The Hbs of *L. reticulatus* and of the gadid *G. morhua* have the tendency to polymerise, which seems disulfide driven in the former species. Polymerisation may be a response to stressful conditions.

The structure and function of Hb 1 of *Raja hyperborea* have been studied in comparison with Hb 1 of the Antarctic skate *Bathyraja eatonii*, both lacking the Bohr-proton and the organophosphate binding sites. Hbs of northern and southern polar skates appeared functionally similar, but there are differences with temperate elasmobranchs, suggesting that in temperate and polar habitats physiological adaptations have evolved along distinct pathways, whereas the effect of the differences characterising the two polar environments appears negligible (Verde et al., 2005).

The Root effect in polar fish Hbs

The decreased O₂ affinity of Hb at lower pH values in the physiological range is known as the alkaline Bohr effect. In many fish Hbs, at lower pH, the O₂ affinity may greatly decrease and cooperativity is lost. This feature, named Root effect, has great physiological importance because it regulates O₂ binding and release. It is linked to the presence of one of two anatomical structures requiring high O₂ pressure: the *rete mirabile*, (supplying the gland that inflates swim bladders—absent in notothenioids—with O₂, regulating buoyancy) and the choroid *rete*, a vascular structure which supplies O₂ to the poorly vascularised retina (Wittenberg & Wittenberg, 1974).

Crystallography has been a powerful tool for comparative investigations (Mazzarella et al., 1999,

2006a, b; Vergara et al., 2009, 2010). The question whether the Root effect is related or not to environmental conditions remains to be answered. It is as yet impossible to link the presence or absence of the Root effect to life style. It does not depend on substitutions of a few amino-acid residues, and it may be generated by combination and interplay of several factors.

The Root effect (Verde et al., 2003b, 2007b) is extensively discussed in another article of this Special Issue (Giordano et al., 2015).

Adaptive evolution and phylogeny of fish of Southern and Northern Oceans

The current changes make comparative work at the poles a source of invaluable indications on the evolution of adaptations. The Antarctic and the Arctic share some features, e.g. high latitudes and cold climates, however in many respects they are more dissimilar than similar. Faunal composition and diversity are linked to geological history, hence there are historical, physical and biological differences. The modern faunas differ in physiological tolerance to environmental parameters.

Studying the evolution of organisms from both poles is advantageous. Fish offer many opportunities for comparative approaches to understand cold adaptation and how to counteract ongoing climate changes. Zoarcidae and Liparidae are represented in both oceans, but hypotheses of adaptation to a common environmental parameter have greater certainty in phyletically unrelated taxa, available in both faunas. The comparative approach permits molecule-to-organism analysis of convergent and parallel evolutionary trends to similar habitats. An elegant example is the identity of polar AFGPs (Chen et al., 1997).

The molecular evolution of Hb

To address some of the questions regarding the physiological and biochemical adaptations in polar fish, part of this review is focussed on the phylogeny of Hb (Stam et al., 1997, 1998; di Prisco & Verde, 2006; Verde et al., 2004b, 2006a, b, c, 2007a, 2008; Negrisolo et al., 2008; Lecointre, 2012), based on the sequences of the α and β chains. We have elucidated the primary structure of the vast majority of Hbs of Antarctic and Arctic species of Tables 1 and 2. Hb

phylogeny gives an important contribution to elucidating links between evolutionary adaptation and life style of polar fish, and the Ross Sea is an invaluable scenario.

In Arctic and Antarctic fish, molecular phylogenies are complete enough to allow the interpretation of trees based on primary structures of macromolecules of physiological interest (e.g. globins), in the light of trees based on other molecules used as markers of species phylogeny.

Figures 1 and 2 report the Neighbour Joining trees of α and β globin sequences. The obtained topology is in general agreement with the trees inferred by a Bayesian method (di Prisco et al., 2007) and trees obtained by maximum likelihood analysis (Verde et al., 2006c). As a result of the isolation of Antarctica, the genotype of Notothenioidei diverged with respect to other fish groups in a way interpreted as typical of a species flock (Eastman & McCune, 2000). In the molecular phylogenetic analysis, the globins of major and minor Antarctic fish Hbs cluster in two separate, strongly supported groups, with the globins of temperate fish Hbs forming the first divergence lineage (Verde et al., 2006a, b, 2012c, d).

When evolutionary pressures and rates of change are the same across taxa, similarity is proportional to phylogeny, and the gene (or protein) tree reflects the species tree. The topologies suggest different evolutionary histories for the α and β chains. Globin paralogs (gene copies originated by duplication in a given genome) that currently exist in Antarctic fish diverged 250 mya; hence, unlike AFGPs, that appeared with cooling, Hb diversification appears less stringently correlated to changes in the environment.

Presumably, the two clusters of Antarctic major (adult) and minor (embryonic) Hbs were generated by gene-duplication events which occurred independently for the α - and β -globin genes. In the phylogenetic trees, the basal position of *P. urvillii* and *E. maclovinus* Hbs is congruent with the postulated divergence before the appearance of AFGPs (data not shown). The globin sequences of the Arctic zoarcoid *A. minor* follow the track of species history, as *A. minor* appears close to the notothenioid clades. By contrast, Arctic gadid sequences occupy different positions in the two trees with regard to temperate and Antarctic sequences. The basal position of the β^1 sequences of Arctic gadids in the β -globin tree (Fig. 3) is probably artefactual, whereas the α -globin tree

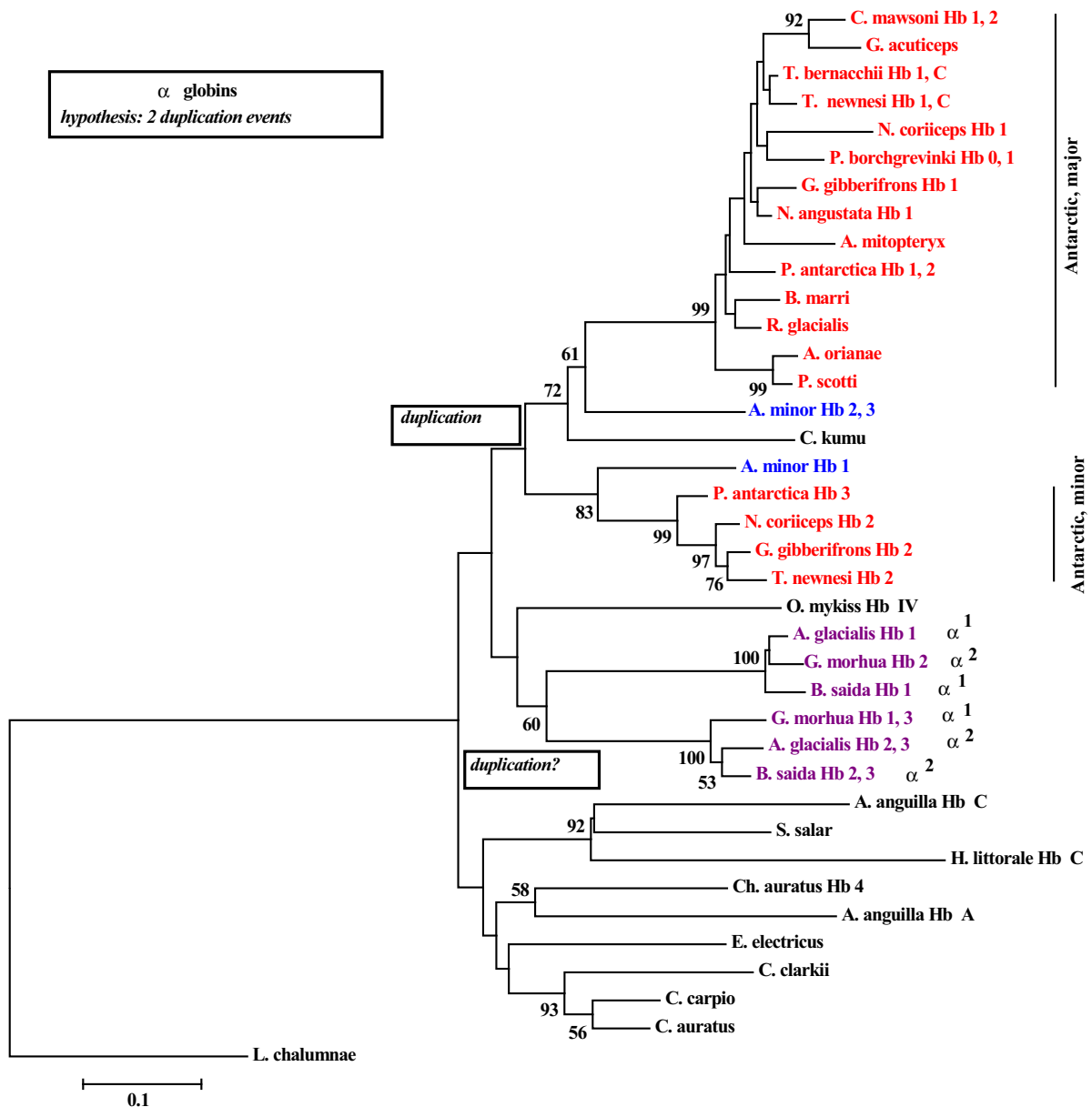


Fig. 2 Neighbour Joining tree of amino-acid sequences of α chains of Arctic, Antarctic and temperate fish Hbs. Bootstrap proportions (BP, percentage of 10,000 replicates) are given at

the nodes. Globin sequences of notothenioids are in red, of zoarcoids in blue, of gadids in purple, of other fishes in black. Adapted from Dettai et al. (2008)

mostly recovers the species tree and includes a few duplications (Fig. 2). Such position may come as an effect of the extreme perturbation of the available mutational space in gadid β^1 -globin sequences, possibly due to the temperature variability experienced by migratory fish in comparison with the thermal stability of notothenioids and zoarcoids, that display

unperturbed phylogenetic signal in β sequences (Dettai et al., 2008).

Other globins

Novel globins, e.g. neuroglobin and cytoglobin, have been recently found in vertebrates (Burmester et al.,

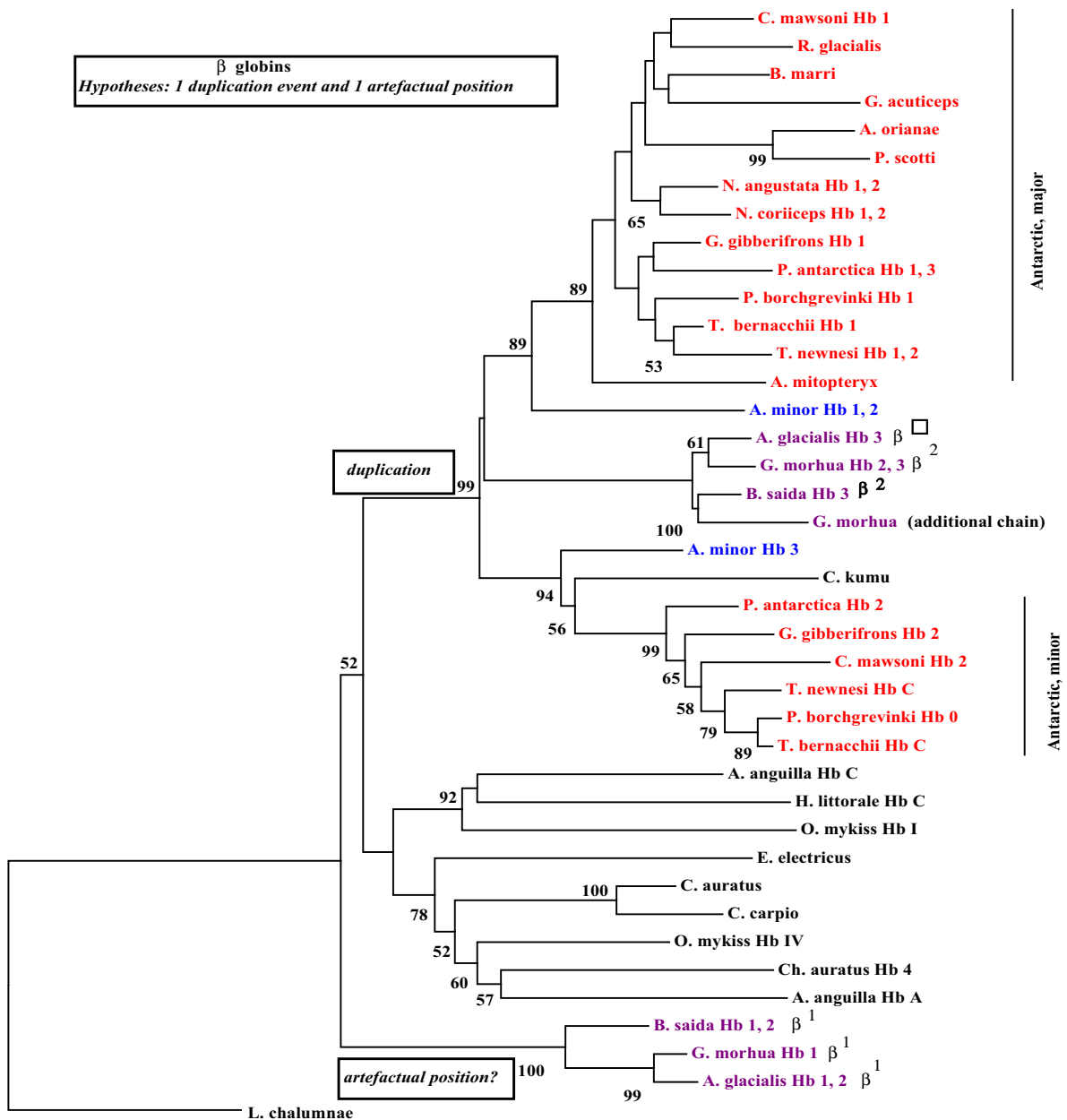


Fig. 3 Neighbour Joining tree of sequences of β chains of Arctic, Antarctic and temperate fish Hbs. For details and colours, see Fig. 2. Adapted from Dettai et al. (2008). In Figs. 2 and 3, besides species mentioned in the text: *G. acuticeps*, *Gymnodraco acuticeps*; *G. gibberifrons*, *Gobionotothen gibberifrons*; *A. mitopteryx*, *Aethotaxis mitopteryx*; *B. marri*, *Bathodracus marri*; *P. scotti*, *Pogonophryne scotti*; *C. kumu*,

Chelidonichthys kumu; *O. mykiss*, *Oncorhynchus mykiss*; *S. salar*, *Salmo salar*; *A. anguilla*, *Anguilla anguilla*; *H. littorale*, *Hoplosternum littorale*; *Ch. auratus*, *Chrysophrys auratus*; *E. electricus*, *Electrophorus electricus*; *C. clarkii*, *Catostomus clarkii*; *C. carpio*, *Cyprinus carpio*; *C. auratus*, *Carassius auratus*; *L. chalumnae*, *Latimeria chalumnae*

2000, 2002). We have recently discovered the neuroglobin gene in the brain of notothenioids, including the icefish family Channichthyidae (Cheng et al.,

2009a, b; Boron et al., 2011; Giordano et al., 2012a). Neuroglobin overexpression produces attenuation of transgenic Alzheimer phenotype in vivo in the

recovery after experimentally induced strokes in mouse, suggesting a role of the protein in Alzheimer's disease (Kahn et al., 2007).

The retention of the neuroglobin gene by icefish, despite the loss of Hb, and of myoglobin in most species, appears very intriguing, and may have important implications in the physiology and pathology of the brain. Whether the gene is expressed is the next question.

Antarctic fish will be a suitable model to learn more about the function of these proteins. The physiological and putative therapeutical importance of these globins is extensively discussed in another article of this Special Issue (Giordano et al., 2015).

Haemoproteins from other marine organisms

Bacteria

With microorganisms, some of the drawbacks in data acquisition have been overcome with the advent of culture-independent molecular tools and large-scale community sequencing. The Southern Ocean is one of the focal regions of microbial ecology; in the Ross Sea, we had the opportunity to investigate a wide variety of extremophiles.

Five non-motile Gram-positive cocci were isolated from saline soils located in geothermally active Mount Erebus (Ross Island) and Mount Melbourne (Terra Nova Bay). The organisms were extremely halotolerant, growing between 0 and 4.2 M NaCl. On the basis of the results of phenotypic characterisations, lipid and quinone analyses and amino-acid composition of the cell wall, the alophiles have been assigned to the genus *Micrococcus* (Nicolaus et al., 1992).

Five isolates of thermophilic bacteria, capable of growing at 65°C were obtained from soil of Cryptogam Ridge (Mount Melbourne) and north of Edmonson Point (Nicolaus et al., 1991). Electron microscopy, morphological and physiological properties, lipid analyses and GC content of the isolates were described. Most features, e.g. optimal growth temperature, some physiological properties and membrane lipids, permit to assign the isolates to the genus *Bacillus*.

We have discovered and characterised a novel thermophilic Gram-positive bacterium, *Bacillus thermoantarcticus* (DSM 9572), isolated from geothermal

soil near the crater of Mount Melbourne (Nicolaus et al., 1996). Taxonomical work has been carried out, as well as characterisation of enzyme content and membrane lipids. It is oxidase-positive, catalase-negative and produces an exopolysaccharide, an exocellular xylanase, an intracellular alcohol dehydrogenase and exo- and endocellular α -glucosidase(s). It optimally grows at 63°C at pH 6.0. It shows an unusually wide range of growth temperature, perhaps an adaptation strategy to extreme cold.

Extensive investigations are ongoing on psychrophilic bacteria. One of the main developments of our work refers to the hemoproteins of Antarctic marine *Pseudoalteromonas haloplanktis* TAC125, whose genome has been sequenced and annotated (Médigue et al., 2005), shedding light on molecular features developed in cold environments. These studies may afford unequivocal conclusions more easily than in vertebrates, that have much more complex physiology and metabolism. Evolution allowed Antarctic marine microorganisms to grow successfully at low temperature and high O₂ content, through a variety of structural and physiological adjustments in their genomes and development of programmed responses to strong oxidative and nitrosative stress (di Prisco et al., 2012b). The availability of genomic sequences from an increasing number of cold-adapted species is providing insights to understand the molecular mechanisms underlying crucial physiological processes.

The genome of *P. haloplanktis* TAC125 contains multiple genes encoding three distinct truncated globins exhibiting the 2/2 α -helical fold. One of them, *Ph-2/2HbO*, displays hexacoordination of the ferric and ferrous haem-Fe atom, and has been extensively characterised by spectroscopic analysis, kinetic measurements and computer simulation. Unique adaptive structural properties enhance the overall flexibility of the protein, so that the structure appears resistant to pressure-induced stress. A genomic mutant strain shows involvement of the cold-adapted globin in the protection against stress induced by high O₂ concentration. Moreover, the protein catalyses peroxynitrite isomerisation in vitro. We analysed (i) how cold temperatures affect physiology of microorganisms, focussing on the molecular mechanisms of adaptation, and (ii), since the physiological role of truncated globins has been demonstrated only in few cases, the structural and functional features of the cold-adapted

globin as an example of a protein having a role in the biology of cold-adapted microorganisms (Giordano et al., 2007a, 2011, 2012b, 2013, and references therein; Russo et al., 2010a, b, 2013; Howes et al., 2011; Verde et al., 2012b).

An important task has been the attempt to shed light on possible multiplicity of functions of cold-adapted *Ph-2/2HbO*. It exhibits a pseudo-enzymatic function involving O_2 and is available for reactions with NO to produce nitrate anions. Under aerobic conditions, *Ph-2/2HbO* protects cells against NO and related RNS (Coppola et al., 2013); conversely, during the anaerobic phase, it may provide NO via nitrite reduction. Moreover, *Ph-2/2HbO* displays a unique adaptive structure increasing flexibility, thus facilitating function at low temperatures (e.g. by enhancing the capacity for correct positioning of ligand(s), which would be made more difficult by a rigid structure).

Modern concepts in biology support the idea that the physiological role of a given molecule is not restricted to a single aspect, although one aspect can be predominant. *Ph-2/2HbO* is a notable case study of relationship between molecular structure (Fig. 4), cold adaptation and a wide range of equally important biological functions (Giordano et al., 2015). The current knowledge is a useful starting point to achieve progress by expanding our understanding of the biological function of fundamentally important macromolecules such as globins, besides *Ph-2/2HbO*. Neuroglobin and cytoglobin, which have

steadily growing biomedical significance, are pertinent examples (Giordano et al., 2013).

Birds

In the study on structure/function relationships in hemoproteins, we investigated adaptations in sequence and O_2 transport-release in some Antarctic birds (Tamburrini et al., 1994, 1999, 2000a, b).

There are rookeries of the diving bird Emperor penguin (*Aptenodytes forsteri*) in the Ross Sea, e.g. near Cape Washington and McMurdo Station. The functional properties of its major Hb 1 have been investigated at different temperatures as a function of proton and organophosphate concentration (Tamburrini et al., 1994). Compared with human HbA, the amino-acid sequence shows 12 substitutions in the contact regions of $\alpha\beta$ dimers. In addition to overall similarities shared with most of the avian Hbs, this Hb shows significant differences, which can be related to life style. In particular (i) the shape of the Bohr-effect curve at 37°C seems well adapted for gas exchange during very prolonged dives, preserving Hb from sudden and uncontrolled stripping of O_2 in response to acidosis. Hence, during diving, O_2 delivery should be essentially modulated by the gas partial pressure at the specific tissue; (ii) the very small enthalpy change observed at lower pH could be another strategy of molecular adaptation, through which O_2 delivery becomes essentially insensitive to exposure to the extreme thermal conditions, and is not impaired at flippers and feet (which experience lower temperature and great muscular activity), allowing the bird to sustain prolonged anaerobiosis. The small alkaline Bohr effect is only chloride-linked, since the pH dependence of the O_2 affinity is totally abolished in the absence of this ion. These functional characteristics appear related to the primary structure of α and β chains.

Similar work was performed on Hbs of chicks and adults of the Adélie penguin (*Pygoscelis adeliae*), also very common in Terra Nova Bay (di Prisco et al., 2000a). The blood of two- to three-week-old Adélie penguin contains six Hbs in approx. equal amounts, five gradually disappearing during development (di Prisco et al., 1997). The adult blood has only one component, Hb 1. A reduced Bohr effect was found in all Hbs. The six Hbs have different O_2 affinities, reduced to a similar extent in the entire pH range by

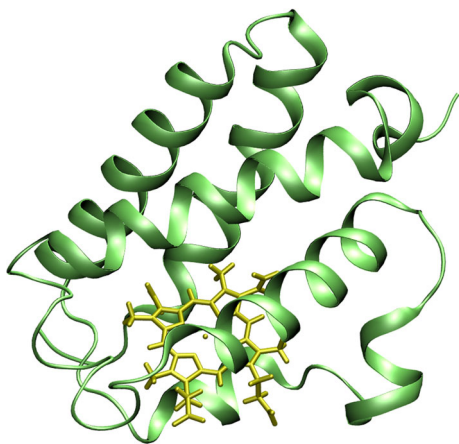


Fig. 4 Structure of *Ph-2/2HbO* (Boechi, unpublished), built by homology modelling. The haem is in yellow

temperature increase. The complete amino-acid sequences of the α and β chains of Hb 1 and chick Hb 4 (having the β chain in common) have been established. Hb 4 can still be found in the blood of two- to three-month-chicks in approx. 10% of the total, comparable with Emperor penguin Hb 2.

In studying molecular adaptations of O₂ storage to the extreme Antarctic conditions, we have investigated the structure/function relationship in *A. forsteri* myoglobin, in search of correlation with the bird life style (Tamburrini et al., 1999). The revised sequence contains one additional residue and 15 differences. The O₂-binding parameters seem well adapted to diving and environmental conditions. Addition of lactate has no major effect on myoglobin oxygenation over a large temperature range, hence metabolic acidosis does not impair myoglobin function under conditions of a prolonged physical effort such as diving. Emperor penguins can dive more than 300-m deep, and remain under water for as long as 20–30 min. Myoglobin significantly contributes to O₂ storage, as also evidenced by its very high level.

Another seabird breeding in coastal Antarctic regions, and very common in the Ross Sea, is the south polar skua (*Catharacta maccormicki*). The other common species, the more aggressive brown skua (*C. antarctica*) commonly breeds in the Antarctic Peninsula. Skuas are known for their aggressive rapacious behaviour. They have a rapid, sustained and powerful flight, enabling them to overtake many birds, and prey on fish and krill and also on chicks and eggs, particularly those of penguins. Their behavioural and physiological characteristics differ from those of penguins.

Catharacta maccormicki has two Hbs with identical β chains. Their amino-acid sequence and O₂-binding properties have been investigated (Tamburrini et al., 2000b; Riccio et al., 2000b, 2001). The two Hbs show peculiar functional features, probably acquired to meet special needs in relation to extreme conditions. Both Hbs showed a weak alkaline Bohr effect which, during prolonged flight, may protect against sudden and uncontrolled stripping of O₂ in response to acidosis due to the temperature increase brought about by the great muscular activity. In thermodynamic behaviour, the different values of heat of oxygenation of the two Hbs suggest different functional roles and reflect the need for specialised O₂ carriers during migrations through environments

characterised by varying temperatures. In contrast to Emperor penguin Hb, which has a chloride-dependent, weak Bohr effect (Tamburrini et al., 1994), the skua Hbs show the Bohr effect also in the absence of chloride. This feature is in common with Adélie Hb (di Prisco et al., 2000a).

Both Hbs bind phosphate at two distinct sites. An additional site is located between the two α chains, increasing the probability of capturing phosphate and transferring it to the main site located between the two β chains, thereby favouring O₂ release. This study on interactions between phosphate and Hb, analysed for the first time at the molecular level also by molecular dynamics simulation (Riccio et al., 2001), highlights the role of Lys99 α and the N and C α -termini in the additional site, and indicates phosphate migration along the central cavity from the additional to the main site, from which it can be released to return into solution. Thus the central cavity, so far considered devoid of a specific function, would instead have an important functional role.

An additional site for polyphosphates with a role in O₂ transport has been suggested in other Hbs (Amiconi et al., 1985). The role of the additional phosphate binding site may be related to the molecular physiology of Hb. Under physiological conditions, it would enhance the ability of Hb to capture phosphate from the solution and readily transfer it to the main binding site which is not far, by means of a site–site migratory mechanism, thus acting as an entry-leaving site.

The additional phosphate binding site can also contribute to over-stabilise the T structure. In fact, this stabilising effect could partially counterbalance the reduced effect of pH on O₂ affinity, favouring controlled release of O₂ under conditions of physiological stress, for instance during prolonged flights. The observation that Lys99 α is strongly conserved supports the hypothesis that most Hbs possess a potential additional site, whose physiological relevance may be linked to evolutionary adaptations and life style.

Mammals

Balaenoptera physalus (fin whale) and *B. acutorostrata* (minke whale) are migratory mammals which may reach the Ross Sea. We have performed studies on their Hbs and O₂-transport system (di Prisco et al., 1991b; Corda et al., 2003).

The functional features of Hb of *B. physalus* have been studied as a function of heterotropic effector concentration and temperature. Particular attention has been given to the effect of CO₂ and lactate since the animal is specialised for prolonged dives often in cold water. The molecular basis of the functional behaviour and in particular of the weak interaction of Hb with 2,3-diphosphoglycerate has been analysed in the light of the primary structure and of computer modelling. The A2(Pro → Ala) substitution in the β chains may be responsible for the displacement of helix A, known to be a key structural feature in Hbs that display an altered interaction with 2,3-diphosphoglycerate as compared with human Hb. In combination with the study on Hb of *B. acutorostrata*, we obtained further insights into the regulation of the interactive effects of temperature, CO₂ and lactate. The temperature dependence of the interaction with CO₂ is linked to the lower temperature at fins and tail, where there is a great muscular activity and CO₂ facilitates O₂ unloading, as in human HbA. Such behaviour may also occur in the lungs, depending on the temperature of the breathed air. In fact, elimination of CO₂ at the lungs would increase the O₂ affinity, facilitating O₂ loading.

The Weddell seal (*Leptonychotes weddellii*), very common in the Ross Sea, is the most southerly mammal in the world. It lives on or under the Antarctic fast ice, and has an extended diving ability which allows to cover considerable distances underwater without breathing. We have investigated the structure/function of the two almost identical major Hbs (present in similar amounts and sharing the β chain) as a function of pH and temperature, in the absence and presence of organic effectors (di Prisco et al., 2000a).

The effect of CO₂ on O₂ binding has been studied for the first time. Hb 1 and Hb 2 showed similar O₂ affinities in all experimental conditions used, whereas the pH dependence of cooperativity of O₂ binding and of the heat of oxygenation was slightly different. The alkaline Bohr effect of both Hbs was enhanced by organophosphates. A striking feature is the temperature dependence of the CO₂ effect. Addition of CO₂ produced a substantial decrease of O₂ affinity at 20°C, restored to a large extent at 37°C and fully by organophosphates. Thus, within the core of the seal huge body, CO₂ does not display any allosteric effect because at 37°C its differential binding with respect to oxy and deoxy structure is abolished. This allows Hbs

to maintain adequate O₂ delivery to power the fins and tail having temperatures well below 37°C, facilitated by CO₂. The combined effect of CO₂, organophosphates and temperature on Hbs optimises O₂ delivery to all tissues in spite of the seal heterothermia.

Other proteins from fish, echinoderms and bacteria

In addition to hemoproteins, marine organisms of the Ross Sea are a source for investigating many other proteins. We have addressed our interest to enzymes and metallothioneins, also in collaboration with other teams of experts.

Enzymes

Fish

A raise in water temperature will affect metabolism (Somero, 2010, 2012, and references therein). To study molecular cold adaptation, it is essential to understand the biochemical mechanisms enabling efficient enzymatic catalysis under the environmental extreme conditions. Cold-adapted organisms have evolved mechanisms to offset the reduced activity rate due to the Q₁₀ effect, either through higher number of enzyme molecules or higher catalytic rate per enzyme molecule.

Taking advantage of the wealth of organisms that populate the Ross Sea, our research has addressed the structural and functional characterisation of enzymes having key roles in the metabolism of cold-adapted Antarctic organisms, with special attention to thermodynamics. Two notothenioid enzymes we studied are glucose-6-phosphate dehydrogenase (G6PD) from the erythrocytes of red-blooded *Dissostichus mawsoni* and blood cells of the icefish *Chionodraco hamatus*, and L-glutamate dehydrogenase (GDH) from the liver of the icefish *Chaenocephalus aceratus* (Ciardiello et al., 1995, 1997a, b, c, 1999a, b, 2000).

G6PD catalyses the first reaction of the pentose-phosphate pathway (oxidation of D-glucose-6-phosphate to D-glucono-δ-lactone-6-phosphate by reduction of NADP⁺). It controls the rate of producing NADPH (used in lipid biosynthesis and in protecting the cell from oxidative stress) and 5-phosphoribosyl pyrophosphate (used in RNA synthesis). In erythrocytes glucose is the primary energy source, and G6PD

is involved in the production of metabolites which can enter the glycolytic pathway, and of NADPH, necessary as coenzyme of glutathione reductase and essential for the activity of catalase.

In icefish G6PD, the temperature effect has found compensation through both higher enzyme level and higher catalytic rate per enzyme molecule. Some levels of the molecular structure have been conserved during cold adaptation (similar molecular mass, number of subunits, amino-acid sequence of the active site, temperature of irreversible heat inactivation). However, the analysis of catalytic and thermodynamic properties reveals a range of differences, some of which (higher catalytic efficiency, lower activation energy, shift toward low temperatures of the apparent optimum activity) are in common with other cold-adapted enzymes (Feller et al., 1992; Davail et al., 1994) and show that over the range 0–15°C Antarctic G6PD is a better catalyst than mesophilic G6PD.

GDH has a key regulatory function in cellular metabolism: it catalyses the reversible oxidative deamination of L-glutamate to α -ketoglutarate and ammonia through reduction of NAD^+ or NADP^+ . It has an important role in controlling levels of ammonia and glutamate and in feeding amino groups into the urea cycle. In the brain, where glutamate is an important neurotransmitter, glutamate production and degradation must be finely regulated.

Compared to mesophilic counterparts, catalytic and thermodynamic properties reveal important differences also in GDH, e.g. the shift of apparent optimum temperature to a lower value, the higher k_{cat} at low temperature, and resistance to hydrostatic pressure, suggesting that Antarctic GDH is cold adapted. The sequence of the active site reveals strikingly high identity with bovine, chicken and tuna GDH, including essential Lys126. With both enzymes, evolution has made enzymatic catalysis suitable for the low temperature of the Antarctic, at the same time ensuring conservation of molecular structure.

A few other investigated enzymes having key metabolic roles in cold-adapted Antarctic fish (icefish in particular) are simply mentioned here: (i) Cu, Zn superoxide dismutase (Natoli et al., 1990), a ubiquitous enzyme dismuting O_2^- peroxide into O_2 and hydrogen peroxide; phosphorylase *b* (Ciardiello et al., 1997c), which breaks up glycogen releasing glucose-1-phosphate, then converted to glucose-6-phosphate by phosphoglucomutase, establishing the metabolic

link with G6PD; (ii) a novel aspartic proteinase (Capasso et al., 1998) showing unique features (the physiological functions include digestion of proteins); and (iii) gill carbonic anhydrase (Rizzello et al., 2007), a family of zinc enzymes catalysing reversible hydration of CO_2 , linked to a role in the defence against oxidative damage; in fact during exercise lack of Hb may produce release of ROS during hypoxia and re-oxygenation, also counteracted by glutathione bound to Cys residues.

Bacteria

Psychrobacter sp. TAD1 is a psychrotolerant bacterium from Antarctic frozen water, growing from 2 to 25°C (optimum at 20°C). It has two GDHs, with different cofactor specificities, subunit sizes/arrangements and thermal properties. NADP^+ -dependent GDH is a hexamer of 47 kDa subunits, comparable to other hexameric GDHs of bacteria and lower eukaria. NAD^+ -dependent GDH has a subunit mass of 160 kDa and belongs to the large-subunit class. It is a dimer: such an oligomeric arrangement has never been reported. Both enzymes have optimum activity at 20°C, but their activities and thermal stabilities are different. NAD^+ -dependent GDH is more cold-active: at 10°C it retains 50% of its maximal activity, compared with 10% for the NADP^+ -dependent enzyme; the temperature optimum is shifted towards lower temperature; it has higher thermostability. NADP^+ -dependent GDH is more heat stable, and apparently less cold adapted, losing only 10% of activity after heating for 30 min, compared with 95% of NAD^+ -dependent GDH. Thus, not only does NAD^+ -dependent GDH have a novel subunit molecular mass and arrangement, but its polypeptide chains are folded differently, thus the two enzymes have distinct cold-active properties (Di Fraia et al., 2000; Camardella et al., 2002).

Aspartate transcarbamylase is the first enzyme of the pyrimidine pathway, catalysing carbamylation of the amino group of Asp at low temperature. It is allosterically regulated. In contrast to the enzyme from *Escherichia coli*, in TAD1 it is not stimulated by ATP and shows an impressive activity at low temperature (Sun et al., 1998). At 0°C, where *E. coli* has no activity, its activity is 26% of its maximal at 30°C (25°C lower than the optimal temperature of the *E. coli* enzyme). In comparison with the latter, and similar to

GDH and G6PD of cold-adapted fish, kinetic and thermodynamic characterisation indicate that high activity at low temperature is linked to increased catalytic efficiency, possibly due to a modification at the active site.

Enzymes from psychrophiles are receiving growing attention due to their relevance for both basic and applied research. Indeed, cold-adapted enzymes may offer novel opportunities in many industrial processes in which high activity at low temperature and low thermostability are required. There is need for cold-adapted bacterial enzymes, suitable for exploitation in industrial processes, e.g. as additives in detergent and food industries, biocatalysts, and in bioremediation processes, minimising energy consumption, reducing the risk of microbial contamination and avoiding temperature instability of reactants or products. One hundred and fifty-five strains were isolated in Terra Nova Bay, and screened for lipolytic activity as a function of temperature (Lo Giudice et al., 2006; Tutino et al., 2009). Most of the isolates have esterase and lipase activities with different responses with various lipids. Lipases and esterases catalyse hydrolysis (and synthesis) of an ester bond. They have wide-ranging versatility in industrial applications. For instance, cold-active lipases are expected to be applicable as additives to detergents used at low temperatures and biocatalysts for biotransformation of heat-labile compounds. The psychrotolerant nature of the isolates allows them to be used for bioremediation of fat-contaminated aqueous systems at low and moderate temperature (4–30°C). They can be distinguished on the basis of substrate specificity. Lipases are esterases displaying maximal activity towards water-insoluble long-chain acylglycerols ($\geq C10$); esterases are able to hydrolyse ester substrates with short-chain fatty acids ($\leq C10$) at least partly soluble in water. Lipases act in a wide range of salt concentration, pH and temperature, with differences in lipolytic activity between strains. Thus, the isolates can provide enzymes suitable for specific industrial processes whose conditions cannot be modified at leisure.

By sequencing the 16S rDNA gene fragment of six γ -proteobacteria of the genera *Pseudoalteromonas*, *Psychrobacter* and *Vibrio*, we identified optimal conditions for expression of lipolytic activity of marine bacteria at low temperature. The genome of *P. haloplanktis* TAC125 was searched for genes encoding ester-hydrolysing enzymes. We cloned and

over-expressed a gene encoding a putative esterase/lipase; the recombinant product (*Ph*TAC125 Lip1) is associated to the cell outer membrane. Sequence alignment showed that it is related distantly to other lipolytic enzymes, but closely to other psychrophilic esterases/lipases. The common features of the aligned proteins strongly suggest that *Ph*TAC125 Lip1 is the first functionally characterised member of a novel lipase family of cold-adapted marine γ -proteobacteria (de Pascale et al., 2008). Membrane fluidity is a key factor in cold adaptation, and psychrophiles may have evolved *ad hoc* enzymes responsible for adaptation to temperature fluctuations, having the role of regulating such fluidity.

Metallothioneins

Metallothionein (MT) is a low-molecular-mass protein rich in thiol groups, considered involved in the protection from the effects of toxic heavy metals, e.g. cadmium and mercury, and used as an environmental marker for metal pollution. It can also act as scavenger of free hydroxyl and superoxide radicals. Biosynthesis can be induced by oxidative stress.

Organisms from yeast to mammals contain MT. MTs have been studied in relation to their putative roles in heavy-metal toxicology, but they are implied in various functions besides detoxification, e.g. protection against stress factors and having a role in mineral metabolism.

The most striking peculiarity of MT is the particular arrangement of the twenty Cys residues, organised in two distinct metal-thiolate domains (a, b). The highly conserved chemical structure of mammalian MTs suggests similar features. But what about other organisms? We have studied MTs of red-blooded and Hb-less Notothenioidei, and showed that MTs of this fish group display remarkably unique features in evolution, expression pattern, structure and function.

MT was not found in icefish liver (Scudiero et al., 1992a), which has a low-molecular-mass zinc protein displaying totally different features, i.e. low Cys and high Glu and Asp content, probably involved in metal-complex formation, but it is not clear whether this protein can fulfil some, if not all, MT functions. In contrast, MT was found in the liver of red-blooded notothenioids.

Although the lack of MT in icefish (Scudiero et al., 1997) with concomitant expression of non-translated

mRNA was intriguing, induction of MT synthesis by cadmium (Carginale et al., 1998) indicated that MT production is only temporarily suppressed. Presumably, owing to reduced oxygenation due to the lack of an O₂ carrier, metabolism is slowed down and production of free radicals occurs at a lower rate. In addition, the Antarctic environment with its low pollution is expected to have little or no effect on MT induction by heavy metals.

That MTs play an important role in Antarctic fish is demonstrated by the presence of a tissue-specific expression of the two isoforms found in all the notothenioid species examined.

The structural similarity of the a- and b-domains in MT of different origins suggests that the MT gene evolved by duplication of an ancestral gene encoding a polypeptide folding one of the two domains. The evolution of two domains may be due to differences in function, the more stable a-domain being involved in metal detoxification, and the more labile b-domain in zinc homeostasis (Zangger et al., 2001).

The chemical, structural and dynamic features of *N. coriiceps* MT differ markedly from those of its mammalian counterpart (Scudiero et al., 2000; Capasso et al., 2006, and references therein). In general, fish and mammal MT show different flexibilities, possibly linked to differences in location of a Cys in the a-domain and in electrostatic charge.

In the eggs of the Antarctic sea urchin *Stereochinus neumayeri*, heat stable, low-molecular-mass zinc-containing polypeptides with unusual primary structure were found (Capasso et al., 1997; Scudiero et al., 1992b): (i) the Cys content is lower than that of vertebrate MTs, but higher than that of non-MTs from vertebrate gonads; (ii) the His, Asp and Glu contents are high, suggesting that these residues may join Cys as additional metal-binding sites.

Concluding remarks

Polar organisms are exposed to strong environmental constraints. We need to understand how they have adapted in the past to cope with these challenges, and to what extent current climate changes will impact on adaptations in the future. The recognition of the important role of the poles in Global Change, and of the control that Antarctica exerts on climate and ocean circulation of the Earth, has awakened great interest in the evolutionary

biology of the organisms that live there, leading to many important SCAR programmes (see Foreword).

The Antarctic is a natural laboratory and the Ross Sea is one of its most important sectors, open to a vast array of disciplines. In contrast to the Arctic and the Peninsula, the Ross Sea is not hit by warming (Bracegirdle et al., 2008; Turner et al., 2009b), but this might only be temporary.

Marine biology has easy access to complex ecosystems and richness of organisms, from mammals to microbes, and the scenario is ideal for integrating biological and physical aspects of oceanography. The Ross Sea is endowed with an impressive density of logistic facilities. The year-round McMurdo Station and Scott Base became active in the 50's and MZS in 1986; in recent years, the Ross Sea is being selected by other nations to install their stations. Thanks to investigations facilitated by this infrastructure network, the suborder Notothenioidei is one of the best known fish groups in the world for many aspects, in particular the molecular bases of adaptations to the extreme conditions of this marine environment.

This article summarises the extensive 30-year-contribution offered by the authors' team in the Ross Sea. Fundamental questions have been addressed in a wide array of marine organisms, related to molecular cold adaptations evolved along with progressive cooling in this geographic area. When relevant, comparisons have been conducted with other areas of primary importance, such as the Peninsula, the Weddell Sea, the sub-Antarctic and the Arctic. In fact, in recent years, the urge to extend these studies to the north has become stronger; comparison with the Arctic is developing in the framework of the IPY programme Team-Fish (Christiansen, 2012).

There is already compelling evidence for widespread changes in polar ecosystems due to climate change (Turner et al., 2009a; Convey et al., 2009). The study of organisms adapted to extreme polar conditions will allow to look at the impact and consequences of anthropogenic challenges and the role played by temperature in establishing species distribution (Kennicut et al., 2014).

The challenging agenda for the next decade will be to incorporate thinking along the physiological/biochemical viewpoint into evolutionary biology. Such an integrative approach can provide answers to the question of how polar marine organisms will respond, and whether they will be able to develop resilience, to

ongoing Global Warming, already in full action in the Antarctic Peninsula and in the Arctic, and foreseen to occur soon in the Ross Sea. The latter is the only region that exhibits some increase in sea-ice extent, while other regions experience a decrease. The importance of comparing the resilience of organisms thriving in the as yet unimpacted Ross Sea with those of the warming Antarctic Peninsula (and with the Arctic) will steadily increase, also because of possible predictions regarding lower latitudes.

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