Cold-Driven Hemoglobin Evolution in Antarctic Notothenioid Fishes Prior to Hemoglobin Gene Loss in White-Blooded Icefishes

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Abstract

Expression of multiple hemoglobin isoforms with differing physiochemical properties likely helps species adapt to different environmental and physiological conditions. Antarctic notothenioid fishes inhabit the icy Southern Ocean and display fewer hemoglobin isoforms, each with less affinity for oxygen than temperate relatives. Reduced hemoglobin multiplicity was proposed to result from relaxed selective pressure in the cold, thermally stable, and highly oxygenated Antarctic waters. These conditions also permitted the survival and diversification of white-blooded icefishes, the only vertebrates living without hemoglobin. To understand hemoglobin evolution during adaptation to freezing water, we analyzed hemoglobin genes from 36 notothenioid genome assemblies. Results showed that adaptation to frigid conditions shaped hemoglobin gene evolution by episodic diversifying selection concomitant with cold adaptation and by pervasive evolution in Antarctic notothenioids compared to temperate relatives, likely a continuing adaptation to Antarctic conditions. Analysis of hemoglobin gene expression in adult hematopoietic organs in various temperate and Antarctic species further revealed a switch in hemoglobin gene expression underlying hemoglobin multiplicity reduction in Antarctic fish, leading to a single hemoglobin isoform in adult plunderfishes and dragonfishes, the sister groups to icefishes. The predicted high hemoglobin multiplicity in Antarctic fish embryos based on transcriptomic data, however, raises questions about the molecular bases and physiological implications of diverse hemoglobin isoforms in embryos compared to adults. This analysis supports the hypothesis that the last common icefish ancestor was vulnerable to detrimental mutations affecting the single ancestral expressed alpha- and beta-globin gene pair, potentially predisposing their subsequent loss.

Key words: **LA and MN hemoglobin clusters, cryonotothenioid, Eleginopsioidea, *Eleginops maclovinus*, adaptive evolution, thornfish.**

Introduction

Oxygen is essential for energy metabolism in most living organisms, and, in vertebrates, oxygen circulates throughout the organism bound to heme groups in hemoglobin proteins that are packed into red blood cells (erythrocytes). Hemoglobin proteins are heterotetrameric proteins composed of 2 alpha-globin and 2 beta-globin subunits (Storz 2018). In teleost fishes, hemoglobin genes are organized in 2 separate gene clusters, each containing mostly paired alpha- and beta-globin genes, in contrast to mammals, in which 1 locus contains alpha-globin genes and a second locus contains beta-globin genes. The teleost "LA" and "MN" clusters originated by duplication of a single ancestral gene cluster with the teleost genome duplication (Amores et al. 1998; Postlethwait et al. 1998; Taylor et al. 2003) and were named based on genes that lie on one side of the clusters in teleosts: *lcmt1* and *aqp8* for the LA cluster and *mpg* and *nprl3* for the MN cluster (Hardison 2008; Opazo et al. 2013). Hemoglobin genes are presumably under strong natural selection that allows species to adapt to different environmental and physiological conditions due to biochemical adaptations such as temperature dependence of oxygen affinity and sensitivity to pH enabling the Bohr and Root effects (Verde et al. 2008; Wells 2009; Storz 2016).

Antarctic fish of the suborder Notothenioidei (notothenioids) inhabit the icy, hyperoxygenated waters of the Southern Ocean. According to the most recent genome-wide, time-calibrated molecular phylogeny, notothenioids

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first evolved approximatively 47 million years ago (MYA) (Bista et al. 2023). A few families of notothenioids diverged before the establishment of freezing conditions in the Southern Ocean and are nowadays represented by the sub-Antarctic families Bovichtidae (thornfishes), and the monotypic families Pseudaphritidae and Eleginopidae composed of *Pseudaphritis urvillii* off southern Australia and *Eleginops maclovinus* off southern South America, respectively (Near et al. 2012). After the Southern Ocean reached its current constantly cold conditions after the mid-Miocene climate transition (MMCT) 13 to 15 MYA (Leutert et al. 2020, 2021; Westerhold et al. 2020), Antarctic notothenioids, or cryonotothenioids, underwent an adaptive radiation starting about 10.7 MYA (Bista et al. 2023). The evolutionary innovation of antifreeze glycoproteins (AFGPs) permitted cryonotothenioids to survive and thrive in the Southern Ocean in near-freezing waters vacated by most competitors and predators (DeVries 1988; Eastman 1993; Chen et al. 1997; DeVries and Cheng 2005).

Among cryonotothenioids, the white-blooded icefishes of the Channichthyidae family are evolutionary oddities that have fascinated evolutionary biologists and the general public for decades because they are the only known vertebrate species living without hemoglobin (Ruud 1954; Sidell and O'Brien 2006; Cheng and Detrich 2007; Garofalo et al. 2009). While the loss of hemoglobin genes happened in the icefish common ancestor after its divergence from Antarctic dragonfishes (Eastman 1993; Near et al. 2006), we still do not know the underlying mechanisms that led to this unique phenotype. Without hemoglobin, icefish blood is translucent white (see Sidell and O'Brien 2006; Beck et al. 2022 for images) and carries oxygen only by dissolution in the blood plasma, which represents only ~10% of the oxygen carried in the blood of their red-blooded relatives that express hemoglobin (Ruud 1954; Holeton 1970). Whether the loss of hemoglobin in icefishes was an adaptive trait or a maladaptive accidental evolutionary event remains a mystery (Sidell and O'Brien 2006; Daane et al. 2020). Nevertheless, this profound state of anemia required physiological compensations, including changes in the vascular system leading to an enlarged heart pumping a much larger blood volume throughout an extended vasculature with large bore capillaries compared to red-blooded relatives of similar size (Sidell and O'Brien 2006; Garofalo et al. 2009; Beck et al. 2022) and was accompanied by changes in conserved non-coding elements located next to anemia-related genes (Daane et al. 2020).

Even among red-blooded Antarctic notothenioids, however, the oxygen transport system already presents unusual characteristics, presumably linked to cold adaptation. Antarctic red-blooded notothenioids generally have lower hematocrit, reduced hemoglobin concentrations, lower hemoglobin affinity for oxygen, and reduced root effect compared to their non-Antarctic sister species (Eastman 1993; di Prisco et al. 2007; Verde et al. 2007, 2008). These evolved physiological characteristics have been proposed to be adaptive by lowering the viscosity of the blood permitted by high levels of ambient dissolved oxygen and low metabolic rates associated with life in the icy Southern Ocean (Wells et al. 1990; di Prisco et al. 1991; Eastman 1993). Although these physiological parameters distinguish cold adapted from temperate notothenioids, we still lack a full understanding of the genomic evolution of hemoglobin genes in notothenioids and an actual demonstration that hemoglobin genes indeed evolved along with the advent of cold adaptation to reveal the molecular bases of these evolved phenotypes.

Most strikingly, while teleost fish generally express multiple hemoglobin protein isoforms with differing physiochemical properties (hemoglobin multiplicity, e.g. Verde et al. 2008; Wells 2009; Verde et al. 2012; Storz 2018; Andersen 2020), cryonotothenioids display a widespread reduction in hemoglobin multiplicity (di Prisco et al. 1990, 1991, 2007; Kunzmann 1991; Verde et al. 2012; Giordano et al. 2015). Hemoglobin protein multiplicity has often been proposed to be an adaptive trait enabling appropriate oxygenation in changing environments (e.g. seasonally hypoxic or fluctuating temperatures), changing physiological conditions (e.g. acidosis following muscular effort), and life stages with embryos, larvae, and adults usually expressing different hemoglobin paralogs leading to the so-called embryonic, larval, and adult hemoglobin denominations (e.g. Powers 1980; Verde et al. 2012; Opazo et al. 2013; Baalsrud et al. 2017; Pan et al. 2017; Barts et al. 2018; Storz 2018; Andersen 2020). Thus, the blood of non-Antarctic notothenioids and other Perciformes, including Antarctic eelpouts (di Prisco et al. 1990), contains multiple major hemoglobin isoforms (di Prisco et al. 1990, 1991, 2007; Kunzmann 1991; Giordano et al. 2015). In contrast, most cryonotothenioids of the paraphyletic group Nototheniidae (notothens) express a single major hemoglobin isoform, which generally represents about 95% of total hemoglobin molecules, and 1 or a few minor isoforms representing a low percentage of total hemoglobin molecules. Plunderfishes (Harpagiferidae and Artedidraconidae) and dragonfishes (Bathydraconidae), the lineages most closely related to icefishes, display a further reduction in hemoglobin multiplicity and greater decrease in the affinity of hemoglobin for oxygen (Kunzmann 1991; di Prisco et al. 1991; Tamburrini et al. 1998; Verde et al. 2006; Giordano et al. 2015). Adults of all studied species in these groups, except for the dragonfish *Cygnodraco mawsoni*, also have a single hemoglobin isoform in circulation (di Prisco et al. 1990; Caruso et al. 1992; Verde et al. 2008). The loss of hemoglobin multiplicity in these high-Antarctic fishes was thus proposed to be related to reduced selective pressure for different hemoglobin subtypes with different capacities in the thermally stable and highly oxygenated Antarctic environment (Verde et al. 2006, 2008; di Prisco et al. 2007). The molecular genetic bases for the reduction in hemoglobin multiplicity in cryonotothenioids remain, however, unknown, and we also do not know how this stepwise reduction went the ultimate step further to complete abrogation of hemoglobin multiplicity in icefishes.

Phylogenetically broad genomic analysis of hemoglobin gene evolution in notothenioids has been stymied until

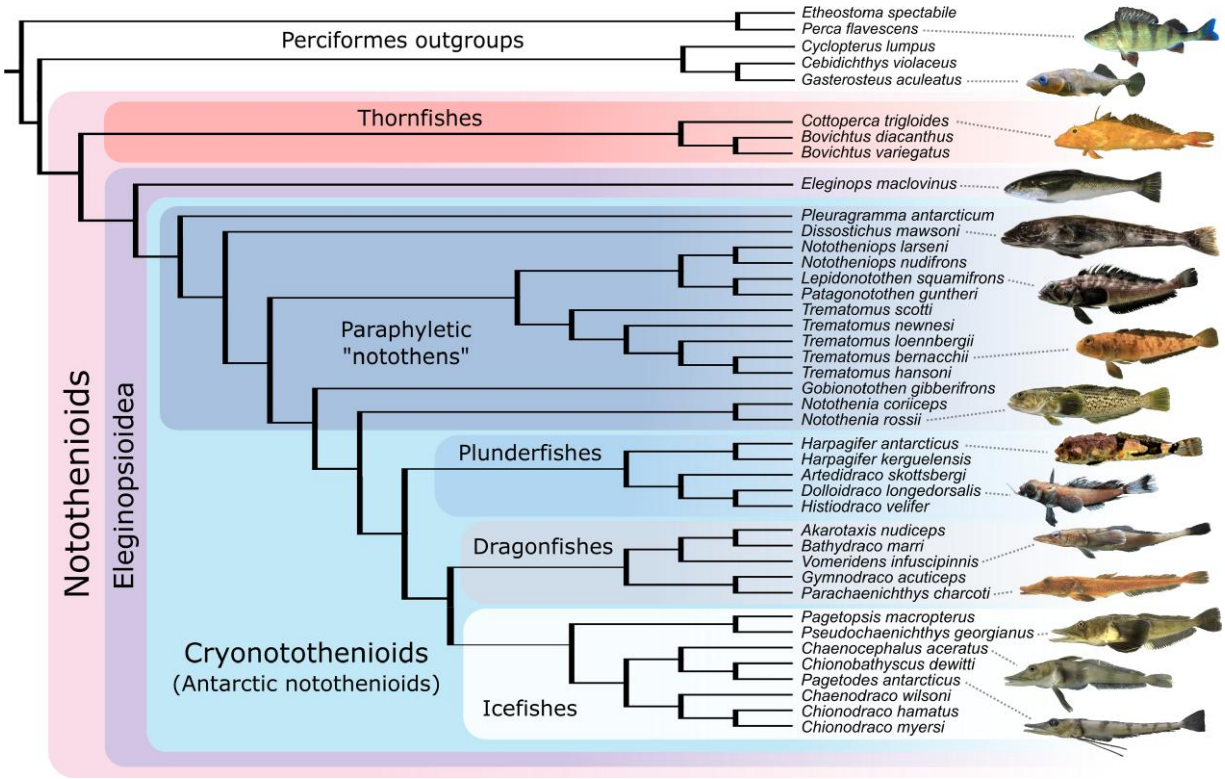

Fig. 1. Notothenioid phylogeny. Phylogenetic relationships of the 36 notothenioids and 5 nonnotothenioid Perciformes studied adapted from (Near et al. 2018).

now by the lack of genomic resources permitting the clear establishment of gene orthologs across species. We recently generated new genome assemblies throughout the notothenioid radiation and investigated the structure of hemoglobin loci based on the first complete reconstruction of both hemoglobin loci across multiple species (Bista et al. 2023). Here, we further studied the evolution of hemoglobin genes in 36 notothenioids broadly distributed across the radiation and 5 perciform outgroups (Fig. 1) to address 3 hypotheses: (i) that life in the frigid Antarctic environment exerted selective pressure on hemoglobin gene content and evolution, (ii) that the observed decrease in hemoglobin multiplicity in cryonotothenioids and further in plunderfishes and dragonfishes is due to either loss of hemoglobin genes or loss of hemoglobin gene expression, and (iii) that mutations leading to alterations in hemoglobin protein function in icefish ancestors predisposed their subsequent loss. To test these 3 independent and nonexclusive hypotheses, we retraced the genomic evolution of hemoglobin genes and their sequences in both hemoglobin clusters along the notothenioid radiation from early diverging temperate notothenioid species to icefishes and surveyed hemoglobin gene expression patterns in hematopoietic organs at the transcriptomic level in embryonic and adult life stages. We further analyzed the rates of evolution of each orthologous gene at different branches of the phylogeny, between different groups of species, and at the level of amino acid sequence and the level of 3D structure.

Results showed that hemoglobin genes evolved substantially during the period of cold adaptation in cryonotothenioid ancestors and that, compared to temperate environments, the icy Antarctic environment further exerted pervasive natural selection on hemoglobin genes. Furthermore, results establish a clear relationship between embryonic and adult hemoglobin gene expression patterns and hemoglobin isoforms, thus uncovering the molecular genetic bases for the long-standing question concerning the reduction of hemoglobin multiplicity observed in cryonotothenioid lineages. Finally, our results provide clues supporting the hypothesis that detrimental alterations to hemoglobin genes could have set suboptimal conditions potentially contributing to subsequent hemoglobin gene loss in the icefish ancestor after it diverged from dragonfishes.

Results

Relative Stability of the LA Cluster

In red-blooded notothenioids, the LA cluster is composed of 2 alpha-globin genes and 1 or 2 beta-globin genes (Bista et al. 2023). Here, we analyzed the evolution of the notothenioid LA cluster in 22 species with contiguous cluster assemblies and included individual gene sequences from up to 13 additional species depending on assembly contiguity (supplementary tables S1 and S2, Supplementary Material online). Hemoglobin gene nomenclature follows the system proposed by Bista et al. (2023) (see Materials and Methods for a summary of the nomenclature system). No LA cluster hemoglobin genes were found in the yellowfin notie

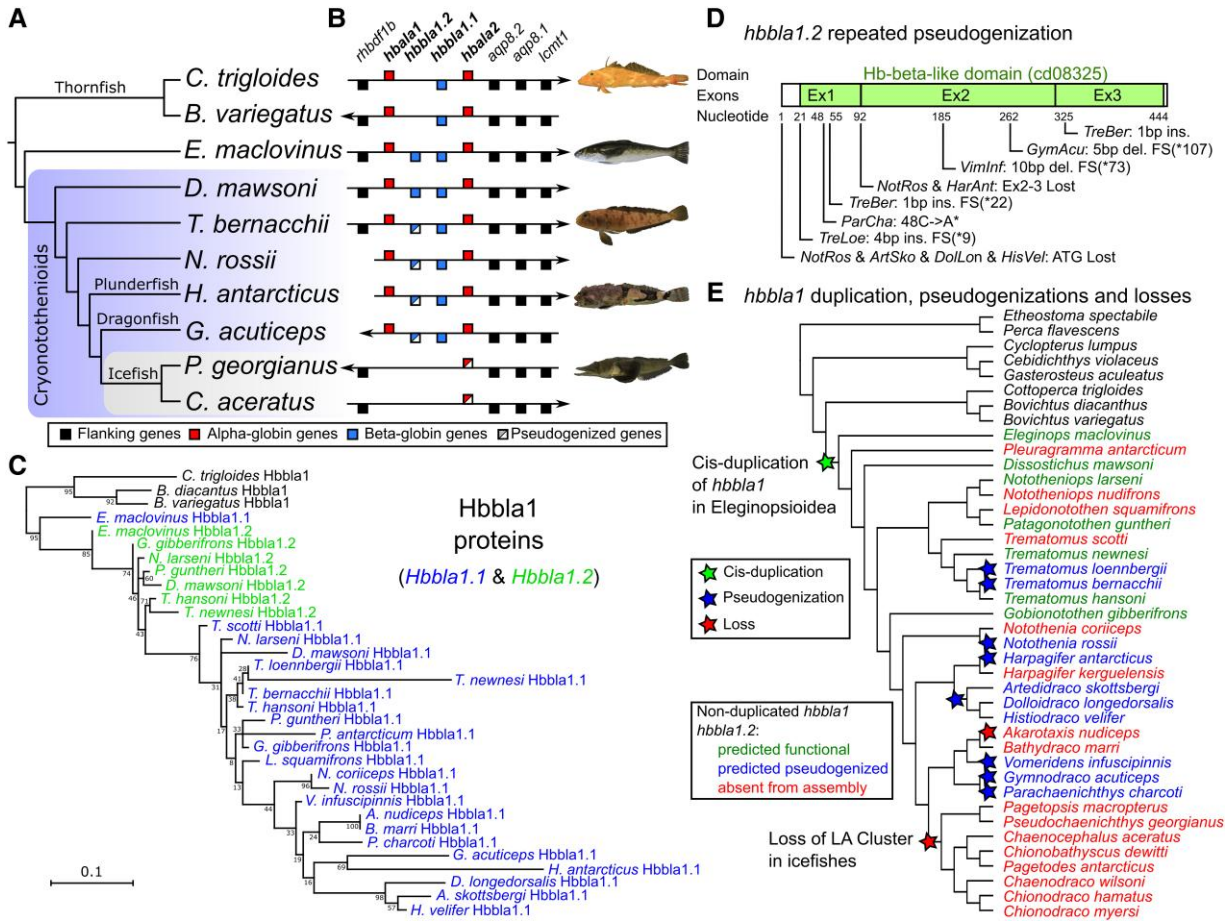

FIG. 2. Evolution of the LA cluster. A) Phylogenetic relationships and B) schematic representation of the genomic organization of the LA cluster and neighboring genes in selected species covering the major notothenioid families (not to scale). Alpha-globin genes (*hbala*) and beta-globin genes (*hbbla*) are represented along with genes flanking the clusters. Pseudogenes are represented with half-filled squares. Genes above the line are transcribed toward the right, and genes below the line are transcribed toward the left. Arrows for the entire cluster represent orientations in each assembly. C) In Eleginopsioidea, the protein Hbbla1.1 evolved rapidly but was retained in all studied species. In contrast, Hbbla1.2 did not substantially evolve, but the gene *hbbla1.2* was repeatedly pseudogenized or lost in many lineages and species. D) Repeated pseudogenization of *hbbla1.2* in cryonotothenioids. E) Duplication, pseudogenization, and losses of *hbbla1* genes in notothenioids.

Notothenios nudifrons genome assembly, likely due to extensive fragmentation of this assembly rather than true gene loss. In temperate thornfish species, including *Cottoperca trigloides*, 1 LA cluster beta-globin gene, *hbbla1* (abbreviated as *β-la1* for ease of reading), is positioned between the 2 alpha-globin genes *hbala1* (*α-la1*) and *hbala2* (*α-la2*) but on the opposite strand (Fig. 2A and B) (Bista et al. 2023), as in other Perciformes (Hotaling et al. 2023). In the Eleginopsioidea (*E. maclovinus* and cryonotothenioids), however, we recently revealed a previously unknown tandem duplication of *β-la1* that became *β-la1.1* and *β-la1.2* in the common ancestor of Eleginopsioidea after divergence from thornfishes (Fig. 2A and B and supplementary fig. S1A, Supplementary Material online) (Bista et al. 2023). In the 8 icefish species studied here, the entire LA cluster except the third exon of the *α-la2* gene was lost, as shown previously (Zhao et al. 1998; di Prisco et al. 2002; Near et al. 2006; Bargelloni et al. 2019; Kim et al. 2019; Bista et al. 2023).

Protein sequences for the alpha-globin subunits from the LA cluster, α-la1 and α-la2, are well conserved across red-blooded notothenioids, with strong sequence identity especially among cryonotothenioids for α-la1 (supplementary fig. S1A, Supplementary Material online) and seemingly higher sequence divergence for α-la2 in plunderfishes and dragonfishes compared to other cryonotothenioids (supplementary fig. S1B, Supplementary Material online) (Bista et al. 2023).

In contrast, while the *E. maclovinus* tandemly duplicated β-la1 proteins remained relatively similar to each other in sequence and to the nonduplicated thornfish ortholog, as seen by both duplicates branching near the base of the phylogenetic tree, tandemly duplicated β-la1.1 and β-la1.2 proteins diverged significantly from one another in cryonotothenioids and group by paralogs (Fig. 2C and supplementary fig. S1A, Supplementary Material online). Cryonotothenioid β-la1.2 proteins show relatively low sequence evolution compared to their *E. maclovinus* and thornfish orthologs as seen by overall relatively short branches, while β-la1.1 proteins

display substantial sequence evolution compared to the *E. maclovinus* ortholog and greater variation among cryonotothenioid species, with especially long branches in plunderfishes and dragonfishes and in the notothen *Trematomus newnesi* (Fig. 2C and supplementary fig. S2, Supplementary Material online). Notably, none of the divergent cryonotothenioid *β-la1.1* genes were predicted to be nonfunctional (e.g. no frameshift or premature nonsense mutations) and *β-la1.1* was present in all studied genome assemblies of red-blooded notothenioids, except for the fragmented assemblies of *Harpagifer kerguelensis* and *N. nudifrons*. In contrast, genes encoding the otherwise relatively stable β-la1.2 proteins are absent from the assemblies or display various deleterious mutations in many cryonotothenioids (Fig. 2D), revealing repeated independent pseudogenization events and losses of *β-la1.2* in many lineages (Fig. 2E) (Bista et al. 2023).

In summary, the study of genome assemblies from 35 notothenioid species shows that the evolution of the LA cluster involved (i) the tandem duplication of *β-la1* in the common ancestor to Eleginopsioidea after divergence from thornfishes as shown in Bista et al. (2023); (ii) the widespread sequence divergence of cryonotothenioid β-la1.1 during and following cold adaptation and the sequence stability of retained *β-la1.2* genes despite their repeated loss or pseudogenization in many cryonotothenioids; and (iii) the genomic deletion of the LA cluster preserving only the third exon of *α-la2* in the icefish ancestor after it diverged from dragonfishes (Near et al. 2006; Bargelloni et al. 2019; Kim et al. 2019; Bista et al. 2023).

Dynamic Evolution of the MN Cluster

In notothenioid evolution, the content of the MN cluster varied more than the content of the LA cluster. Here, we analyzed the evolution of the notothenioid MN cluster in the 12 species that have contiguous cluster assemblies and included gene sequences from up to 20 additional species depending on the hemoglobin gene and assembly contiguity (supplementary table S3, Supplementary Material online). As we previously observed in Bista et al. (2023), the least developed notothenioid MN cluster, found in *E. maclovinus*, contains 3 alpha-globin and 3 beta-globin genes and the most extended MN cluster, present in the dragonfish *Gymnodraco acuticeps*, has 11 alpha-globin and 13 beta-globin genes (Fig. 3A and B and supplementary table S3, Supplementary Material online). In icefishes, hemoglobin genes of the MN cluster are absent from all 8 studied genome assemblies, although 6 of the 8 studied genome assemblies are contiguous in that region and show the flanking genes *kank2* and *nprl3* present and in conserved synteny, as previously shown for 4 of them (Bargelloni et al. 2019; Kim et al. 2019; Bista et al. 2023).

In red-blooded notothenioids, MN cluster genomic evolution is marked by numerous gene duplications and pseudogenization events (Fig. 3A and B) as we previously observed in Bista et al. (2023). Here, we discuss these results further and make additional observations on the structure of the MN cluster. Among nonnotothenioid Perciformes, MN clusters also generally display large variations in gene copy number with tandem duplications of any gene of the cluster although most frequently of the *hbamn1* (*α-mn1*) and *hbbmn1* (*β-mn1*) gene pair (Hotaling et al. 2023). In notothenioids, detected tandem gene duplications were, however, restricted to the *α-mn1* and *β-mn1* gene pair (Fig. 3A and B). In notothenioids (Bista et al. 2023) and in other Perciformes (Hotaling et al. 2023), several tandem duplicates underwent independent pseudogenizations (Fig. 3A and B). Notably, in all species for which multiple *α-mn1* and *β-mn1* paralogs could be retrieved, α-mn1 and β-mn1 tandemly duplicated protein sequences group by species instead of by orthologs in protein phylogenetic trees (supplementary figs. S3 and S4, Supplementary Material online), suggesting lineage-specific tandem duplications or concerted evolution among hemoglobin paralogs.

A few additional gene losses also characterize the evolution of the MN cluster in cryonotothenioids. Notably, all cryonotothenioids with contiguous assemblies lack an alpha-globin *α-mn1* gene between the flanking gene *kank2* and the first beta-globin *β-mn1* gene (Fig. 3A and B) as observed in Bista et al. (2023). This shared absence suggests that this *α-mn1* gene was lost in the cryonotothenioid ancestor after it diverged from the *E. maclovinus* lineage. A few other losses also merit a comment because they concerned *hbamn2* (*α-mn2*) and *hbbmn2* (*β-mn2*) that otherwise were conserved and unduplicated in notothenioids and did not show substantial sequence divergence based on protein phylogenetic trees (supplementary figs. S3 and S4, Supplementary Material online). In the spiny plunderfish *Harpagifer antarcticus*, the *β-mn2* gene is absent, and in the dragonfish *G. acuticeps*, both *α-mn2* and *β-mn2* are absent (Fig. 3A and B) (Bista et al. 2023). However, *β-mn2* is present in all 3 studied barbeled plunderfishes and in 3 other dragonfishes (supplementary fig. S4, Supplementary Material online); therefore, *β-mn2* losses in *H. antarcticus* and in *G. acuticeps* are independent and convergently occurred in the *H. antarcticus*' lineage and in the *G. acuticeps*' lineage. Contiguous genome assemblies of additional plunderfish and dragonfish species are necessary to reveal if these losses were species specific or shared by other members of these 2 groups. Similarly, *α-mn2* is present in the genome assemblies of 3 studied dragonfishes (supplementary fig. S3, Supplementary Material online), but its presence is uncertain in the dragonfish *Parachaenichthys charcoti*. Therefore, the loss of *α-mn2* in *G. acuticeps* could either be species specific or shared by other dragonfish species.

Despite the independent loss of *α-mn2* and *β-mn2* in the plunderfish *H. antarcticus* and the dragonfish *G. acuticeps*, these losses focus attention on the fact that among studied cryonotothenioids, only plunderfish and dragonfish species display alterations to the MN cluster at these genes. Notably, while β-mn1 and β-mn2 paralogous proteins are highly similar and group by species in perciform outgroups and in thornfishes, in Eleginopsioidea, β-mn1 and β-mn2 proteins group by paralogs instead of by

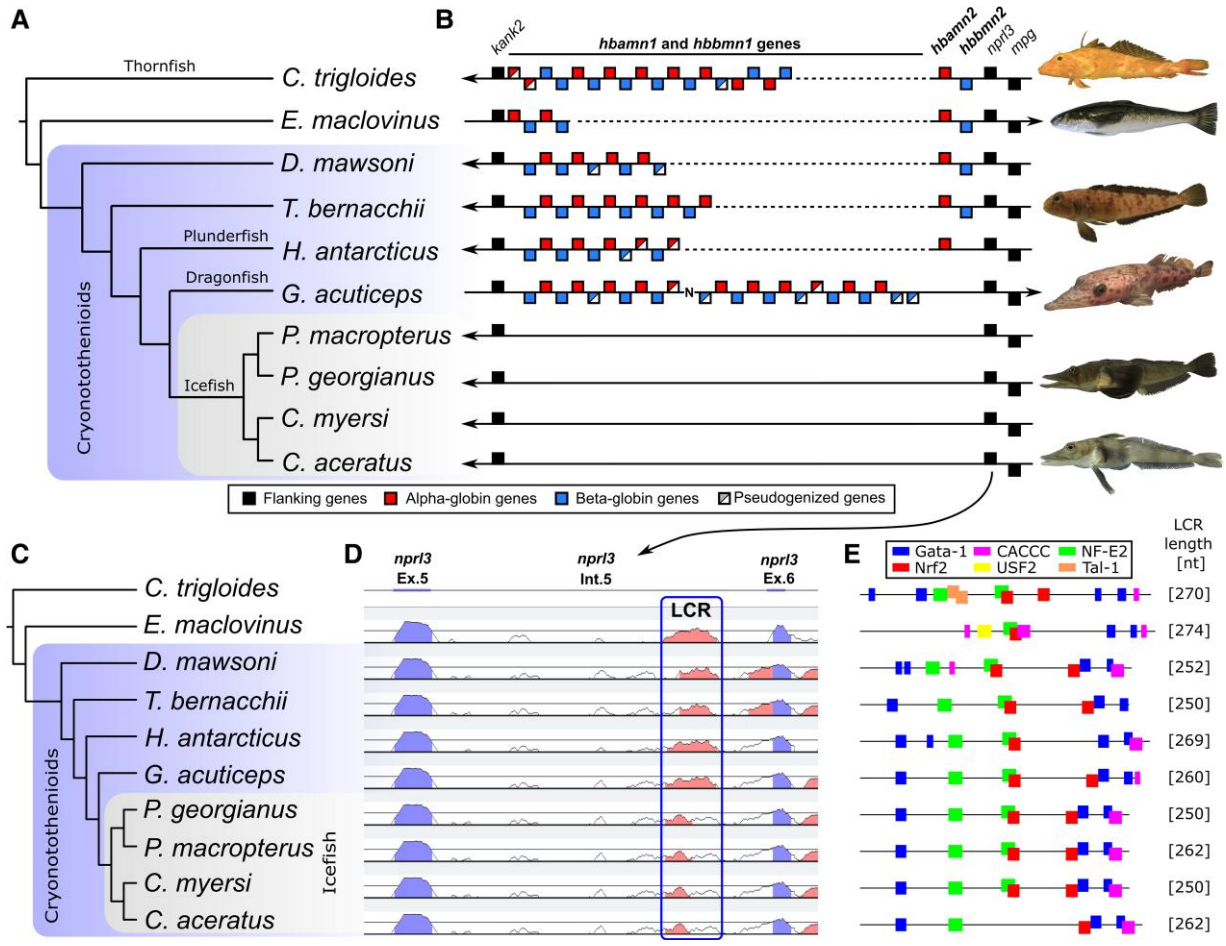

FIG. 3. Evolution of the MN cluster. A) Phylogenetic relationships and B) schematic representation of the genomic organization of the MN cluster and neighboring genes in selected species covering the major notothenioid families (not to scale). Alpha-globin genes (*hbamn*) and beta-globin genes (*hbbmn*) are represented along with genes flanking the clusters. Pseudogenes are represented with half-filled squares. Genes above the line are transcribed toward the right, and genes below the line are transcribed toward the left. Arrows for the entire cluster represent orientations in each assembly. C) Phylogenetic relationships and D) VISTA alignments of the MN cluster LCR located between exons 5 and 6 of the neighboring gene *nprl3* in selected species using the Channel bull blenny *C. trigloides* as reference. E) Despite less global sequence identity in icefishes, most major transcription factor binding sites present in the LCR of red-blooded Antarctic species are also present in icefish LCRs. The length of the LCR in nucleotides for each species is provided in brackets and is drawn to scale.

species, revealing the divergence of β-mn paralogs in the *E. maclovinus* and cryonotothenioid common ancestor after its divergence with thornfishes. Protein sequence divergence is particularly marked for β-mn2 in the Eleginopsioidea ancestor and further in plunderfishes and in dragonfishes as seen by relatively longer phylogenetic branches for β-mn2 proteins in these groups (supplementary fig. S4, Supplementary Material online), indicating rapid evolution potentially culminating in the loss of the *β-mn2* gene in *H. antarcticus* and *G. acuticeps*.

In summary, the study of the MN cluster in notothenioid genome assemblies expands on previous observations (Bista et al. 2023) and shows that (i) the gene content of the MN cluster dynamically evolved in all notothenioid lineages with numerous lineage-specific duplications and pseudogenizations of the *α-mn1* and *β-mn1* gene pairs; (ii) β-mn2 sequences diverged from their β-mn1 counterparts in the common ancestor of Eleginopsioidea after their lineage diverged from thornfishes; (iii) *β-mn2* genes evolved rapidly or were independently lost in some plunderfish and dragonfish lineages; and (iv) the entire MN cluster was deleted in the icefish ancestor after it diverged from dragonfishes.

Together, these results reveal that the LA and the MN clusters evolved dramatically differently with the MN cluster experiencing numerous lineage-specific tandem duplications compared to the relative stability of the LA cluster and the LA cluster retaining a hemoglobin gene fragment in icefishes while losing the entire MN cluster.

Sequence Conservation in the Icefish MN Cluster LCR

While regulatory elements controlling expression of the LA cluster hemoglobin genes are not well conserved in teleost fish in general and in notothenioids specifically (Lau et al. 2001, 2012; Philipsen and Hardison 2018), the expression of

MN cluster hemoglobin genes is regulated by a highly conserved *cis*-acting noncoding regulatory element, the MN locus control region (LCR), located between exons 5 and 6 of the flanking gene *nprl3* (Ganis et al. 2012; Hardison 2012; Philipsen and Hardison 2018). One hypothesis to explain the loss of hemoglobin genes in icefishes could be that regulatory elements driving the expression of hemoglobin genes were altered or lost to cause the expression of hemoglobin in inappropriate cells or amounts in the icefish ancestor and that subsequent hemoglobin gene deletion was a way to relieve that hypothesized detrimental misexpression. Using the zebrafish LCR sequence (Ganis et al. 2012) as reference in an mVISTA alignment (Frazer et al. 2004), we identified and retrieved LCR sequences from phylogenetically diverse notothenioid species, including 4 icefish species (Fig. 3C to E). The sequences of putative LCRs in notothenioids remained rather stable in sequence and in length, ranging from 250 nucleotides in *Trematomus bernacchii*, *Pseudochaenichthys georgianus*, and *Chionodraco myersi*, to 274 nucleotides in *E. maclovinus* (Fig. 3E). Analysis of transcription factor binding sites for major hematopoietic regulators in the notothenioid LCR revealed, surprisingly, that most, if not all, transcription factor binding sites present in the LCRs of red-blooded notothenioids were conserved in the corresponding LCR sequence in icefishes (Fig. 3E). Functional experiments are needed to test the hypothesis that the icefish LCR sequence has the regulatory capacity to drive gene expression in red blood cells. Nonetheless, strong sequence conservation suggests that the functional conservation of the MN cluster LCR was not dramatically altered in the icefish ancestor and therefore makes the hypothesis unlikely that LCR loss precipitated loss of the MN cluster in icefishes. These results also raise the question of what mechanism led to the retention of the LCR sequence in all studied icefish species even though no hemoglobin genes remain to respond to that regulation.

Adult Hemoglobin Gene Expression

To understand the contribution of each hemoglobin gene to functional hemoglobin tetramers in adults, we reanalyzed publicly available RNA-sequencing data from 6 notothenioids and the European perch *Perca fluviatilis* as a Perciformes outgroup (Fig. 4 and supplementary tables S4 and S5, Supplementary Material online) (Xu et al. 2015; Pasquier et al. 2016; Berthelot et al. 2019; Chen et al. 2019; Bista et al. 2020).

We first observed that expression of *α-la1*, *α-mn1*, and *β-mn1* was not detectable in the head kidney or spleen of adult Perciformes, including the European perch or any notothenioids (Fig. 4), despite the *α-mn1* and *β-mn1* gene pair being present in numerous copies in all studied species. Perciformes generally express multiple hemoglobin isoforms (Verde et al. 2008), consistent with our observation of the expression in adult perch of 2 alpha-globin genes, *α-la2* and *α-mn2*, and 2 beta-globin genes, *β-la1* and *β-mn2*, thus potentially producing at least 4 tetrameric hemoglobin protein isoforms: $\alpha_2^{la2}\,\beta_2^{la1}$ (i.e. hemoglobin tetramers with 2 α subunits from the *α-la2* gene and 2 β subunits from the *β-la1* gene), $\alpha_2^{la2}\,\beta_{2,}^{mn2}$ $\alpha_2^{mn2}\,\beta_{2,}^{la1}$ and $\alpha_2^{mn2}\,\beta_2^{mn2}$. In the thornfish *C. trigloides*, expression of only 2 alpha-globin genes and 1 beta-globin gene was abundantly detected in the adult spleen (Fig. 4), suggesting the formation of 2 major hemoglobin isoforms: $\alpha_2^{la2}\,\beta_2^{mn2}$ and $\alpha_2^{mn2}\,\beta_{2,}^{mn2}$ consistent with previous observations reporting the presence of only 2 hemoglobin isoforms sharing the same beta-subunits in adults of this species (Giordano et al. 2009).

In *E. maclovinus* and all cryonotothenioids (i.e. Eleginopsioidea) with available data, adult hemoglobin gene transcripts are largely dominated by the *α-la2* alpha-globin gene and the *β-la1.1* beta-globin gene (Fig. 4), consistent with the presence of a single major hemoglobin isoform representing from 70% up to 99% of expressed hemoglobin in all previously studied adult Eleginopsioidea species (Verde et al. 2008; Coppola et al. 2010). The absence of transcripts from the *β-la1.2* duplicate in all species agrees with its pseudogenization or loss in many Eleginopsioidea species (Fig. 4). In adult *E. maclovinus*, 3 hemoglobin isoforms were previously observed with the predominant isoform sharing the beta chain with a second isoform and the alpha chain with a third isoform (Coppola et al. 2010). The major isoform therefore likely corresponds to $\alpha_2^{la2}\,\beta_2^{la1.1}$, and because *α-mn2* and *β-mn2* are the only 2 hemoglobin genes other than *α-la2* and *β-la1.1* with transcripts detected in adult spleen and head kidney, the 2 minor isoforms thus likely correspond to $\alpha_2^{la2}\,\beta_2^{mn2}$ and $\alpha_2^{mn2}\,\beta_2^{la1.1}$. In adult *Dissostichus mawsoni* and *T. bernacchii* head kidneys, *α-mn2* and *β-mn2* transcripts are further reduced in quantity relative to *α-la2* and *β-la1.1* transcripts (Fig. 4), in agreement with the report of the major isoform accounting for over 95% of the total hemoglobin in these species (di Prisco et al. 1991; Camardella et al. 1992), which therefore logically corresponds to the $\alpha_2^{la2}\,\beta_2^{la1.1}$ isoform. In adult spiny plunderfish *H. antarcticus* and adult dragonfish *G. acuticeps*, only *α-la2* and *β-la1.1* transcripts were detected in head kidneys (Fig. 4), in agreement with the presence of a single hemoglobin protein isoform in adults of these species (di Prisco et al. 1991; Tamburrini et al. 1992), thus logically $\alpha_2^{la2}\,\beta_2^{la1.1}$ as well.

Embryonic Hemoglobin Gene Expression

Because embryos and adults usually express different hemoglobin paralogs leading to the so-called embryonic and adult hemoglobin denominations (Opazo et al. 2013; Storz 2018), we analyzed RNA-sequencing data from whole embryos of the European perch *P. fluviatilis* and of the dragonfish *G. acuticeps* (Fig. 5) (Flynn et al. 2015; Pasquier et al. 2016), the only 2 species of our study for which such data are available. In striking contrast to the expression of hemoglobin genes in adults (Fig. 5A and B) and in embryos of both perch and dragonfish, *α-mn1* and *β-mn1* paralogs are highly expressed (Fig. 5C and D). In perch embryos, *α-mn1* and *β-mn1* paralogs

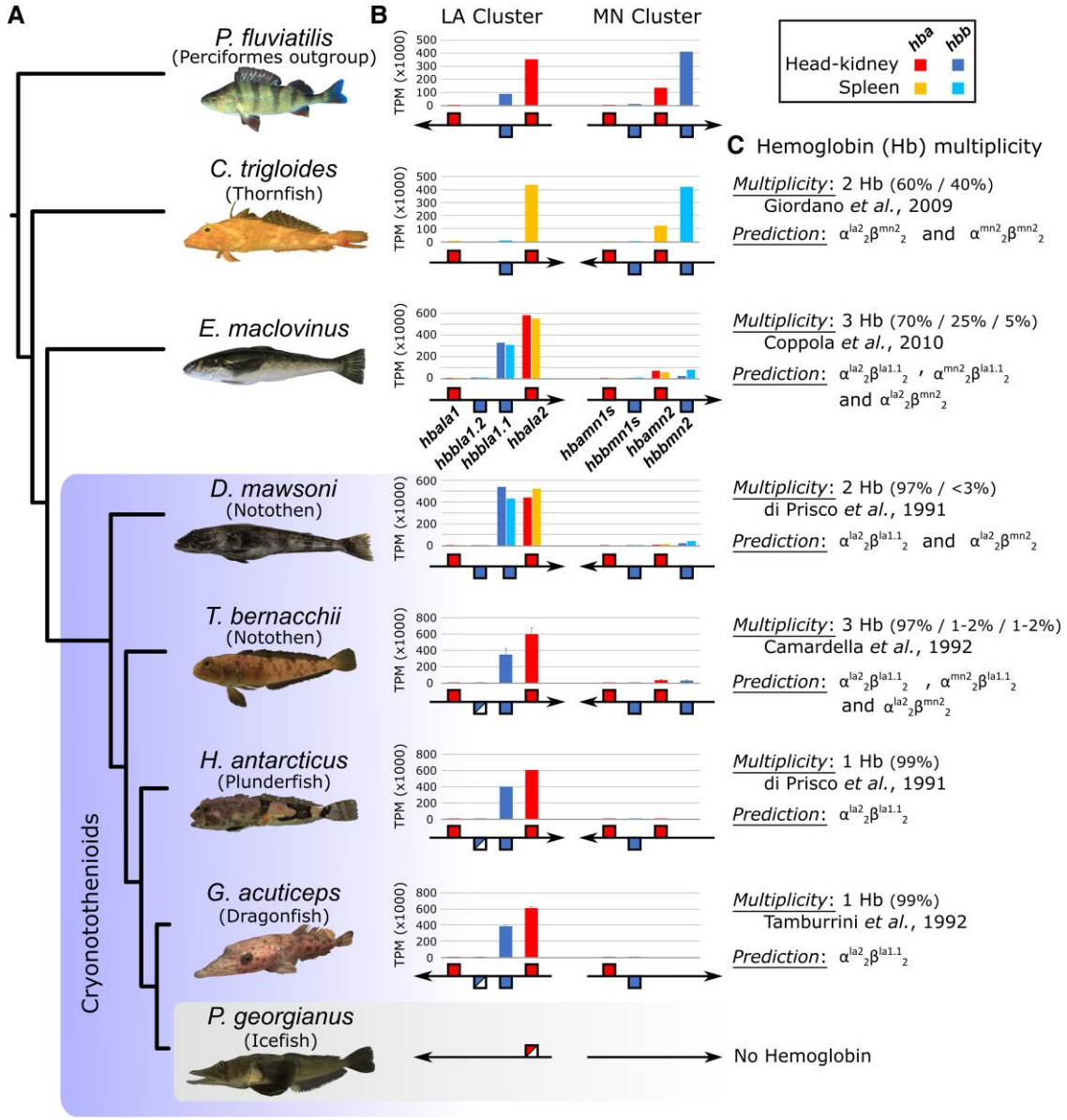

FIG. 4. Evolution of hemoglobin gene expression in adult notothenioids explains the reduction in hemoglobin protein multiplicity. A) Phylogenetic relationships and B) expression patterns of hemoglobin genes in adult head kidney and/or spleen of notothenioids. Expression level is provided in TPM, and error bars represent the standard deviation when multiple comparable sequencing libraries were available (see supplementary table S4, Supplementary Material online). Expression levels are represented in the order in which hemoglobin genes are organized in the LA and MN clusters. Because tandem duplicates of *hbamn1* and *hbbmn1* are highly similar to each other, heterogenous in number across species, and lowly expressed in adult organs, fragment counts for each paralog were combined for display purposes. Fragment counts for each individual gene are provided in supplementary table S5, Supplementary Material online. C) Previously published differing hemoglobin protein multiplicity and relative proportions of observed protein isoforms in each of the studied notothenioid species, and the prediction of expressed hemoglobin isoforms based on hemoglobin gene expression patterns. No hemoglobin multiplicity information could be found for the European perch *P. fluviatilis*.

are the most expressed genes, with each paralog approximatively equally expressed, except for *β-mn1.3* and *β-mn1.4*, which are less expressed (Fig. 5C and E). In embryos compared to adults, the relative expression of *α-la2* and *β-mn2* is dramatically reduced and *α-mn2* is barely detected (Fig. 5A and C). In perch embryos and adults, *α-la1* expression was not detected (Fig. 5A and C). Perch embryos thus likely express up to 6 or more hemoglobin isoforms: $\alpha_2^{mn1}\ \beta_2^{mn1}$, $\alpha_2^{mn1}\ \beta_2^{la1}$, $\alpha_2^{mn1}\ \beta_2^{mn2}$, $\alpha_2^{la2}\ \beta_2^{la1}$, $\alpha_2^{la2}\ \beta_2^{mn1}$, and $\alpha_2^{la2}\ \beta_2^{mn2}$.

In embryos of the dragonfish *G. acuticeps*, *α-mn1* and *β-mn1* paralogs account for almost half of the total hemoglobin gene transcripts (Fig. 5D), but not all paralogs contribute equally (Fig. 5F). Instead, the first and last genes of the tandem array contribute the majority of the *α-mn1* and *β-mn1* paralog transcripts (Fig. 5F). As in perch embryos, the relative expression of *α-la2* in dragonfish embryos is dramatically reduced compared to adults (Fig. 5A to D); however, in contrast to perch embryos, *α-la1* is significantly expressed and represents approximatively a third of total

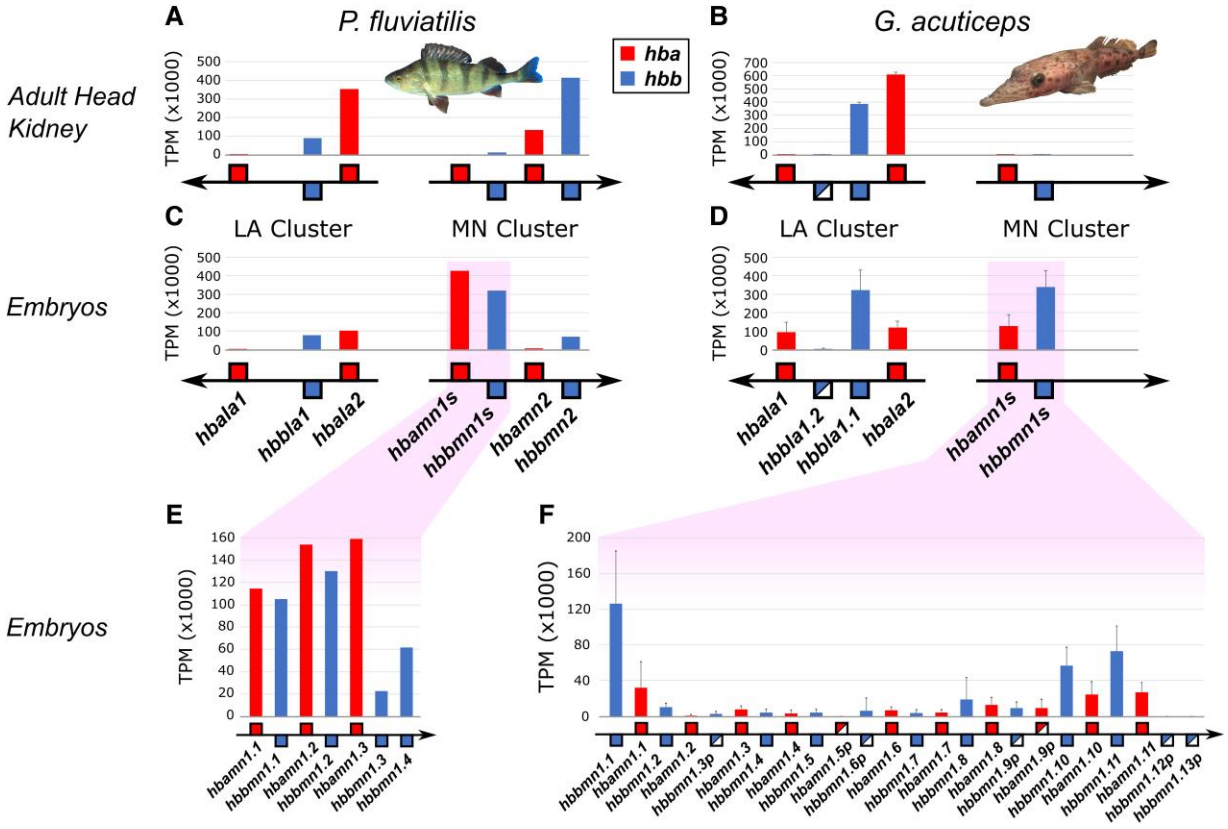

Fig. 5. Gene expression in embryos and adults reveals embryonic and adult hemoglobin genes. Expression patterns of hemoglobin genes in A) and B) adult head kidney and C to F) embryos of A, C, and E) the European perch *P. fluviatilis* and B, D, and F) the dragonfish *G. acuticeps*. Expression level is provided in TPM, and error bars represent the standard deviation when multiple comparable sequencing libraries were available (see supplementary table S4, Supplementary Material online). Expression levels are represented in the order in which hemoglobin genes are organized in the LA and MN clusters. Because tandem duplicates of *hbamn1* and *hbbmn1* are highly similar to each other, heterogenous in number across species, and lowly expressed in adult organs, fragment counts for each paralog were combined for display purposes in A) to D) and are detailed in E) and F).

alpha-globin gene transcripts in dragonfish embryos (Fig. 5C and D). Dragonfish embryos thus potentially express also up to 6 or more hemoglobin isoforms: $\alpha_2^{la1}\beta_2^{la1.1}$, $\alpha_2^{la1}\beta_2^{mn1}$, $\alpha_2^{la2}\beta_2^{la1.1}$, $\alpha_2^{la2}\beta_2^{mn1}$, $\alpha_2^{mn1}\beta_2^{la1.1}$, and $\alpha_2^{mn1}\beta_2^{mn1}$.

Together, our analysis of these expression data revealed the dynamic change in hemoglobin gene expression between embryos and adults and showed that (i) *α-la1*, *α-mn1*, and *β-mn1* are embryonic hemoglobin genes, (ii) *α-mn2* and *β-mn2* are predominantly adult hemoglobin genes, and (iii) *α-la2* and *β-la1* are expressed both in embryos and adults. Furthermore, these data established a clear relationship between the number and nature of hemoglobin gene paralog expression and hemoglobin protein multiplicity. This analysis revealed that, despite the retention of multiple genes in the genome, the reduction or loss of expression of *α-mn2* and *β-mn2* explains how the major hemoglobin isoform $\alpha_2^{la2}\beta_2^{la1.1}$ represents ~95% of hemoglobin tetramers in most cryonotothenioid species and is the single hemoglobin isoform in plunderfishes and dragonfishes (Giordano et al. 2015).

Episodic Diversifying Selection on Hemoglobin Genes

To distinguish among the nonexclusive hypotheses that notothenioid hemoglobin gene evolution occurred (i) at times of climatic shifts and (ii) in the ancestors to icefishes, we performed 4 independent HyPhy aBSREL tests (Smith et al. 2015) to search for episodes of diversifying selection in hemoglobin genes during the notothenioid radiation (Fig. 6A).

At the branch leading to Eleginopsioidea, which was previously shown to be characterized by an increased rate of genome-wide evolution (Daane et al. 2019), results showed that only the 2 dual embryonic and adult hemoglobin genes *α-la2* and *β-la1.1* underwent episodic diversifying selection ($P = 0.0474$ and $P = 0.0392$, respectively; Fig. 6A1 and supplementary table S6, Supplementary Material online). This result is consistent with the embryonic-to-adult switch in gene expression and the predominant expression of *α-la2* and *β-la1.1* in Eleginopsioidea species compared to perch and thornfish. At the branch leading to cryonotothenioids, *α-la2*, *β-la1.1*, and *β-la1.2* experienced episodic diversifying selection ($P = 0.0030$, $P = 0.0233$, and $P = 0.0412$, respectively; Fig. 6A2 and supplementary table S6, Supplementary Material online), which coincides with the period of cold adaptation and the emergence of cryonotothenioids as well as a period involving a high rate of genome-wide molecular evolution (Daane et al. 2019). Furthermore, among cryonotothenioids, at the branch

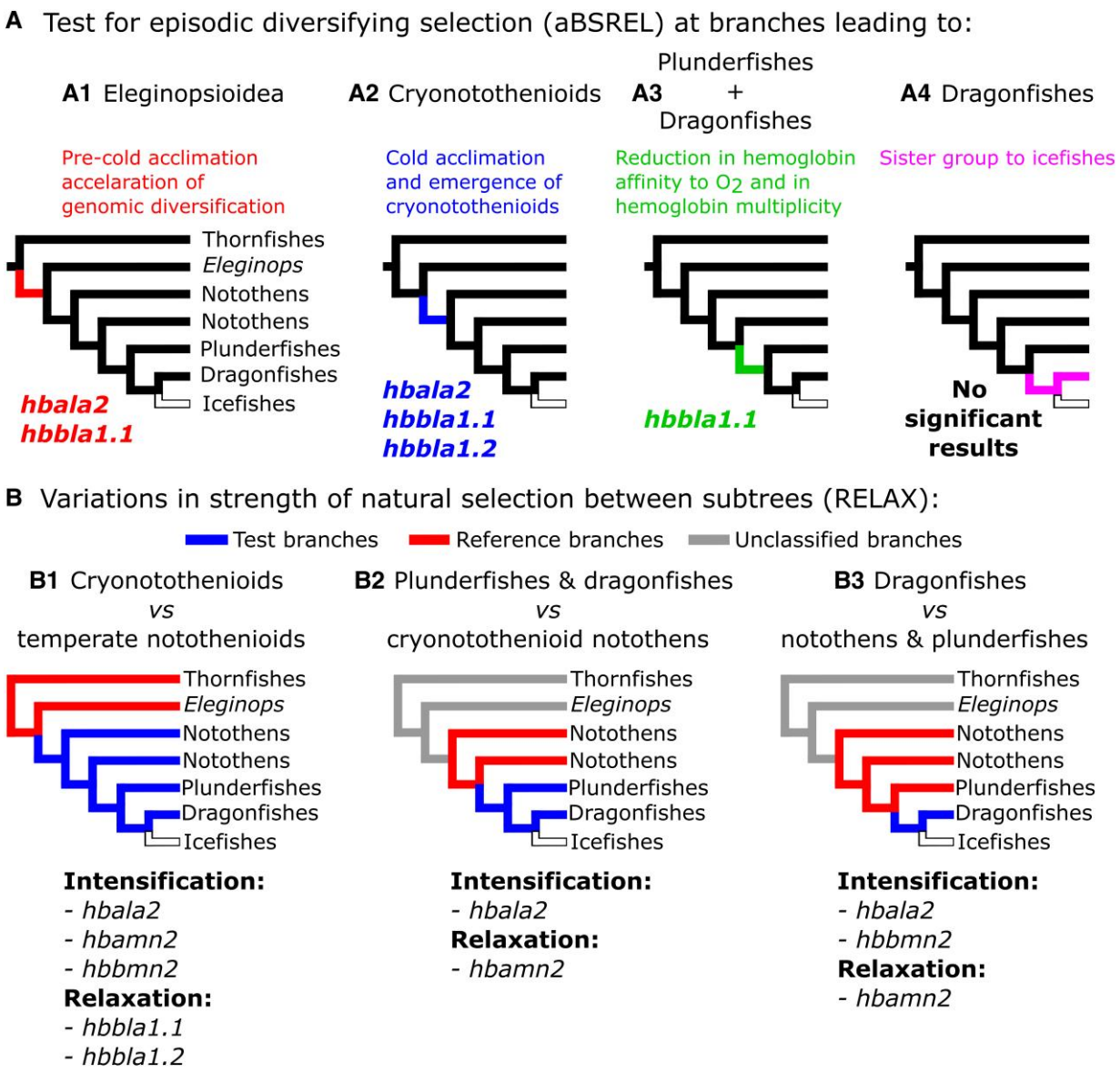

FIG. 6. Tests for change in selective pressure on hemoglobin genes. A) Results of independent aBSREL tests for episodic diversifying selection at branches leading to Eleginopsioidea, to cryonotothenioids, to the plunderfish and dragonfish group, and to dragonfishes. B) Results of RELAX tests for variations in the strength of natural selection between 2 subtrees. Three tests were conducted: cryonotothenioids compared to non-cryonotothenioid notothenioids, the plunderfish and dragonfish group compared to other cryonotothenioids, and dragonfishes compared to other cryonotothenioids. Icefishes do not have complete hemoglobin sequences to compare against, but they are represented in the schematic phylogeny to help understand group relationships.

leading to the plunderfish and dragonfish group, which coincides with the generalized reduction in hemoglobin multiplicity to a single isoform and its lower affinity for oxygen in extant species compared to other cryonotothenioid notothens (Giordano et al. 2015), only the predominantly expressed beta-globin gene *β-la1.1* was found to have undergone episodic diversifying selection ($P = 0.0493$; Fig. 6A3 and supplementary table S6, Supplementary Material online). Finally, at the branch leading to dragonfishes, the sister group to icefishes, none of the hemoglobin genes were found to have undergone further accelerated evolution (Fig. 6A4 and supplementary table S6, Supplementary Material online). Together, this analysis revealed that only the 2 major adult Eleginopsioidea hemoglobin genes (*α-la2* and *β-la1.1*) experienced episodic diversifying selection, mostly before the divergence of *E. maclovinus* and cryonotothenioids, and with the divergence of cryonotothenioids, therefore during periods of climatic shifts. These episodes of diversifying selection on the 2 most-expressed hemoglobin genes were thus potentially part of the process of cold adaptation to maintain appropriate oxygenation as water temperatures dropped.

Pervasive Changes in Strength of Natural Selection on Hemoglobin Genes

Because gene evolution may have occurred not only episodically and maybe only in some lineages of the

notothenioid radiation but also by pervasive intensification or relaxation of selective pressure among a subset of lineages, we hypothesized that hemoglobin genes evolved under different selective pressures in cryonotothenioids compared to temperate notothenioids and differently in sister lineages to icefishes compared to other cryonotothenioids. To test these nonexclusive hypotheses, we employed the HyPhy test RELAX to detect variations in the strength of natural selection between 2 subtrees (Fig. 6B) (Wertheim et al. 2015).

While RELAX tests did not reveal any significant pervasive change in the strength of natural selection for the embryonic globin genes *α-la1*, *α-mn1*, and *β-mn1* in any of the 3 tests performed (supplementary table S7, Supplementary Material online), all 4 globin genes expressed in adults (*α-la2*, *α-mn2*, *β-la1.1*, and *β-mn2*) and the repeatedly pseudogenized or lost *β-la1.2* seem to have undergone pervasive changes in the strength of natural selection. Hemoglobin genes *α-la2*, *α-mn2*, and *β-mn2* displayed intensified selection ($P = 0$, $P = 0.002$, and $P = 0.001$, respectively; Fig. 6B1 and supplementary table S7, Supplementary Material online), and *β-la1.1* and *β-la1.2* displayed relaxed selection in cryonotothenioids compared to the noncryonotothenioid notothenioids ($P = 0$ and $P = 0.020$, respectively; Fig. 6B1 and supplementary table S7, Supplementary Material online). This result is consistent with the long branches observed in the β-la1.1 protein tree (Fig. 2C) and the repeated losses and pseudogenization of *β-la1.2* (Fig. 2D and E) due to reduced selection. In plunderfishes and dragonfishes compared to other cryonotothenioids, *α-la2*, which was the only alpha-globin subunit gene strongly expressed in adults, displayed intensified selection ($P = 0.022$; Fig. 6B2 and supplementary table S7, Supplementary Material online), and *α-mn2*, which was lost or expressed dramatically less in plunderfishes and dragonfishes, displayed relaxed selection ($P = 0.007$; Fig. 6B2 and supplementary table S7, Supplementary Material online). Finally, comparing dragonfishes (the sister group to icefishes) to other cryonotothenioids, *α-la2* and *β-mn2* displayed further pervasive intensified selection ($P = 0.033$ and $P = 0.019$, respectively; Fig. 6B3 and supplementary table S7, Supplementary Material online), while *α-mn2* displayed further relaxation ($P = 0.046$; Fig. 6B3 and supplementary table S7, Supplementary Material online), consistent with a reinforced reliance on *α-la2* in the single hemoglobin protein isoform and the loss of expression of *α-mn2* and *β-mn2* in plunderfishes and dragonfishes.

Together, all hemoglobin genes expressed in adults (i.e. *α-la2*, *β-la1.1*, *α-mn2*, and *β-mn2*), plus the repeatedly pseudogenized or lost *β-la1.2* gene, evolved under different strengths of natural selection in cryonotothenioids compared to noncryonotothenioid notothenioids. This finding suggests that the frigid Antarctic environment has been exerting different evolutionary pressures on gene evolution compared to the temperate environment in which early diverging temperate notothenioids currently live. Interestingly, among cryonotothenioids, the predominantly expressed *α-la2* gene and the adult *α-mn2* and *β-mn2* genes, which lost expression in plunderfishes and dragonfishes, display signs of intensified or relaxed natural selection in plunderfish and/or dragonfishes compared to the other cryonotothenioids. These results demonstrate that most major adult hemoglobin genes pervasively evolved in cryonotothenioids, including in the sister groups to icefishes. In contrast, embryonic hemoglobin genes *α-la1*, *α-mn1*, and *β-mn1*, surprisingly, do not show patterns of changes in the strength of natural selection between any of the studied subgroups. The number of species from which *α-mn1* and *β-mn1* gene sequences could be retrieved (12 and 10, respectively; supplementary table S7, Supplementary Material online), however, may have been too small to detect changes in the strength of natural selection.

Variable Rates of Synonymous and Nonsynonymous Mutations among Hemoglobin Genes

To compare the rate of hemoglobin evolution among genes and across notothenioid species, we calculated the frequencies of synonymous (dS) and nonsynonymous (dN) mutations and the nonsynonymous-to-synonymous mutation ratio ($\omega = dN/dS$) in available notothenioid hemoglobin genes using the early diverging temperate thornfish *C. trigloides* as reference (Fig. 7).

In cryonotothenioids, *α-la2* and *β-la1.1*, which dominate expression in adults, as well as *β-la1.2*, which was repeatedly pseudogenized or lost, evolved significantly more than embryonic and lowly expressed *α-la1* and all genes of the MN cluster (Fig. 7A to H). The *α-la2*, *β-la1.1*, and *β-la1.2* genes display a much higher level of synonymous mutations ($dS = 0.65 \pm 0.07$) (Fig. 7B, C, and H) compared to the other hemoglobin genes ($dS = 0.27 \pm 0.08$) (Fig. 7A, D to G, and H). Similarly, *α-la2*, *β-la1.1*, and *β-la1.2* display a higher level of nonsynonymous mutations ($dN = 0.14 \pm 0.02$) (Fig. 7B, C, and H) compared to the other hemoglobin genes ($dN = 0.08 \pm 0.02$) (Fig. 7A, D to G, and H).

Most rates of sequence evolution, however, were equivalent among notothenioid hemoglobin genes, except for the strictly embryonic *α-mn1*/*β-mn1* gene pairs, which had a higher nonsynonymous-to-synonymous mutation ratio ω than all other hemoglobin genes (Fig. 7D and E). In cryonotothenioids, all LA cluster hemoglobin genes and the MN cluster *α-mn2* and *β-mn2* genes display a nonsynonymous-to-synonymous mutation ratio of $\omega = 0.23 \pm 0.02$, ranging from 0.19 for *β-la1.2* to 0.26 for *α-la1* (Fig. 7A to C and F to G). In contrast, the embryonic *α-mn1* and *β-mn1* genes display a much higher nonsynonymous-to-synonymous mutation ratio with $\omega = 0.59$ for *α-mn1* and $\omega = 0.34$ for *β-mn1* (Fig. 7D and E). It is thus possible that the repeated duplications that characterize these embryonic genes permitted a significant increase in nonsynonymous mutation rates compared to synonymous mutation rates and provided a ready source of adaptive evolution for sensitive embryonic stages by constantly and in a lineage-specific manner, diversifying in gene copy number and protein sequence.

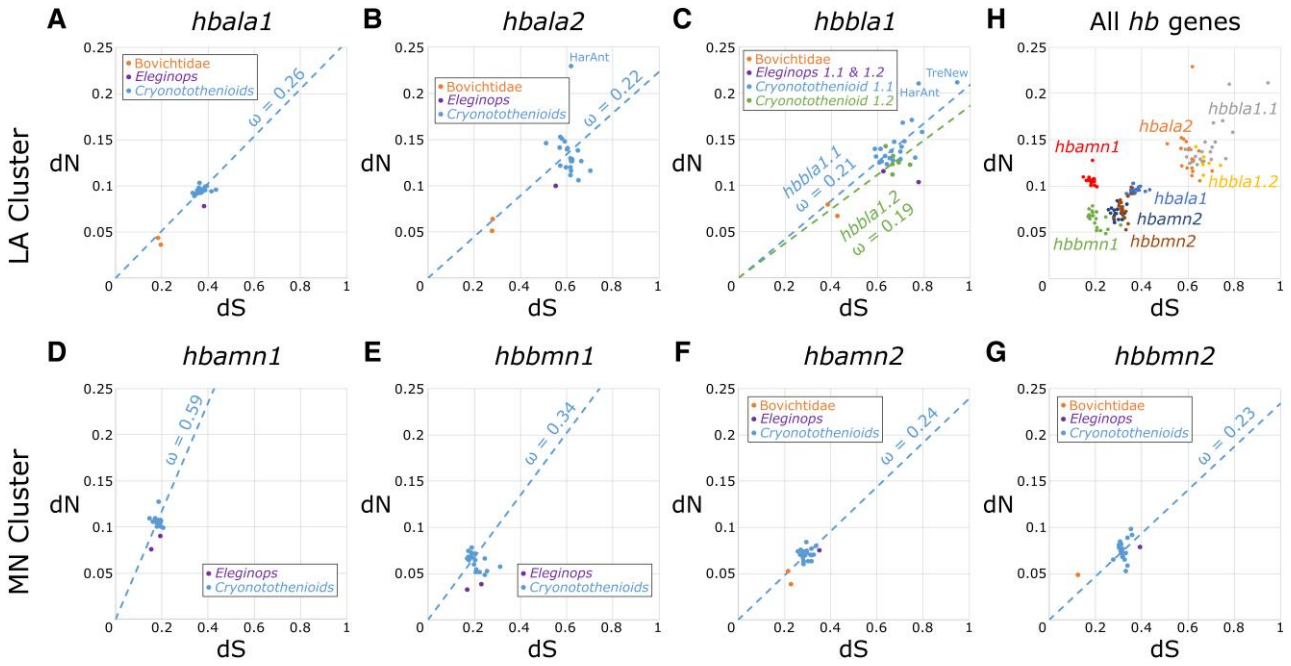

FIG. 7. Synonymous and nonsynonymous mutations in hemoglobin genes in notothenioids. Thornfish *C. trigloides* sequences were used as reference. A to G) All genes were studied separately but also combined in H) a single graph revealing diverging rates of evolution between genes. Dashed lines represent the average $\omega = dN/dS$ ratio in cryonotothenioids.

Site-Specific Evolution of Hemoglobin Proteins in Notothenioids

We then questioned whether specific sites within each hemoglobin protein underwent diversifying selection and thus might have contributed to adaptation to the frigid Antarctic environment or led to deleterious mutations that allowed the deletion of genes in icefishes and related species. We analyzed each set of orthologous genes with the HyPhy test MEME (Murrell et al. 2012) to detect sites that evolved under episodic diversifying selection (supplementary table S8, Supplementary Material online), cross-compared them to the NCBI Conserved Domain Database (CDD) (supplementary table S9, Supplementary Material online) (Lu et al. 2020), modeled each hemoglobin protein using SWISS-MODEL (Waterhouse et al. 2018) and iCn3D (supplementary table S10, Supplementary Material online) (Wang et al. 2022), and tested for potential deleterious effects on hemoglobin function using PROVEAN (supplementary table S11, Supplementary Material online) (Choi and Chan 2015).

Within *α-la1* and all genes of the MN cluster, which includes all the lowly or unexpressed genes in adult cryonotothenioids, MEME detected a few sites that evolved under episodic diversifying selection in some notothenioid lineages (Fig. 8A and E to H and supplementary table S8, Supplementary Material online). Among sites detected by MEME, 1 amino acid site in the α-mn1 protein (D29A, supplementary table S10, Supplementary Material online; Fig. 8E) shared by almost all Eleginopsioidea and 1 site in the α-mn2 protein (A98V, Fig. 8F) shared by all plunderfishes were predicted by PROVEAN to cause deleterious effects. In the α-mn1 protein, 1 diversifying site (T114V) shared by all cryonotothenioids is predicted to be functionally important at the tetramer interface; however, this substitution is predicted to have a neutral effect on the function (Fig. 8E). This T114V site may therefore reveal an important adaptive change in maintaining the tetrameric embryonic hemoglobin structure in response to cold adaptation.

In contrast, MEME identified numerous sites evolving under episodic diversifying selection in the α-la2 and β-la1.1 proteins that are encoded by the 2 genes predominantly expressed in adult cryonotothenioids (Fig. 8B and C and supplementary table S8, Supplementary Material online). A few diversifying sites were further predicted to have deleterious consequences on protein functions. In the α-la2 protein, site K59 is changed to 1 of 5 different amino acids in most Antarctic species and PROVEAN predicts that each substitution should have deleterious consequences on protein function (supplementary table S11, Supplementary Material online). In the 3 closely related dragonfish species *Akarotaxis nudiceps*, *Bathydraco marri*, and *Vomeridens infuscipinnis*, the substitution of K62 with an isoleucine in α-la2 is also predicted to be deleterious. In the β-la1.1 protein, 2 diversifying sites also involved in the tetramer interface are predicted to have deleterious mutations: T112V in *H. antarcticus* and A129G in about half of other cryonotothenioid species of various families. One substitution at a site involved in heme binding, L142A, is predicted to be deleterious in *T. newnesi* (Fig. 8C). In addition, the diversifying site K83 in the β-la1.1 protein is changed to 1 of 4 different other amino acids in

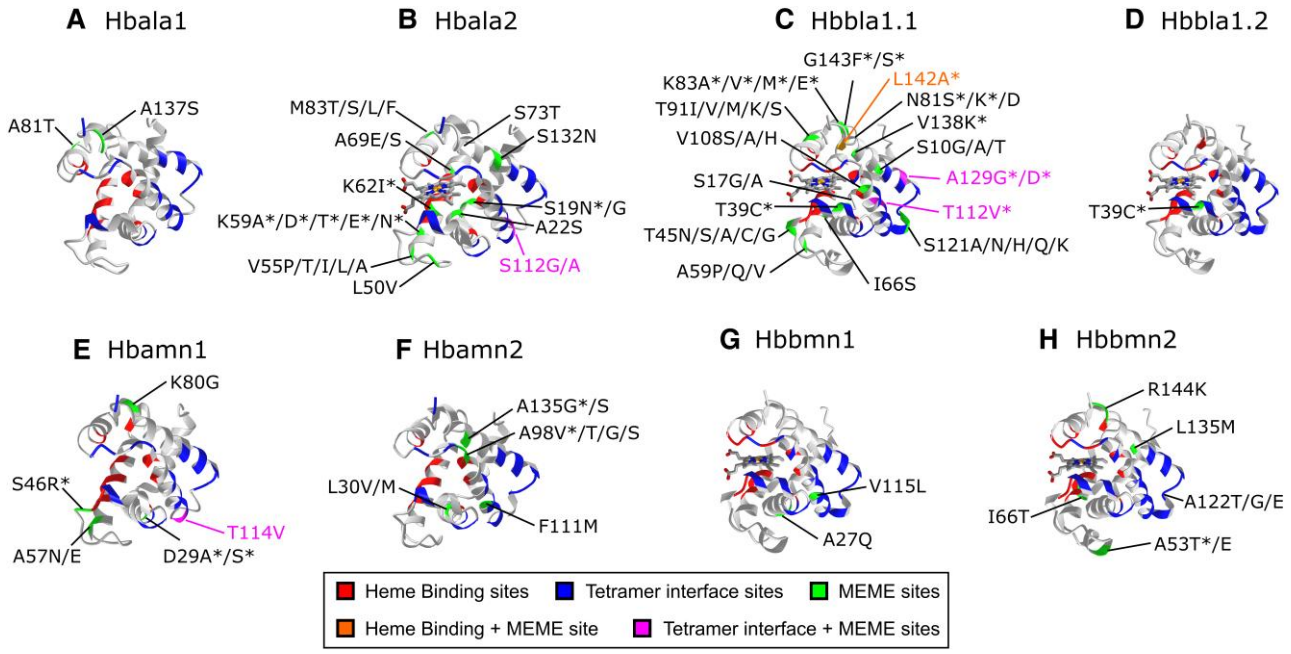

FIG. 8. 3D models of hemoglobin subunits and sites under diversifying selection in notothenioids. Each protein was modeled using SWISS-MODEL and visualized with iCn3D with custom tracks showing sites known to be involved in heme binding and tetramer interface based on NCBI CDD. See supplementary table S10, Supplementary Material online for detailed information on input sequences, templates, and models. Individual sites detected to have been subject to episodic positive or diversifying selection using MEME are labeled with their amino acid in the reference sequence and their conversions in other sequences. The site detected by MEME and involved in heme binding, the sites detected by MEME and involved in tetramer interface, and the other sites detected by MEME are highlighted on the protein model according to the legend on the figure. Amino acid changes predicted by PROVEAN to have deleterious effect are marked with an asterisk (*).

cryonotothenioids, and all 4 mutations are predicted to be deleterious (supplementary table S11, Supplementary Material online).

Overall, in the 3D models, most diversifying sites appear located at the surface of the subunits. Interestingly, sites located at the surface of the protein are more likely to contribute to adaptive evolution because they are expected to affect the protein's affinity for oxygen, temperature-sensitive bonds mediating interactions between subunits, and the action of allosteric effectors such as protons and CO_2 (reviewed in Storz 2018); therefore, variable sites MEME identified here are plausible sites of adaptive evolution.

Discussion

Our evolutionary genomic analyses revealed that the cooling of the Southern Ocean and the following frigid Antarctic conditions drove the evolution of notothenioid hemoglobin genes and that a few hemoglobin genes suffered deleterious mutations or were lost in lineage-specific manners in cold-adapted cryonotothenioid species. Analysis of hemoglobin gene expression in adult hematopoietic organs in various temperate and Antarctic species further revealed a switch in hemoglobin gene expression underlying the long-standing problem of the origin of the hemoglobin multiplicity reduction in Antarctic fish, including the expression of a single protein isoform in adult plunderfishes and dragonfishes. Finally, our results support the hypothesis that plunderfish, dragonfish, and icefish ancestors may have become overly specialized in their hemoglobin systems, leaving the icefish ancestor vulnerable to deleterious mutations in the only 2 genes that provided adult hemoglobin expression.

Adaptive Evolution of Notothenioid Hemoglobin Genes to the Cold

While it has long been proposed that the cold Antarctic environment affected blood parameters and hemoglobin characteristics of cryonotothenioids (Eastman 1993; di Prisco et al. 2007; Verde et al. 2007, 2008), we demonstrate here how the cold Antarctic environment shaped the evolution of hemoglobin genes.

Hemoglobin gene evolution was relatively stable in notothenioid ancestors before the cooling of Antarctica, but the onset of cold conditions in the Southern Ocean was accompanied by a burst of genomic evolution regarding hemoglobin genes. According to the most recent genome-wide time-calibrated molecular phylogeny, the divergence of *E. maclovinus* from the cryonotothenioid lineage occurred about 26 MYA (Bista et al. 2023), thus after the onset of Antarctic glaciation and the formation of ice sheets in East Antarctica following the Eocene–Oligocene transition about 34 MYA (Westerhold et al. 2020; Hutchinson et al. 2021) (Fig. 9). It is, therefore, possible that the increased rate of genome evolution observed at the branch leading to Eleginopsioidea (i.e. *E. maclovinus* and cryonotothenioids) (Daane et al. 2019) was driven by the onset of

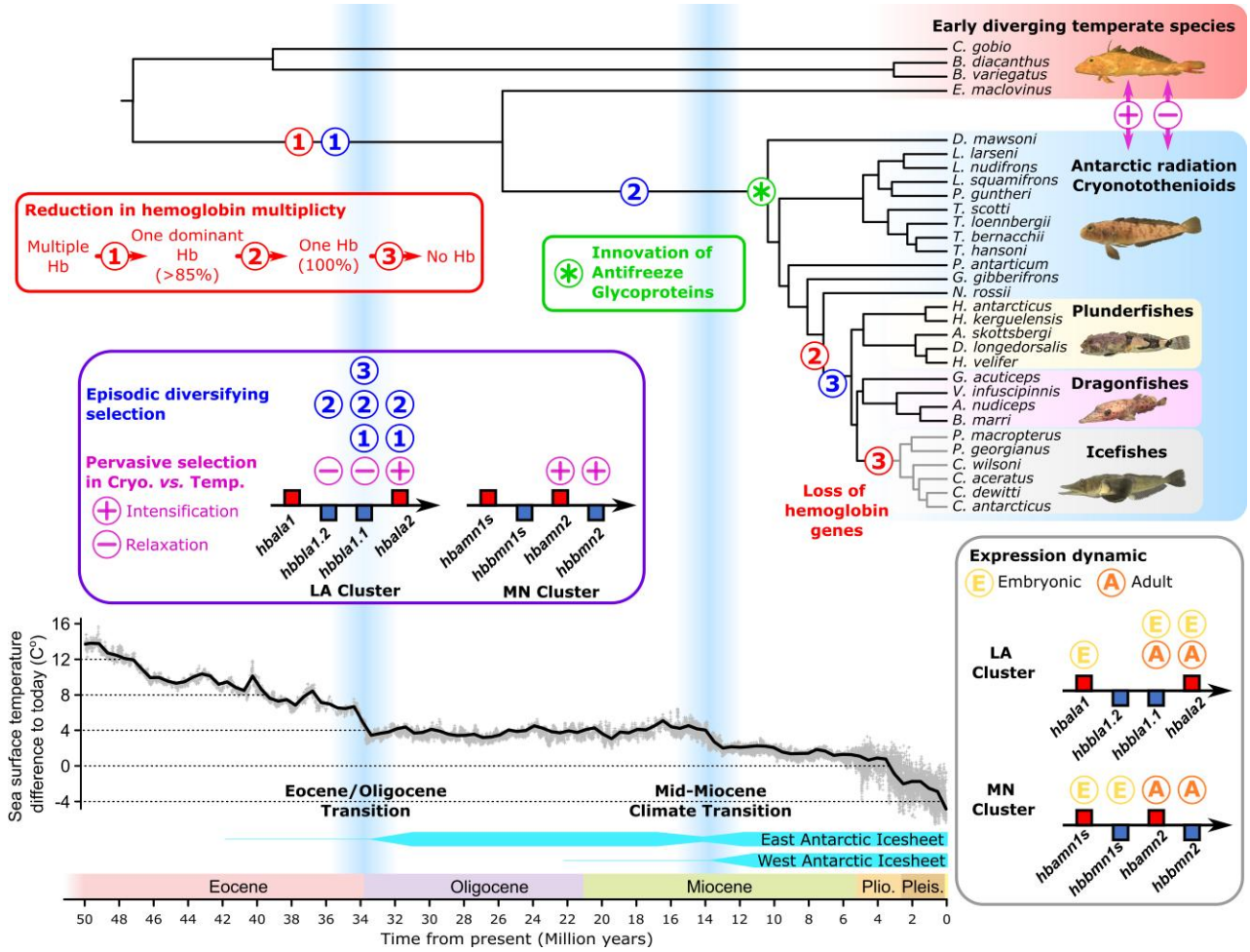

Fig. 9. Hemoglobin multiplicity reduction and cold-driven gene evolution in Antarctic notothenioids prior to the loss of hemoglobin in icefishes. Time-calibrated phylogeny (Bista et al. 2023) highlighting early diverging notothenioids and the Antarctic clade (cryonotothenioids), as well as plunderfishes, dragonfishes, and icefishes within cryonotothenioids. East and West Antarctic ice sheet extent shown along with paleoclimatic data (Westerhold et al. 2020). Benthic $\delta^{18}O$ (‰) values were converted to temperature anomalies in degrees Celsius with respect to average global temperature from 1961 to 1990 following (Westerhold et al. 2020). The solid line represents a lowess smoothed curve of sea surface temperature evolution with $f = 0.005$. The Eocene/Oligocene transition and the MMCT, which led to major global sea surface temperature drops and the formation of ice sheets in Antarctica, are indicated by vertical columns. Steps of hemoglobin multiplicity reduction and episodes of diversifying selection detected on hemoglobin genes are plotted at corresponding branches. The relative placement of numbers representing episodic diversifying selection periods and steps of reduction in hemoglobin diversity do not reflect the exact timing of these events, which likely occurred over the length of the branch. Pervasive selection on hemoglobin genes in cryonotothenioids versus early diverging temperate notothenioids is represented using "+" and "−" signs for intensified and relaxed selections, respectively. The innovation of AFGPs in the cryonotothenioid ancestor (Chen et al. 1997) is shown at the base of the Antarctic radiation due to the impossibility to precisely date its emergence along the branch. The dynamic variation of hemoglobin gene expression is summarized in the bottom right box with letters referring to the developmental stages at which expression is detected: embryonic, adult, or embryonic and adult. Cryo., cryonotothenioids; Pleis., Pleistocene; Plio., Pliocene, Temp., early diverging temperate notothenioids.

cold, although not yet freezing, conditions around the continent. In this context, the tandem duplication of *β-la1* in Eleginopsioidea (Fig. 2) and the episodic diversification of *α-la2* and *β-la1.1* (Figs. 6A1 and 9) could be related to the initial cooling of Antarctic waters. Furthermore, the switch in expression pattern with *β-la1.1* becoming the predominantly expressed beta-globin gene over *β-mn2* (Fig. 4), which started to diverge in sequence from its *β-mn1* paralogs after the divergence of thornfishes (supplementary fig. S4A, Supplementary Material online), might also reveal evolutionary adaptations in response to the early cooling of the Southern Ocean.

Despite rapid change leading to the Eleginopsioidea, most evolutionary changes in hemoglobin genes in our 28 red-blooded species data set occurred in the cryonotothenioid ancestor after divergence from the *E. maclovinus* lineage, therefore concomitant with the generalized icy polar conditions around Antarctica. According to the same recent notothenioid phylogeny, the diversification of the cryonotothenioid radiation occurred around 10.7 MYA (Bista et al. 2023), thus after the MMCT around 14 MYA, which led to the formation of ice sheets in West Antarctica and the rapid establishment of freezing polar conditions around the entire Antarctic continent

(Leutert et al. 2020; Westerhold et al. 2020) (Fig. 9). The innovation of AFGPs, the hallmark character of cryonotothenioids (Chen et al. 1997; DeVries and Cheng 2005; Bista et al. 2023), is thus concomitant with the establishment of persistent icy conditions in the Southern Ocean following the MMCT (Fig. 9). These new frigid conditions also influenced the evolution of notothenioid genomes (Chen et al. 2019; Daane et al. 2019; Daane and Detrich 2022; Bilyk et al. 2023; Bista et al. 2023), including, likely, their hemoglobin genes, in ways that maintained physiologically appropriate protein function in this icy and hyperoxygenated environment. In the MN cluster, the genomic loss of the first embryonic *α-mn1* gene can also be retraced to the cryonotothenioid ancestor (Fig. 3), although the physiological consequences of this loss are unknown. At this period of evolution in the cryonotothenioid ancestor, the predominantly expressed genes in adults *α-la2* and *β-la1.1*, as well as the repeatedly pseudogenized *β-la1.2*, underwent episodic diversifying selection (Figs. 5A2 and 8). These 3 genes, along with the *α-mn2*/*β-mn2* gene pair in the MN cluster, also evolved under different natural selective pressures in cryonotothenioids compared to noncryonotothenioid notothenioids (Figs. 5B1 and 8). Further, these observations are consistent with generally longer branches for this ancestor in the protein phylogenetic trees (supplementary figs. S1 to S4, Supplementary Material online) and numerous amino acid changes shared by all cryonotothenioids (see supplementary protein alignment files deposited in the dedicated United States Antarctic Program Data Center [USAP-DC] project page). Together, these results demonstrate that the cold Antarctic environment has been exerting strong selective pressure on organisms and shaping the evolution of adult hemoglobin genes in the cryonotothenioid radiation.

Hemoglobin Gene Expression Patterns Reveal the Molecular Genetic Basis for the Reduction of Hemoglobin Protein Multiplicity in Cryonotothenioids

Hemoglobin multiplicity (i.e. the presence of multiple hemoglobin protein isoforms with differing physiochemical properties) is generally regarded as an adaptive trait enabling appropriate oxygenation in changing environments (e.g. seasonally hypoxic or fluctuating temperatures), changing physiological conditions (e.g. acidosis following muscular effort), and life stages (embryonic, larval, and adult forms) (Verde et al. 2012; Baalsrud et al. 2017; Pan et al. 2017; Barts et al. 2018; Andersen 2020); thus, most fish species present multiple hemoglobin isoforms. Cryonotothenioid adults, however, were first described in 1980 as having only 1 major hemoglobin isoform and 1 or few minor isoforms (Wells et al. 1980). In the following decades, several studies confirmed and expanded these observations by revealing that plunderfishes and dragonfishes generally display a single hemoglobin isoform and no minor isoforms (Di Prisco 1988; di Prisco et al. 1990; Kunzmann 1991; Tamburrini et al. 1998). Gradually, an evolutionary picture has emerged that this characteristic reduction in hemoglobin multiplicity observed in cryonotothenioids compared to temperate species is due to relaxed selection for multiple hemoglobin isoforms stemming from the extreme physicochemical stability and high oxygenation of Antarctic waters (Verde et al. 2006, 2008; di Prisco et al. 2007). The molecular genomic basis of this 40-year long-standing question of the reduction in hemoglobin multiplicity in cryonotothenioids, however, remained unexplained.

Our detailed survey of hemoglobin genes among extant notothenioids and of their expression in adult hematopoietic organs revealed that the reduction in hemoglobin multiplicity in cryonotothenioids is attributable to a switch in gene expression characterized by reduced expression of hemoglobin genes of the MN cluster and increased relative expression of hemoglobin genes of the LA cluster. Temperate water adult perch and thornfish strongly express the *α-mn2* and *β-mn2* gene pair, which contributes to hemoglobin multiplicity in combination with the expression of *α-la2* and *β-la1.1* (Fig. 4). In contrast, Eleginopsioidea species predominantly express only the *α-la2* and *β-la1.1* gene pair, which provides the major hemoglobin isoform $\alpha_2^{la2}\ \beta_2^{la1.1}$ that usually represents ~95% of total hemoglobin protein in the blood, and lowly express or do not express the *α-mn2* and *β-mn2* gene pair, whose products are likely incorporated into the minor isoforms $\alpha_2^{mn2}\ \beta_2^{la1.1}$ and $\alpha_2^{la2}\ \beta_2^{mn2}$, which would represent just a few percent of total hemoglobin when expressed (Fig. 4). Notably, the major switch in beta-globin gene expression in Eleginopsioidea species (i.e. reduced *β-mn2* expression relative to increased *β-la1.1* expression) and the divergence of β-mn2 sequences from their β-mn1 paralogs both occurred in the common ancestor of Eleginopsioidea after their divergence from thornfishes. The relative timing and potential correlation of these 2 events are, however, unknown. If the switch in beta-globin gene expression occurred first, the selective pressure on *β-mn2* could have been relaxed and led to *β-mn2* divergence in Eleginopsioidea. Alternatively, if the divergence of *β-mn2* occurred first due to fixation in small populations despite potentially accumulating slightly deleterious mutations, the switch in beta-globin gene expression could have been a compensatory mechanism to preserve hemoglobin function. The monotypic family Pseudaphritidae, represented by the temperate notothenioid *P. urvillii*, diverged from the main notothenioid lineage after the divergence of thornfishes and before the divergence of Eleginopsioidea (Near et al. 2012; Daane et al. 2019). Notably, *P. urvillii* also presents reduced hemoglobin multiplicity with a predominant isoform representing over 95% of the total hemoglobin (D'Avino and di Prisco 1997; Verde et al. 2004). It is therefore likely that the initial switch in gene expression that led to reduced hemoglobin multiplicity occurred in the ancestor of Pseudaphritidae and Eleginopsioidea after its divergence from thornfishes. Genome sequencing and expression analysis of hemoglobin genes in *P. urvillii* are, however, necessary to confirm this prediction in the future.

Furthermore, the loss of expression of *α-mn2* and *β-mn2* in *H. antarcticus* and *G. acuticeps* revealed the genomic basis for a single hemoglobin isoform in adults of these species, which would be genetically $\alpha_2^{la2}\ \beta_2^{la1.1}$, in plunderfishes and dragonfishes (Fig. 4). We therefore here establish for the first time a clear relationship between hemoglobin gene repertoire and expression and hemoglobin protein multiplicity in notothenioids. According to this relationship, the hemoglobin multiplicity observed in *Pleuragramma antarcticum*, *T. newnesi*, and *Trematomus borchgrevinki*, with 3, 3, and 5 hemoglobin isoforms, respectively (D'Avino et al. 1994; Tamburrini et al. 1996; Riccio et al. 2000), is probably related to independently evolved lineage-specific increases in *α-mn2* and *β-mn2* gene expression. Contiguous genome assemblies and transcriptomic data of hematopoietic organs from these 3 species were, however, not available to test these predictions.

Our analyses of hemoglobin gene expression in embryos compared to adult hematopoietic organs revealed that *α-la1*, *α-mn1*, and *β-mn1* are embryonic hemoglobin genes, that *α-mn2* is an adult hemoglobin gene, and that *β-la1*, *α-la2*, and *β-mn2* are both embryonic and adult hemoglobin genes (Fig. 5). Given the hemoglobin gene expression pattern observed in *G. acuticeps* embryos, we predict that, in contrast to adult individuals displaying a single hemoglobin $\alpha_2^{la2}\ \beta_2^{la1.1}$, *G. acuticeps* embryos likely display much greater hemoglobin multiplicity with potentially up to 6 or more hemoglobin isoforms. This putative high hemoglobin multiplicity in dragonfish embryos compared to adults raises the hypothesis that, in variable conditions, the sensitive benthic embryonic and planktonic larval phases survive best when multiple hemoglobin isoforms are expressed, while the more robust adults may not require multiple hemoglobin isoforms to handle minor variations in their consistent benthic environment. Future studies on the expression of hemoglobin genes in additional Antarctic and temperate notothenioid embryos and larvae will further reveal the molecular bases and physiological implications of hemoglobin multiplicity in embryos compared to adults and the underlying molecular switch in hemoglobin gene expression patterns during notothenioid life histories.

Were Icefishes Predisposed to Lose Hemoglobin Genes?

Because hemoglobin affinity for oxygen and hemoglobin multiplicity in adult plunderfishes and adult dragonfishes are reduced compared to those of other adult cryonotothenioids (Giordano et al. 2015), we hypothesize that these changes in hemoglobin physiological properties predisposed the icefish lineage to subsequent hemoglobin gene losses.

Our genomic, phylogenomic, and expression analyses demonstrated that among notothenioids, only plunderfish and dragonfish species lost *α-mn2* and/or *β-mn2* genes (Fig. 3) and that species with predicted functional *α-mn2* and *β-mn2* genes lost expression of minor hemoglobin isoforms that incorporate α-mn2 and β-mn2 subunits (Fig. 4). These gene losses in some plunderfish and dragonfish species and their loss of expression in other adult plunderfish and dragonfish species represent the molecular bases of the lack of hemoglobin multiplicity in this group. In agreement, these 2 adult globin genes, which are otherwise lowly expressed in other adult cryonotothenioids, evolved under different selective pressures in plunderfishes and dragonfishes compared to other cryonotothenioids (Figs. 6B and 9), and several amino acid changes at sites under diversifying selection, including some predicted to be deleterious, are shared between plunderfishes and dragonfishes (Fig. 8 and supplementary table S11, Supplementary Material online). It is therefore likely that the icefish ancestor also relied on the expression of a single hemoglobin isoform, $\alpha_2^{la2}\ \beta_2^{la1.1}$, and that *α-mn2* and/or *β-mn2* had accumulated deleterious mutations in their coding and/or regulatory regions, rendering these genes unexpressed, nonfunctional, or pseudogenized as in today's plunderfishes and dragonfishes.

In this context, the icefish ancestor might have been vulnerable to function-altering mutations affecting its only 2 remaining expressed adult globin genes *α-la2* and *β-la1.1*. Indeed, the highly expressed paralog *β-la1.1* underwent episodic diversifying selection in the last common ancestor of plunderfish, dragonfish, and icefish, consistent with the high sequence divergence in these genes across this group demonstrated by long branches in phylogenetic trees. And the most highly expressed hemoglobin gene *α-la2* evolved under intensified selection in plunderfishes and dragonfishes compared to other cryonotothenioids and further in dragonfishes compared to other cryonotothenioids. Thus, with only 1 alpha-globin and 2 beta-globin genes actively expressed in the adult icefish ancestor, the probably small population was likely vulnerable to any mutations affecting gene expression, tetramer assembly, or oxygen-binding function for any of the 2 simultaneously necessary genes required for adult hemoglobin. Evolutionary changes seen in hemoglobin genes and their expression in plunderfishes and dragonfishes may therefore represent a precondition for the loss of hemoglobin genes in the icefish ancestor.

Conclusions

We demonstrate here that notothenioid hemoglobin genes evolved before, during, and following adaptation to the icy cold Antarctic environment (Fig. 9 and supplementary table S12, Supplementary Material online). These results also reveal that hemoglobin genes and their expression patterns likely started to evolve after the divergence of the thornfish lineage from other notothenioids, thus before frigid polar conditions were widely reached around the continent but simultaneously with the initial cooling of the Antarctic continent at the Eocene–Oligocene transition (Fig. 9). We further show that the 2 most strongly expressed hemoglobin genes in adult

cryonotothenioids, *α-la2* and *β-la1.1*, underwent episodic diversifying selection at the branches corresponding to periods of Antarctic glaciation (Fig. 9 and supplementary table S12, Supplementary Material online) and that all hemoglobin genes expressed in adult cryonotothenioids have been pervasively diversifying compared to their temperate relatives (supplementary table S12, Supplementary Material online), pointing to continuing adaptive processes supposedly gradually bringing physiology closer to a functional optimum in response to the constantly frigid environmental conditions in the Southern Ocean (Fig. 9). Additionally, we reveal that Antarctic dragonfish embryos express an embryonic hemoglobin gene set comparable to perch embryos and suggest the expression of numerous hemoglobin isoforms in dragonfish embryos, raising questions on the molecular basis and physiological implications of hemoglobin multiplicity in embryos compared to adults. Finally, we provide clues supporting the hypothesis that the icefish ancestor relied on a single alpha-globin gene and a single beta-globin gene in adults, which left this lineage at the mercy of any detrimental alterations to hemoglobin genes, setting suboptimal conditions potentially predisposing for their loss in the icefish ancestor after it diverged from dragonfishes.

While our results illuminate the evolutionary forces that shaped the evolution of hemoglobins in notothenioids, the cause and genetic processes leading to the loss of hemoglobin genes in icefishes and how those presumably somewhat deleterious mutations were able to become fixed in ancestral, perhaps small, icefish populations remain unanswered. What genetic mechanisms deleted all hemoglobin genes from the icefish ancestor genome? Is life without hemoglobin and mature red blood cells an adaptive trait to cope with high blood viscosity in this cold environment (Wells et al. 1990; Egginton 1996) or in response to iron deficiency in the Southern Ocean (Corliss et al. 2019; Laptikhovsky 2019)? Is it possible that hemoglobin is generally dispensable in Antarctic notothenioids? Indeed, a 10-day treatment with phenylhydrazine, which selectively destroys red blood cells (Shetlar and Hill 1985), reduced the red-blooded bullhead notothen *Notothenia coriiceps* hematocrit by over 90% and hemoglobin concentration by over 70% without killing the animals (Borley et al. 2010), and red-blooded *T. bernacchii* can survive for few days when 95% of its hemoglobin is bound to toxic carbon monoxide (di Prisco et al. 1992). Hematological research showed that the plasma of the red-blooded Antarctic dragonfish *B. marri* carries up to one-sixth of the total oxygen transported by the blood, "a value which is certainly not negligible" (Kunzmann et al. 1991). In this context, the loss of hemoglobin in icefishes may have been a nonadaptive but nonlethal accidental event not strongly selected against due to the paucity of competition and predators and small population sizes in the Southern Ocean. Perhaps existing traits that partially adapted the icefish ancestor to already somewhat degraded hemoglobin capacity in the plunderfish, dragonfish, and icefish ancestor also permitted the icefish ancestor to survive, reproduce, and evolve additional compensatory physiological traits to this seemingly maladaptive condition as hemoglobin gene losses became fixed in the population. Additional physiological and phenotypic studies, prerequisites for linking phenotypes to genotypes, and additional contiguous genome assemblies of diverse cryonotothenioids are needed to better connect notothenioid hemoglobin physiological functions, such as oxygen-binding capacity, Bohr and Root effects, and hemoglobin diversity and multiplicity to their genetic underpinnings.

Materials and Methods

Genome Assemblies, Species Names, and Phylogeny

Published genome assemblies of 36 notothenioids and 6 nonnotothenioid Perciformes were searched for hemoglobin genes (Ahn et al. 2017; Baalsrud et al. 2018; Bargelloni et al. 2019; Kim et al. 2019; Bista et al. 2020, 2023; Catchen et al. 2020; Feron et al. 2020; Heras et al. 2020; Moran et al. 2020; Lee et al. 2021; Rivera-Colón and Catchen 2022). In these assemblies, the diversity of notothenioids is represented by 3 thornfishes (Bovichtidae), the Patagonian blennie *E. maclovinus* (Eleginopidae), 14 notothens (cryonotothenioids of the paraphyletic group Nototheniidae), 2 spiny plunderfishes (Harpagiferidae), 3 barbeled plunderfishes (Artedidraconidae), 5 dragonfishes (Bathydraconidae), and 8 Antarctic icefishes (Channichthyidae). The full list of genome assemblies used in this study is provided in supplementary table S1, Supplementary Material online. Species names follow the currently recognized taxonomy (Sheiko 2019; Eastman and Eakin 2021). Notothenioid phylogenetic relationships used throughout the study follow the recent broad species tree topology recovered using RADseq (Near et al. 2018) with the addition of *H. kerguelensis* as sister species to *H. antarcticus*, and of *Pagetodes antarcticus* as sister species to *Pagetodes atkinsoni*. The resulting species tree used in the study is available on the dedicated USAP-DC project page.

Hemoglobin Gene Retrieval

In teleost fish, hemoglobin genes occur in 2 distinct and unlinked clusters (LA and MN), each with generally tandem pairs of an alpha and a beta chain gene (Hardison 2008; Opazo et al. 2013). We first located each cluster in each assembly by searching for their flanking genes (i.e. *rhbdf1b*, *aqp8*, and *lcmt1* for the LA cluster and *kank2*, *nprl3*, and *mpg* for the MN cluster). Hemoglobin gene names follow a recently proposed nomenclature system based on genomic position, which is available directly from genome assemblies, instead of inferred expression patterns, which must be experimentally determined and are available for only a subset of species (Bista et al. 2023). Alpha- and beta-globin chain names (i.e. *hba* and *hbb*) are appended with a suffix indicating their origin in the LA or the MN cluster (e.g. *hbala* and *hbamn*). A numeral suffix further reflects the relative position of the gene within each cluster, with the orientation of the

cluster determined by the most upstream alpha-globin gene, which is arbitrarily named *hbala1* nearest the *rhbdf1b* gene and *hbamn1* nearest the *kank2* gene in the LA and MN clusters, respectively. The neighboring beta-globin gene is then called *hbbla1* and *hbbmn1* for the LA and MN clusters, respectively. Genes further along in the locus contain suffixes with larger numbers (e.g. *hbala2* and *hbamn2*). Tandemly duplicated genes use a decimal with incrementally higher numbers (e.g. *hbamn1.1* and *hbamn1.2*) and pseudogenes use a "*p*" (e.g. *hbamn1.3p*) to follow zebrafish gene nomenclature guidelines (Bradford et al. 2022). Sequence, length, position, and strand of individual exons of each alpha and beta gene were manually retrieved from each assembly by performing BLASTN searches using the yellow perch *Perca flavescens* as reference for exon boundaries. Exons of each gene were concatenated and translated into proteins to verify their accuracy. If a gene was incomplete (e.g. a verified missing exon) or displayed a premature stop codon, the gene was considered pseudogenized. Positions and orientation of all hemoglobin and flanking genes for the LA and MN clusters in the studied species are provided in supplementary tables S2 and S3, Supplementary Material online, respectively.

Hemoglobin Gene Expression Analysis

The NCBI Sequence Read Archive (SRA) was searched for transcriptomic sequencing libraries from hematopoietic organs (head kidney and spleen) for notothenioid species with contiguous LA and MN clusters. The NCBI SRA was also searched for sequencing data from notothenioid embryos to study embryonic hemoglobin gene expression; data were found for only the dragonfish *G. acuticeps*. Head kidney and embryo libraries from the European perch *P. fluviatilis* were used as outgroups to notothenioids. Analyzed paired-end libraries are listed in supplementary table S4, Supplementary Material online (Flynn et al. 2015; Xu et al. 2015; Pasquier et al. 2016; Berthelot et al. 2019; Chen et al. 2019; Bista et al. 2020). For each library, adapters were removed from the raw reads using cutadapt 4.1 (Martin 2011) and quality trimmed using Trimmomatic 0.39 (Bolger et al. 2014) with a quality score of 20 on a 5-nucleotide sliding window. The resulting quality-filtered libraries were verified using FastQC v0.11.9 (Andrews 2010). Genome annotations for each species were updated or created for all hemoglobin genes based on the position of each exon identified during the hemoglobin gene retrieval. Each genome was then indexed and reads were aligned using STAR 2.7.10a (Dobin et al. 2013). Because *α-mn1* duplicates often display long stretches of perfect nucleotide sequence identity to each other, as do *β-mn1* duplicates, paired reads were allowed to map to multiple positions and were sorted by name using SAMtools 1.15.1 (Danecek et al. 2021). Finally, fragment counts for each hemoglobin gene were obtained with featureCounts v2.0.1 (Liao et al. 2014), and multimapping read counts were evenly split among mapped genes. Read counts were finally normalized in transcripts per million (TPM). Because some genomes are not annotated across their entire length, only reads mapping to hemoglobin genes were used for the TPM normalization. Because expression of all duplicated *α-mn1* and *β-mn1* genes was low in adult organs and because the number of *α-mn1* and *β-mn1* duplicates varies across species, fragment counts of paralogs were combined for display purposes. Fragment counts for each individual gene are, however, provided in supplementary table S5, Supplementary Material online.

Analysis of the MN Cluster LCR

The MN cluster LCR was identified by aligning the zebrafish *Danio rerio* LCR sequence (Ganis et al. 2012) to the fifth intron of the gene *nprl3* in the thornfish *C. trigloides* using BLASTN. Notothenioid LCR sequences were then extracted using a mVISTA LAGAN alignment of the MN cluster (Frazer et al. 2004). Transcription factor binding sites in the LCR of the MN cluster of selected species were predicted using PROMO and TRANSFAC v.8.3 (Farré et al. 2003) for the canonical erythrocyte transcription factors Gata-1, CACCC, Nf-E2, Nrf2, Usf2, and Tal-1 (Ganis et al. 2012; Philipsen and Hardison 2018).

Protein Phylogenetic Trees

Individual hemoglobin protein sequences from all available notothenioid species and 5 nonnotothenioid Perciformes outgroups were sorted per gene and analyzed separately. Two additional trees combined the tandemly duplicated β-la1 proteins and all the β-mn proteins, respectively. Protein sequences were aligned using MUSCLE and visually inspected, and the best-fit model was determined using ModelFinder based on Bayesian information criterion (Kalyaanamoorthy et al. 2017). RAxML-NG (Kozlov et al. 2019) was then run with the best-fit model, 50 parsimony and 50 random starting trees, and 200 bootstraps or bootstrapping at the default cutoff of 0.03. Phylogenetic trees were visualized and edited using MEGA-X (Kumar et al. 2018). All alignments and ModelFinder and RaxML results are available on the dedicated USAP-DC project page.

Selection on Hemoglobin Genes

Synonymous and nonsynonymous substitutions (dS and dN, respectively) were calculated using KaKs_Calculator 2.0 (Wang et al. 2010) with the MS method and the thornfish *C. trigloides* sequences as reference. All input files and results of the dN/dS analysis are available on the dedicated USAP-DC project page.

HYpothesis testing using PHYlogenies (HyPhy) tests (i.e. aBSREL, RELAX, and MEME) were conducted on the Datamonkey web server (Weaver et al. 2018; Kosakovsky Pond et al. 2020). Four independent aBSREL tests for episodic diversifying selection (Smith et al. 2015) were performed on each gene to test, for each branch of interest in the phylogeny, whether a proportion of sites have evolved under positive selection. The aBSREL test models both site-level and branch-level nonsynonymous-to-synonymous

mutation ratio ω heterogeneity but does not test for selection at specific sites. The tests analyzed the branches leading (i) to Eleginopsioidea (Fig. 4A1) because it was previously shown that the rate of evolution increased genome wide on this branch (Daane et al. 2019), (ii) to cryonotothenioids (Fig. 4A2) because this branch corresponds to the period of cold adaptation in cryonotothenioids and also a period of high rate of molecular evolution (Daane et al. 2019), (iii) to the plunderfish and dragonfish group (Fig. 4A3) because hemoglobin multiplicity and hemoglobin affinity for oxygen are further reduced in plunderfishes and dragonfishes compared to cryonotothenioids (Giordano et al. 2015), and (iv) to dragonfishes (Fig. 4A4) because they form the sister group to icefishes (Near et al. 2018; Bista et al. 2023). All input files and results of the aBSREL tests are available on the dedicated USAP-DC project page and a summary of results is available in supplementary table S6, Supplementary Material online.

Three RELAX tests for variations in strength of natural selection between 2 subtrees of the species tree (Wertheim et al. 2015) were performed on each gene as depicted in Fig. 4B. The RELAX test asks whether the strength of natural selection has been relaxed or intensified along a specified set of test branches by comparing a set of "test" branches to a second set of "reference" branches. The tests compared (i) the cryonotothenioid subtree (including the branch leading to the cryonotothenioids) to the early-diverging temperate notothenioid subtree (Fig. 4B1), (ii) the plunderfish and dragonfish subtree (including the branch leading to the group) to the subtree encompassing all other cryonotothenioids (excluding the branch leading to cryonotothenioids) (Fig. 4B2), and (iii) the dragonfish subtree (including the branch leading to the group) to the subtree encompassing all other cryonotothenioids (including plunderfishes) (Fig. 4B3). All input files and results of the RELAX tests are available on the dedicated USAP-DC project page, and the summary of results is available in supplementary table S7, Supplementary Material online.

MEME tests for sites that have been subject to episodic positive or diversifying selection (Murrell et al. 2012) were performed on each gene using all available notothenioid sequences. MEME employs a mixed-effects maximum likelihood approach to test the hypothesis that individual sites have been subject to episodic positive or diversifying selection. For each site, MEME infers 2 nonsynonymous-to-synonymous mutation ratio ω rate classes and corresponding weights representing the probability that the site evolves under each respective ω rate class at a given branch. All input files and results of the MEME tests are available on the dedicated USAP-DC project page and a summary of results is available in supplementary table S8, Supplementary Material online.

Because of frequent duplications of *α-mn1* and *β-mn1* genes among notothenioids and the duplication of the *β-mn2* gene in the Antarctic silverfish *P. antarcticum*, for all 3 HyPhy tests (i.e. aBSREL, RELAX, and MEME), which allow only 1 sequence per species, these 3 genes were run first using the least divergent protein sequence for each species and second with the most divergent protein sequence for each species. The least and most divergent sequences for each species were defined based on their branch lengths in the protein trees. Because results were similar when studying the least or the most divergent data set, we do not expand on these tests; however, results are provided in supplementary tables S6 to S8, Supplementary Material online and on the dedicated USAP-DC project page.

Functional Sites and Protein 3D Modeling

For each protein of the LA and MN clusters, the position of the alpha- or beta-globin domains and the sites known to be involved in heme binding and tetramer interface were determined using NCBI CDD (Lu et al. 2020). The reference sequences used for the NCBI-CDD search, the recovered CDD domain, and predicted functional sites are provided in supplementary table S9, Supplementary Material online.

3D protein structures were then modeled with SWISS-MODEL using the fish template ranked highest based on GMQE and QMEANDisCo Global scores (Waterhouse et al. 2018; Studer et al. 2020). Resulting models in PDB format were loaded in iCn3D 3.14.0 (Wang et al. 2022: 3), and custom annotation tracks were added to highlight sites identified by MEME tests for episodic positive or diversifying selection, sites identified by NCBI-CDD as involved in heme binding or tetramer interface, and the overlaps between MEME- and CDD-predicted sites. Input sequences for modeling, best template ID and names, GMQE and QMEANDisCo Global scores of resulting models, and custom annotation tracks for iCn3D are provided in supplementary table S10, Supplementary Material online. All models are available on the dedicated USAP-DC project page in PDB and PNG formats, both loadable in iCn3D.

Each site identified by MEME to have been subjected to episodic positive or diversifying selection among notothenioids was further examined for variants in each species. Each variant was then analyzed by PROVEAN (Choi and Chan 2015) to predict neutral or deleterious consequences of the amino acid change on the protein function. Results of the PROVEAN tests are reported in supplementary table S11, Supplementary Material online.

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online.

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Author Contributions

Conceptualization: T.D. and J.H.P. Methodology: T.D. Validation: T.D. Formal analysis: T.D. Investigation: T.D., K.H., and A.L. Resources: T.D., I.B., and J.H.P. Data curation: T.D. and I.B. Writing—original draft: T.D. Writing—review & editing: T.D., I.B., K.H., A.L., and J.H.P. Visualization: T.D. Supervision: T.D. and J.H.P. Project administration: T.D. and J.H.P. Funding acquisition: T.D. and J.H.P.

Conflict of interest statement. None declared.

Data Availability

All data generated or analyzed during this study are included in the published article (and its Additional Information files), are publicly available in NCBI, or are deposited in the dedicated United States Antarctic Program Data Center (USAP-DC) project repository p0010417 (https://www.usap-dc.org/view/project/p0010417).

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