Title

Positive and relaxed selective pressures have both strongly influenced the evolution of cryonotothenioid fishes during their radiation in the freezing Southern Ocean

Authors

Kevin T. Bilyk*, Xuan Zhuang, Chiara Papetti*

* authors of correspondence

Kevin T. Bilyk* (bilykk@montclair.edu)

ORCID ID: 0000-0002-2262-2703

Department of Biology, Montclair State University, 1 Normal Ave., Montclair, NJ, 07043, USA

Xuan Zhuang (xz036@uark.edu)

ORCID ID: 0000-0002-1919-9764

Department of Biological Sciences, University of Arkansas, Fayetteville, AR, 72701, USA

Chiara Papetti* (chiara.papetti@unipd.it)

ORCID ID: 0000-0002-4567-459X

Department of Biology, University of Padova, Padova 35121, Italy

Abstract

Evolution in the chronic cold of the Southern Ocean has had a profound influence on the physiology of cryonotothenioid fishes. However, the suite of genetic changes underlying the physiological gains and losses in these fishes is still poorly surveyed. By identifying the genomic signatures of selection, this study aims to identify the functional classes of genes that have been changed following two major physiological transitions: the onset of freezing temperatures, and the loss of hemoproteins. Looking at the changes that followed the onset of freezing temperatures, positive selective pressure was found among a set of broadly acting gene regulatory factors, suggesting a route through which cryonotothenioid gene expression has been retooled for life in the cold. Further, genes related to the cell cycle and cellular adhesion were found under positive selection suggesting that both present key challenges to life in freezing waters. By contrast, genes showing signatures of the relaxation of selective pressure showed a narrower biological impact, acting on genes related to mitochondrial function. Finally, while chronic cold-water temperatures appear correlated with substantial genetic change, the loss of hemoproteins resulted in little observable change in protein-coding genes relative to their red-blooded relatives. Combined, the influence of positive and relaxed selection shows that long-term exposure to cold has led to profound changes in cryonotothenioid genomes that may make it challenging for them to adapt to a rapidly changing climate.

Keywords

cold adaptation, cold specialization, molecular evolution, notothenioids

Significance Statement

Evolution in the isolation of the cold Southern Ocean have led to widespread physiological change in the cryonotothenioid fishes, but how this is reflected in changes among their pool of protein coding genes remains poorly described. Here, we comprehensively investigated changes in the suite of protein coding genes to identify which genes and associated functional categories have been changed following evolution in the chronic cold.

INTRODUCTION

Today, the waters of the Antarctic shelf are dominated by the members of a single taxonomic group, the cryonotothenioid fishes, comprised of the five Antarctic families of the perciform suborder Notothenioidei (Dornburg et al, 2017; Eastman, 2005; Near et al, 2018). The origin of this group is closely linked to the onset of freezing conditions in Antarctic waters (Matschiner et al, 2011), and their subsequent radiation driven by continued changes to the region's climate (Near et al, 2012). Evolution in this isolated, frigid environment has resulted in a diverse group of fishes that now share a profound specialization to life in the cold (Beers and Jayasundara, 2015; Daane and Detrich, 2022). However, the genetic consequences of evolution in chronic cold remain poorly surveyed in this group.

The most obvious shift in selective pressure experienced by the cryonotothenioids during the geological evolution of the Southern Ocean came from the region's cooling. In contrast to the cold-temperate waters inhabited by their closest non-Antarctic relatives, surface water temperatures around Antarctica remain below -1.5°C, and freezing water temperatures define the most species-rich waters along the Antarctic coast, even to great depths (DeVries and Steffensen, 2005). In addition to being cold, the waters around Antarctica are remarkable for stability, with only modest thermal variability compared to temperate and tropical waters (Barnes et al, 2006). This reaches an extreme in high-latitude habitats, such as the waters of McMurdo Sound, which are characterized by water temperatures that remain near their freezing point throughout the year (Cziko et al, 2014).

Although the origin of the cryonotothenioid clade is estimated at 22.4 million years ago (Ma) (Near et al, 2012), most of the living diversity of Antarctic notothenioids is believed to have originated more recently, with many speciation events taking place within the last 5 million years (Bista et al. 2022). We expect that evolution in these freezing waters would have imposed strong selective pressures to deal with the biological challenges and opportunities that came with life in the cold, but it would also have exposed endemic fishes to an important relaxation of selective pressure for some biological traits less impacted by cold adaptation. Specifically, the persistence of cold-stable water temperatures and high dissolved oxygen levels would be expected to relax selective pressures across biological systems that previously dealt with temperature and oxygen variability (Somero, 2010).

In addition to the influence of temperature, the isolation of the Southern Ocean has allowed the evolution of lineages characterized by substantial, and in some cases unique, physiological reorganization. This is exemplified by the members of the notothenioid family Channichthyidae (the icefishes) that are

extraordinary for the absence of the respiratory pigment hemoglobin (Sidell and O'Brien, 2006). Oxygen is found solely in physical solution in icefish blood, and these fishes are thought capable of surviving in the Southern Ocean because the cold, stable, and well-mixed Antarctic waters create a marine environment rich with dissolved oxygen. While icefishes have undergone widespread and profound anatomical and physiological reorganization following the loss of hemoglobin, whether this is mirrored in large changes to the protein pool and corresponding genes, or if it is compensated through plasticity and gene expression regulation (Bargelloni et al. 2019), is less understood. At a minimum, the loss of hemoglobin would be expected to produce a relaxation of purifying selection on its former partnering genes, and prior work has suggested that at least some genes with roles focused solely on supporting hemoglobin may be heading towards becoming pseudogenes (Bilyk et al. 2019).

Several prior studies have aimed to develop a broad understanding of the changes to protein-coding genes in the cryonotothenioids associated with polar evolution. Looking first at global biases in amino-acid composition that may explain adaptation to polar conditions, Berthelot et al. (2019) found evidence for only limited change, identifying an increase in leucine substitutions for methionine. This change was hypothesized to serve as an adaptation in the cryonotothenioids tied to redox regulation, providing a defense against increased levels of reactive oxygen species in the oxygen-rich waters of the Southern Ocean.

Studies by Daane and colleagues (2020; 2019) have since investigated how genetic mechanisms have shaped evolutionary change in the cryonotothenioids during their radiation in the Southern Ocean. Using the evolution of buoyancy adaptation among several Antarctic lineages (Eastman, 2020) as a model to explore the origins of novel traits in the cryonotothenioids, this was found to result from bone demineralization associated with developmental alterations similar to human skeletal dysplasia. However, the onset of positive diversifying selective pressure that reshaped these pathways was found to precede the origin of the cryonotothenioids, showing the role of historical contingency in shaping the capacity for adaptation in this group (Daane et al, 2019). Contrasting with buoyancy adaptation, Daane et al (2020) investigated the genetic regions controlling erythropoiesis and found evidence that relaxation of selective pressure followed sustained cooling of the Southern Ocean, and acted independently on these genetic elements among several impacted lineages of cryonotothenioids. The influence of this relaxation of selective pressure was further found biased toward conserved noncoding elements (CNEs) rather than to coding regions.

While results by Berthelot et al. (2019) as well as Daane and colleagues (2020; 2019) provide important insight into the timing and mechanisms of adaptive change in the cryonotothenioids, how the suite of protein

coding genes have been changed during their radiation in the freezing Southern Ocean remains poorly surveyed. The aim of this study is therefore to identify how changes in selective pressure following the onset of chronic cold conditions and following the loss of hemoproteins have shaped the suite of protein coding genes of modern cryonotothenioid fishes.

RESULTS

To investigate how the shifts in selective pressure from evolution in chronic cold and loss of hemoproteins have affected the pool of protein coding genes among the cryonotothenioids, we obtained and analyzed 3,453 orthogroups that contained all species in a 19-taxa dataset (Fig 1). These taxa included seven red-blooded cryonotothenioids and five hemoglobin-lacking icefishes where public genomic and transcriptomic resources were available, along with a background set of seven temperate and tropical fishes. The 3,453 orthogroups that were used in the analysis represent 15.3% of the predicted peptides identified in the annotated *D. mawsoni* genome. Metrics on the filtered genomic and transcriptomic resources used to identify orthogroups are presented in Fig 1, alongside the phylogenetic framework for evolutionary hypothesis testing (detailed in S Figure 1). Additional information on the tissue content of each transcriptome is presented in S Figure 2 and comparisons between the genomic and transcriptomic derived predicted peptides for species, where both were available, are presented in S Figure 3.

This set of orthogroups was used for alignment construction then evaluated using HyPhy to identify genes showing signatures of either positive or relaxed selective pressures. The goal being to understand whether positive and relaxed selective pressure affected distinct functional categories of protein coding genes.

First, the red-blooded cryonotothenioids were compared to temperate and tropical fishes to identify the suite of changes that have followed their evolution in chronic cold. From this comparison, aBSREL (adaptive branch-site random effects model) and BUSTED (Branch-Site Unrestricted Statistical Test for Episodic Diversification) identified 113 and 89 orthogroups respectively under positive selection, with 160 orthogroups identified as experiencing positive selective pressure from either of the two analyses, and 42 orthogroups consistently identified as experiencing positive selective pressure by both analyses (Fig 2A). RELAX then identified 114 orthogroups as experiencing relaxed selective pressure (Fig 2A). The set of genes showing signatures of positive selective pressure and relaxed purifying selective pressure was largely distinct, but nine genes were found to show signatures of both (S Table 3).

Next, the icefishes were compared against a background set of the red-blooded cryonotothenioids, to identify genes under changed selective pressure following the loss of hemoproteins. This comparison showed a far smaller signature of impacts from both positive and relaxed selective pressures. The signature of significant positive selective pressure was identified on only 15 orthogroups using BUSTED, and 32 orthogroups with aBSREL, resulting in a total of 41 distinct orthogroups identified as experiencing positive selective pressure from either analysis, with 6 orthogroups identified under positive selective pressure by both analyses (Fig 2B). Similarly, relaxed selective pressure was detected for only 16 orthogroups using RELAX (Fig 2B). There was no overlap among these sets of genes showing signatures of positive and relaxed selective pressure.

To put changes in selective pressure into their broader biological context, we tested for functional enrichment of genes showing signatures of changed selective pressure by GO enrichment analysis (S Tables 4-9). Enrichment analysis of the combined set of 160 orthogroups that were identified under positive selection in the red-blooded cryonotothenioids compared to temperate and tropical fishes by either aBSREL (S Table 4) or BUSTED (S Table 5) found a total of 40 significant enriched GO terms at the FDR corrected p-value threshold of 0.1 (S Table 6). Clustering through EnrichmentMap identified three clusters of GO terms connected by shared sets of genes (Fig 3A), identifying functional impacts on the regulation of gene expression, cell cycle progression, and cell adhesion. Looking at the more restricted set of 42 orthogroups where both BUSTED and aBSREL agreed they were under positive selection found 31 significantly enriched GO terms (S Table 7). While this shared set of orthogroups identified by both BUSTED and aBSREL provides a narrower view of changed biological systems in the cryonotothenioids, it continues to highlight a critical role of changed regulation of gene expression. This set of 42 orthogroups identified as experiencing positive selective pressure by both BUSTED and aBSREL are provided in S Table 8 while the larger set of 160 orthogroups identified by either of those analyses is provided in S Table 9.

By contrast with the investigation of orthogroups showing signatures of positive selective pressure, enrichment analysis of the orthogroups under relaxed selective pressure in the red-blooded cryonotothenioids produced a much more restricted set of enriched GO terms after FDR correction (S Table 10). Only a single GO term was enriched, identifying the mitochondria as a key site of relaxed selective pressure. The full set of orthogroups identified by RELAX as experiencing significant relaxation of selective pressure are presented in S Table 11. Further, the set of genes found under positive or relaxed selection are grouped by cluster and GO term in S Tables 12-15.

Unlike the comparison between the red-blooded cryonotothenioids and temperate fishes, only a small number of orthogroups were identified under changed selective pressure in the icefishes relative to the red-blooded cryonotothenioids. At the target FDR corrected p-value these did not result in any enriched GO terms. Instead, the biological impact of changed protein coding sequences among the icefishes was investigated by direct interrogation of the set of orthogroups, and to help characterize their disparate biological roles they were grouped according to their Panther protein classification (Thomas et al. 2003) as presented in Table 1.

Finally, the ability to discriminate selective change on each orthogroup is based on the success of the homology-based approach to pruning paralog contamination used in this study. To evaluate this approach, we compared our findings to an alternate set of orthogroups determined as described in Birkeland et al. (2020). As detailed in the S Material this continued to show the same broad trends in the number of genes under changed selection with cold adaptation and loss of hemoproteins. While the Birkeland et al. (2020) approach showed a reduced ability to identify change in functional classes of genes, it did continue to show significant signatures of positive selection acting on a core group of gene regulatory factors as determined by both BUSTED and aBSREL, consistent with what we have found using our primary approach to paralog pruning.

DISCUSSION

The radiation of the cryonotothenioids in the isolation of the frigid Southern Ocean would have exposed these fishes to distinct selective pressures compared to their cold-temperate precursor. However, the extent to which these shifts in selective pressure are reflected across the pool of protein coding genes has remained unclear. In this study, we found that evolution in the chronic cold is correlated with signatures of positive selective pressure acting heavily on proteins controlling gene expression along with basic cellular functions, suggesting key roles in cold adaptation in cryonotothenioids. This contrasts with the signatures of relaxed selective pressure, where a far narrower biological impact was resolved. Finally, in the icefishes, a small set of protein coding genes were found to experience a change in selective pressure following the loss of hemoproteins despite widespread anatomical and physiological change we see in this cryonotothenioid family.

Genes under positive selective pressure in the red-blooded cryonotothenioids

The freezing temperatures of the Southern Ocean present clear challenges to life. While the most immediate threat these fishes face comes from inoculative freezing, the extreme low water temperatures broadly impact

biological processes. The set of genes showing signatures of positive selective pressure are suggestive of which biological functions may have been involved in polar adaptation in our focal group.

The largest signature of change comes from a collection of genes with roles controlling gene expression. These include varied gene regulatory factors: transcription factors, transcription activators, transcription repressors, histone-modifying enzymes, and signaling proteins. Among protein coding genes, changes to such gene regulatory factors can have particularly wide-ranging impacts, as this can influence expression across the gene regulatory factor's target genes as well (Nowick et al., 2011; Perdomo-Sabogal et al., 2014; Tirosh et al., 2009). Prior work has shown that evolutionary change in gene regulatory factors regularly occur, and that such change may be a driving force behind species diversification and evolutionary innovation (Wagner and Lynch, 2008; Nowick et al., 2013; Perdomo-Sabogal et al., 2014).

In terms of how this may relate to the evolution of the cryonotothenioids, today these fishes show a highly modified transcriptional program compared to temperate fishes (Chen et al., 2008). This includes increased expression of genes with biological roles that would mitigate many cold induced stresses, with roles in protein biosynthesis, protein folding and degradation, lipid metabolism, antioxidation, antiapoptosis, innate immunity, and choriongenesis, among others. Given capacity for widespread influence on the expression of downstream genes, changes to broadly acting gene regulatory factors such as those found under positive selection may thus have played a role in creating the cold-adapted transcriptome now seen among the cryonotothenioids.

In addition to changes that may impact the types of genes that are expressed, the set of genes under positive selection included several involved in the process of gene expression itself. The genes showing signatures of positive selection include those mediating RNA polymerase activity (*MED23*, *TCEA1*, *UTP15*) and mRNA processing (*THOC5*). Prior studies have suggested that nascent protein synthesis (Pace and Manahan 2007) and their folding (Place and Hofmann 2005) are challenges to life in freezing polar waters. Our findings may suggest the efficient transcription came under selection with adaptation to low temperature as well. Transcription has been shown to exhibit reduced efficiency and increased errors as an organism moves from physiological temperatures (Meyerovich et al, 2010). Adaptation to cold temperatures could thus exert selective pressure on the machinery of transcription to ensure continued accurate gene expression with the transition from temperate to polar conditions.

Alongside changes related to the pattern and process of gene expression, the set of genes under positive selection suggests compensatory change to adapt to the rate limiting effects of low temperature on

biological systems. This change is first seen through impacts to genes with essential roles in the cell cycle. A critical constraint on an organism's viable temperature range is the temperature range over which cell division can occur (Begasse et al., 2015), as this dictates the temperature range that growth and development can occur. The cryonotothenioids show change to several components of the machinery of cellular replication (*GNAI1*, *KIF22*, *NUF2*, *RTF2*), suggesting adaptative change has acted on this toolkit to enable a basic activity of life to continue at freezing temperature.

In addition to challenges to the functioning of the cell cycle from the cold, the rate limiting effects of low temperature present a further problem. However, past comparative studies have shown that adaptation can compensate for the rate limiting effect of the cold on growth rates (Clarke 2003), and this seems to extend to the cell cycle itself. Investigation of cell cycle progression in the cryonotothenioid *Harpagifer antarcticus* found this to progress at a faster rate at 0°C than in its sub-Antarctic congener *H. bispinis* at 5°C suggesting cold compensation in the Antarctic species (Brodeur et al. 2003). Here, signatures of positive selective pressure were seen among genes related to cell cycle progression, including genes with a direct role in its regulation and progression (*CC14A*, *GNL2, DRG1*, *PDC6I*, *PRCC*). This could represent parts of the genetic change that allow the cell cycle to progress at a biologically relevant rate at the low temperatures of the Southern Ocean.

Finally, we see signatures of positive selection among genes associated with cell adhesion. These included components of the junctions themselves (*CADH1*, *DSC2L, EPCAM*), that link intercellular connections to the cytoskeleton (*CTNA1*), and that regulate cell junction organization (*TMM47*). The ability of cells to adhere to one another is strongly impacted by temperature, with a reduced capacity for adhesion as temperatures drop as cell-cell linkages lose the ability to organize and maintain cohesion (Attramadal A 1975; Rico et al., 2010; Zieger et al., 2011). Thus, the capacity to organize and maintain adhesion may have come under selective pressure in the cryonotothenioids in order to maintain the integrity of tissues in freezing waters.

Genes under relaxed selective pressure in the red-blooded cryonotothenioids

The Southern Ocean is remarkable for the chronic nature of its cold-water temperatures as much as the severity of this cold. Along with these stable low water temperatures, the surface waters of the Southern Ocean are oxygen rich as the oxygen carrying capacity of water increases with decreasing temperature (DeVries and Steffenson 2005). Wave driven mixing action then ensures that even habitats at depth are saturated with oxygen across much of the Antarctic's continental shelf. The onset of these cold and oxygen rich conditions would have relaxed selective pressure for the ancestor of the cryonotothenioids coming

from an environment that was far more variable in both. Here, enrichment analysis on the set of genes putatively under changed selective pressure suggests that transition from a temperate to a chronic cold environment has left an impact on the genetic toolkit of the cryonotothenioids, in particular through change to genes related to mitochondrial function (Fig 3B, S Table 10).

These genes span a range of roles within the mitochondria, including several components of the electron transport chain *(SDHA, NDUA8, UQCRC2),* genes essential to mitochondrial function *(FASTKD1, MCAT, MPC2b, NNT, PRODHA, SLC25A44),* and genes associated with biosynthesis within the mitochondria *(METTL17, MRPL41, MRPL53, MRPS17, MRPS22, MRPS30, TACO1, TEFM)* or of essential mitochondrial proteins *(COA7, DDX28, TOMM22).* Recent work analyzing the types of selection acting on the protein coding genes in the notothenioid's mitochondrial genome by Papetti et al (2021) concluded that a relaxation of selection was active early in the evolution of the cryonotothenioids, perhaps driven by the initial onset of cooling conditions. The results of this study suggest that the pattern of selective change may extend across the broader set of genes related to mitochondrial function, not just those restricted to the mitochondrial genome itself.

As for the source of changed selective pressure, one explanation may be the increased oxygen availability as the Southern Ocean cooled. Variable oxygen levels are a challenge for living things that rely on aerobic respiration given oxygen's role as the terminal electron acceptor in the mitochondria's electron transport chain. Cells of aerobic organisms maintain a toolkit to ensure oxygen homeostasis to ensure a constant rate of respiratory activity even as oxygen levels may vary (Longmuir 1957; Wilson et al., 1979). The onset of persistent high oxygen levels in the Southern Ocean could thus have reduced the necessity of maintaining such flexibility in mitochondrial function.

Besides oxygen availability, the signature of relaxed selection observed on mitochondrial genes may also be a consequence of the temperature stability of the Southern Ocean's water temperatures. Study of mitochondrial capacities in Antarctic notothenioids by Mark et al., 2012 showed that the mitochondrial capacities of these Antarctic fishes showed thermal limits that were lower than those of temperate species. Relaxed selective pressure broadly across the genetic toolkit of the mitochondria thus may alternatively reflect the lack of demand to maintain mitochondrial function at temperatures that are no longer encountered in cold Antarctic waters.

Genes under changed selective pressure in the icefishes

Among the highly cold-adapted cryonotothenioid fishes, the species of the family channichthyidae are remarkable for their lack of hemoproteins, but has this loss led to extensive change among the pool of protein coding genes? Cold adaptation, which was explored using the red-blooded cryonotothenioids, is a very broad trait with widespread expected consequences across biological systems and therefore we expect there should be many genes involved; in contrast, the loss of hemoproteins is a far more specific trait, so we expected to find fewer genes under selection. Our results tested this hypothesis – we identified a diverse suite of genes under cold-adapted selection; and a few specific genes under hemoprotein-less selection.

While relatively few genes showed signatures of changed selective pressure in the icefishes when compared to the red-blooded cryonotothenioids, this set of icefish genes under changed selective pressure was diverse (Table 1). The small number of genes under positive or relaxed selective pressure did not result in significant enrichment of GO terms after correcting for multiple hypothesis testing, but interrogating this gene list, several of the genes under positive selection have roles that correspond to phenotypic change observed in the icefishes. These included genes with roles in cardiac morphogenesis, myogenesis, and vascular development (*MEF2C*, *NIPLB*), change that is correlated with the highly modified cardiovascular system seen in the icefishes that allows the larger blood volume necessary to transport oxygen in blood plasma without the presence of red blood cells (Sidell and O'Brien, 2006). Interestingly, Bargelloni et al. (2019) found that several genes of the Myocyte enhancer factor (MEF family), including *MEF2C* are largely upregulated in the icefish and these may play an important role in the larger mitochondrial density seen in icefish cells. Similarly, several genes with roles in energy metabolism (*F16P1*, *GTR1*, *PAQR1*) correspond to increases in mitochondrial size and density within icefish cells, and perhaps the need to keep cellular metabolism fueled in these fishes as oxygen availability is more limited. Finally, selection was seen in genes related to cartilage biosynthesis (*CHSTB*) and neural patterning (*IRX3*) both of which would likely be tied to the continued modification of skeletal and skull shape than what is seen in the remaining cryonotothenioids.

Besides the more limited scope of the comparison compared to cold adaptation, are there other reasons why we might observe such muted signatures of genetic change among protein-coding genes following hemoprotein loss? One possibility is that the relatively recent divergence of the icefishes has not allowed sufficient time to see widespread changes in protein-coding genes even if selective pressures have shifted. Alternatively, Bargelloni et al. (2019) suggested that the loss of hemoglobins has been accompanied by change in mechanisms like gene silencing or gene expression regulation more than change to protein coding sequences. This would correspond to recent findings by Daane et al. (2021) and Bista et al. (2022) where

investigation of genetic regions controlling erythropoiesis in the icefishes found clearer signatures of drift acting on the conserved noncoding elements that control gene behavior rather than the coding regions themselves. Finally, prior work has also shown that the oxygen-rich Southern Ocean environment relaxed selective pressure on oxygen carriers before hemoprotein loss. This is observed in the reduction in hemoglobin isoform complement among the red-blooded cryonotothenioids (Eastman, 1993), and in the attenuation of regulatory elements upstream of hemoglobin (Lau et al, 2012) both in the red-blooded cryonotothenioids and the icefishes (Daane et al. 2021). As a result, the icefishes may simply reflect an extreme result of the same selective forces that are acting across the cryonotothenioids as a whole.

Conclusions

The genomes of the cryonotothenioid fishes have been changed during their evolution in the isolation of the Southern Ocean as seen among their suite of protein coding genes. The observed changes reflect not only the abiotic challenges of life in a cold and oxygen-rich environment, but also the stability of the region's waters.

The influence of positive selective pressure was most strongly felt on proteins with roles in gene expression, suggesting one route through which the cryonotothenioid transcriptomic profile was retooled for life in the cold. Further change observed in genes with roles in the machinery of transcription, cell cycle progression, and in cell adhesion, pointing to biological challenges for life in a freezing ocean. Contrasting with the influence of positive selection, the relaxation of selective pressure appears to have exerted a narrower influence. This seems to have driven change to a diverse group of genes related to mitochondrial function, perhaps due to the reduced challenges of carrying out aerobic metabolism given the cold stable temperatures and high oxygen levels found in Southern Ocean waters. Finally, in icefishes, the loss of hemoglobin has resulted in far fewer detected signatures of change in the sequence of protein coding genes, perhaps due to the smaller number of possible target genes compared to cold adaptation, or due to the alternative genetic and physiological mechanisms that may have compensated for this unique loss.

In evaluating these changes, one note of caution is that we are limited by the number of orthologous relationships that we could identify among the protein coding sequences of the target species. It is likely that further biological systems under positive and relaxed selective pressure have yet been discovered in this group as a fuller view of protein coding genes becomes available.

Ultimately though, cryonotothenioid fishes have been exposed to long and intense selective pressure for survival in freezing waters, and now show widespread change when compared to temperate fishes including

long development and generation times, deferred maturity, and extended life spans (Peck 2016). The genetic change we identified in the cryonotothenioids since the onset of freezing conditions may further suggest a genetic specialization to life in the cold that would leave them particularly ill-equipped to deal with a warming world. In light of these changes, it seems unlikely that any reverse adaptation might be possible in the short term of the predicted climatic alteration occurring in Antarctica (Masson-Delmotte et al, 2021).

MATERIALS & METHODS

Genomic resources, sequence, assembly, & filtering

To investigate how the pool of protein-coding genes have been affected by the ecological and physiological transitions experienced during the radiation of the cryonotothenioids, public genomic and transcriptomic resources were compiled for the cryonotothenioids along with a relevant background set of temperate and tropical fishes. In total, protein-coding sequences were compared across 19 species including 7 red-blooded cryonotothenioids, 5 hemoglobin-lacking icefishes, and 7 temperate and tropical fishes. The full list of the species used in this study and accession numbers for the genomic and transcriptomic resources used in the analysis can be found in the supplementary material (S Table 1). Of these, genome-derived predicted protein-coding gene sequences were publicly available at the time of this study for only three red-blooded species: *Dissostichus mawsoni* Norman, 1937 (Nototheniidae; Chen et al, 2019), *Notothenia coriiceps* Richardson, 1844 (Nototheniidae; Shin et al, 2014), and *Parachaenichthys charcoti* Vaillant, 1906 (Bathydraconidae; Ahn et al, 2017). To extend this limited dataset, sequenced transcriptomic reads were downloaded for species where more than one tissue was available, including the three red-blooded species with available sequenced genomes (Bargelloni et al, 2019; Berthelot et al, 2019; Bilyk et al, 2018; Kim et al, 2019; Song et al, 2019).

To provide a baseline for evaluating changes in selective pressure in the cryonotothenioids, predicted peptides and coding domain sequences (CDS) were compiled for a background set of temperate and tropical fishes. These included two extant temperate notothenioid fishes that diverged prior to the adaptation to polar conditions in the cryonotothenioids, the Patagonian blenny *Eleginops maclovinus* Cuvier 1830 (Eleginopidae; Chen et al, 2019) and *Cottoperca gobio* 1861 Günther (Bovichtidae; Bista et al, 2020). The remaining species were selected from the teleost fishes available from Ensembl (Yates et al, 2020), with the aim to minimize phylogenetic distance, avoiding obligatory freshwater species, avoiding species that inhabit freezing environments, and minimizing nested taxa. Species native to freezing habitats were avoided in the background set as this would likely obscure signatures of change among biological systems resulting from adaptation to freezing conditions among the cryonotothenioids.

The transcriptomic reads were assembled for all of the cryonotothenioids. The reads were first cleaned with Fastp v0.20.1 (Chen et al, 2018) then assembled with Trinity v. 2.6.5 (Grabherr et al, 2011) using default parameters. Assemblies then went through preliminary filtering to remove redundancy with CD-hit v. 4.8.1 (Fu et al, 2012), and by removing contigs with less than 1 Transcripts Per Million (TPM) read coverage based on the transcriptome's original sequenced reads. Predicted peptides and their corresponding CDS were then determined for the transcriptomes using TransDecoder v. 5.5.0 (Haas, 2021). These predicted peptides were subjected to a final round of filtering meant to reduce much of the redundancy present in each species' transcriptome. Predicted peptides were mapped against the Swissprot database using BlastP v2.10.1, retaining only the best match for each Swissprot accession number as determined by e-value.

Finally, the relative completeness of species' genome or transcriptome derived set of predicted peptides was evaluated using BUSCO v. 4 (Simão et al, 2015). BUSCO provides a measure of the assembly's gene-completeness by quantifying the presence of conserved single copy orthologous genes. In addition to providing a general measure of quality for each species' gene set, BUSCO scores were used to choose between genomic and transcriptomic derived predicted peptides and CDS when both were available for a given species. The transcriptomic assemblies were generally found to show higher levels of complete genes likely reflecting the oversampling that is inherent to transcriptomic sequencing, though this came at the expense of greater redundancy which had to be filtered.

Phylogenetic reconstruction

To provide a phylogenetic frame of reference for later evolutionary hypothesis testing, we reconstructed the relationship among the 19 investigated species. This phylogeny was constructed using 2 mitochondrial (*16S*, *ND2*) and 11 nuclear genes (*MYH6*, *PKD1*, *SH3PX3*, *HECW2*, *SSRP1*, *PPM1D*, *RPS71*, *TBR1*, *PTR*, *RHO*, and *ZIC1*). Accession numbers for the genes used in this analysis are provided in S Table 2.

The sequences of each gene were aligned using MUSCLE v. 3.8.31 (Edgar, 2004), and the best-fit nucleotide substitution model of each gene was determined using the Akaike information criterion (AIC) in ModelTest-NG v0.1.6 (Darriba et al, 2020). The aligned sequences were concatenated and partitioned according to their best-fit models (GTR + I + gamma, mitochondrial: ND2 and 16S; GTR + I + gamma: MYH6 and PKD1; GTR + gamma: SH3PX3, HECW2, and SSRP1; GTR: PPM1D; HKY + gamma: RPS71, TBR1, PTR, and RHO; HKY + I: ZIC1). These substitution models were then implemented using MrBayes v3.2.6 (Ronquist et al, 2012) to carry out Bayesian phylogenetic analyses. The Markov chain Monte Carlo simulation was run for 100 million generations with four chains and sampled every 100 generations.

MCMC convergence was assessed using the standard deviation of clade frequencies and potential scale reduction factor, and the first 25% of sampled trees were discarded as burn-in. Clade support was evaluated using posterior probabilities for nodes retained in the 50% majority rule consensus tree.

Orthogroup inference, paralog pruning and building the multiple sequence alignments

Orthogroups were inferred across species from their filtered set of predicted peptides using OrthoFinder v. 2.5.1 (Emms and Kelly, 2019). The resulting orthogroups were then filtered to remove potential paralog contamination using a two-step process. First, PhyloTreePruner v. 1.0 (Kocot et al., 2013) was used to isolate the largest monophyletic subtree from the gene trees generated by FastTree 2 (Price et al., 2010) produced for the contigs within each orthogroup. Second, the subtree was collapsed to a set of species-specific putative orthologs using BlastP against the *D. mawsoni* predicted peptide set. In this last step, contigs were only considered to have an orthologous relationship if they were best Blast hits, as determined by e-value, to the same *D. mawsoni* predicted peptide. If more than one contig from a species matched the same *D. mawsoni* predicted peptide then only the best blast hit, as determined by e-value, was retained. If more than one transcript was present from *D. mawsoni* in the orthogroup, the orthogroup was subset for each *D. mawsoni* transcript, and contigs from other species were assigned only to the *D. mawsoni* transcript with the best blast hit. Finally, only filtered orthogroups with representatives of all 19 target species were retained for constructing the multiple sequence alignments (MSAs). To evaluate our approach to paralog pruning, we generated an additional set of filtered orthogroups using the approach described in Birkeland et al. (2020) which is presented in the S Material.

Multiple sequence alignments were then generated for each retained orthogroup. The CDS for each orthogroup's predicted peptides were codon aligned with GUIDANCE2 (Sela et al, 2015) using Prank v.140603 (Löytynoja, 2014), running 25 pseudo replicates per alignment. The alignments were then trimmed to remove any missing sites, and only alignments with all species and a minimum final length of 300nt were retained for evolutionary hypothesis testing.

Identifying orthogroups under changed selective pressure

Orthogroups experiencing positive selective pressure were identified using the phylogeny-based strategy implemented in the Branch-Site Unrestricted Statistical Test for Episodic Diversification (BUSTED; Murrell et al, 2015) and the adaptive Branch-Site Random Effects Likelihood (aBSREL; Smith et al, 2015) methods in HyPhy (Kosakovsky Pond et al, 2020). The contrasting set of orthogroups experiencing a relaxation pressure was identified using the RELAX method in HyPhy (Wertheim et al, 2015).

The orthogroups were first used to compare protein-coding genes in the red-blooded cryonotothenioids as the foreground against the background set of temperate and tropical fishes to identify the set of orthologous genes that have come under changed selective pressure during the cryonotothenioid radiation in the frigid Southern Ocean. Similarly, the icefishes were tested as the foreground against a background of red-blooded cryonotothenioids to identify the set of orthogroups that have come under changed selective pressure specific to the loss of hemoproteins. A False Discovery Rate (FDR) adjusted p-value threshold of 0.1 was used in all tests to identify those orthogroups showing a significant change in selective pressure from the background set.

Testing for functional enrichment

Gene ontology (GO) enrichment analysis was used to place the impacts of positive and relaxed selective pressures into biological context. The *D. mawsoni* predicted peptide set was annotated for GO terms using the Orthologous Matrix fast mapping utility (OMA browser; Altenhoff et al, 2021). The resulting GO annotations was then used to generate a slimmed set of GO terms for *D. mawsoni* using Blast2GO (Conesa et al, 2005). GO slim terms were then applied across an orthogroup if it contained the original annotated *D. mawsoni* predicted peptide.

TopGO v. 2.44 (Alexa and Rahnenfuhrer, 2018) was used to test for functional enrichment among the isolated sets of genes under positive diversifying or relaxed selective pressure within the Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) ontologies from the GO-slim annotation sets. Enrichment was tested using the sets of significant orthogroups identified by BUSTED, aBSREL, and RELAX, against a background set of 3,452 orthogroups with representatives from all 19 species originally used in the analysis. Fisher's exact test was then used to identify enriched terms using an FDR adjusted p-value threshold of 0.1, both on the results of the individual tests, on the intersected set of genes where both BUSTED and aBSREL agreed on signatures of positive selection, and on the combined set of genes identified under positive selection by either BUSTED or aBSREL. The identified set of significant GO terms were then visualized using the EnrichmentMap (Merico et al, 2010) plug-in of Cytoscape network visualization software v. 3.8.2 (Shannon et al, 2003).

ACKNOWLEDGMENTS

The authors would like to thank Dr. Agnès Dettaï (Muséum national d'Histoire naturelle, Paris, France) and Enrico Negrisolo (University of Padova, Padova, Italy) for advice on the phylogenetic markers used in this analysis. The authors would further like to thank Katherine R. Murphy and Angel G. Rivera-Colón for their helpful comments on the manuscript, as well as the thoughtful comments of our reviewers. Many of the selection test methods applied in this study were facilitated using best practices and scripts provided as part of a Next Generation Sequencing-based workshop sponsored by the National Science Foundation (Award # 1744877 to Scott Santagata, Biology Department, Long Island University, New York, USA). KT Bilyk would like to acknowledge partial support for this work from the National Science Foundation (Award # 1341701). CP acknowledges partial support from the University of Padova BIRD funding (grant no. 164793).

AUTHOR CONTRIBUTIONS

KTB and CP developed the original comparison between temperate and polar species, KTB and XZ developed the comparison between the icefishes and red-blooded cryonotothenioids. XZ built the phylogenetic framework for the study and KTB carried out the selective pressure analyses. All three authors contributed to the interpretation of the selective pressure results and writing the manuscript.

DATA AVAILABILITY STATEMENT

All genomic and read data used in this study were downloaded from publicly available repositories as detailed in the supplementary material. The scripts used in this study are available through the GitHub repository: https://github.com/TheOneTrueKevin/2023-Positive-and-Relaxed-Selective-Pressure-on-Cryonotothenioid-Fishes

FIGURES

Figure 1.

Panel A shows the comparative groupings used in the analysis, with species in red highlighting temperate/tropical fishes, species in purple highlighting red-blooded cryonotothenioids, and species in blue highlighting icefishes. Panel B reports BUSCO metrics for the peptide sets used for original ortholog detection in all 19 target species.

Figure 2.

Number and distribution of orthogroups showing changes in selective pressure with the transition to freezing water temperatures and with hemoprotein loss. The phylogenetic trees show the comparison each analysis is based on while the Venn diagrams show the number of genes identified by RELAX as being under relaxation of purifying selective pressure, or by aBSREL and BUSTED as being under positive selective pressure. Estimated divergence times for the two focal clades are from Near et al. (2012).

Figure 3.

GO terms enriched in the Biological Process ontology among the red-blooded cryonotothenioids among genes showing signatures of positive (panel A) and relaxed (panel B) selective pressures.

TABLES

Table 1.

Genes identified under changed selection in the icefishes (family Channichthyidae). This table shows the set of genes that were determined by aBSREL, BUSTED, or RELAX to have come under changed selective pressure in the icefishes compared to the red-blooded cryonotothenioids. Genes are characterized according to their Panther protein classification (Thomas et al. 2003), serving to highlight their diverse range of functions. Genes that were identified as experiencing positive selective pressure by both aBSREL and BUSTED are bolded in the table.

REFERENCES

Ahn D-H, Shin SC, Kim B-M, Kang S, Kim J-H, Ahn I et al (2017). Draft genome of the Antarctic dragonfish, *Parachaenichthys charcoti*. GigaScience 6(8).

Altenhoff AM, Train C-M, Gilbert KJ, Mediratta I, Mendes de Farias T, Moi D et al (2021). OMA orthology in 2021: website overhaul, conserved isoforms, ancestral gene order and more. Nucleic Acids Research 49(D1): D373-D379.

Alexa A, Rahnenfuhrer J (2022). topGO: Enrichment Analysis for Gene Ontology. R package version 2.50.0.

Attramadal A (1975). The effect of temperature on long-term cell adhesion. Acta Pathol Microbiol Scand A. 83(3):323-327

Bargelloni L, Babbucci M, Ferraresso S, Papetti C, Vitulo N, Carraro R et al (2019). Draft genome assembly and transcriptome data of the icefish *Chionodraco myersi* reveal the key role of mitochondria for a life without hemoglobin at subzero temperatures. Communications Biology 2(1): 443.

Barnes DKA, Fuentes V, Clarke A, Schloss IR, Wallace MI (2006). Spatial and temporal variation in shallow seawater temperatures around Antarctica. Deep Sea Research Part II: Topical Studies in Oceanography 53(8): 853-865.

Begasse ML, Leaver M, Vazquez F, Grill SW, Hyman AA (2015). Temperature dependence of cell division timing accounts for a shift in the thermal limits of *C. elegans* and *C. briggsae*. Cell Reports, 10(5): 647-653,

Beers JM, Jayasundara N (2015). Antarctic notothenioid fish: what are the future consequences of 'losses' and 'gains' acquired during long-term evolution at cold and stable temperatures? Journal of Experimental Biology 218(12): 1834-1845.

Berthelot C, Clarke J, Desvignes T, William Detrich HI, Flicek P, Peck LS et al (2019). Adaptation of proteins to the cold in Antarctic fish: A role for Methionine? Genome Biology and Evolution 11(1): 220-231.

Bilyk KT, Vargas-Chacoff L, Cheng CHC (2018). Evolution in chronic cold: varied loss of cellular response to heat in Antarctic notothenioid fish. BMC Evolutionary Biology 18(1): 143.

Bilyk KT, Zhuang, X., Murphy, K.R., Cheng CHC (2019). A tale of two genes: divergent evolutionary fate of haptoglobin and hemopexin in hemoglobinless Antarctic icefishes. J Exp Biol 222 (6): jeb188573

Birkeland S, Slotte T, Krag Brysting A, Gustafsson ALS, Rhoden Hvidsten T, Brochmann C, Nowak M D (2022). What can cold-induced transcriptomes of Arctic Brassicaceae tell us about the evolution of cold tolerance? Molec Ecol, 31, 4271– 4285.

Bista I, McCarthy SA, Wood J, Ning Z, Detrich Iii HW, Desvignes T et al (2020). The genome sequence of the channel bull blenny, *Cottoperca gobio* (Günther, 1861). Wellcome open research 5: 148.

Bista I, Wood JMD, Desvignes T, McCarthy SA, Matschiner M, Ning Z, Tracey A, Torrance J, Sims Y, Chow W, Smith M, Oliver K, Haggerty L, Salzburger W, Postlethwait JH, Howe K, Clark MS, Detrich III WH, Cheng C-HC, Miska EA, Durbin R (2022). Genomics of cold adaptations in the Antarctic notothenioid fish radiation. bioRxiv. https://doi.org/10.1101/2022.06.08.494096.

Brodeur JC, Calvo J, Clarke A, Johnston IA (2003). Myogenic cell cycle duration in *Harpagifer* species with sub-Antarctic and Antarctic distributions: evidence for cold compensation. Journal of Experimental Biology 206(6): 1011-1016.

Chen L, Lu Y, Li W, Ren Y, Yu M, Jiang S et al (2019). The genomic basis for colonizing the freezing Southern Ocean revealed by Antarctic toothfish and Patagonian robalo genomes. GigaScience 8(4).

Chen S, Zhou Y, Chen Y, Gu J (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34(17): i884-i890.

Chen Z, Cheng CHC, Zhang J, Cao L, Chen L, Zhou L et al (2008). Transcriptomic and genomic evolution under constant cold in Antarctic notothenioid fish. Proceedings of the National Academy of Sciences 105(35): 12944.

Clarke A (2003). Costs and consequences of evolutionary temperature adaptation. Trends Eco Evol 18(11): 573-581

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research, Bioinformatics, 21(18): 3674–3676

Cziko PA, DeVries AL, Evans CW, Cheng C-HC (2014). Antifreeze protein-induced superheating of ice inside Antarctic notothenioid fishes inhibits melting during summer warming. Proceedings of the National Academy of Sciences 111(40): 14583.

Daane JM, Auvinet J, Stoebenau A, Yergeau D, Harris MP, Detrich HW, III (2020). Developmental constraint shaped genome evolution and erythrocyte loss in Antarctic fishes following paleoclimate change. PLOS Genetics 16(10): e1009173.

Daane JM, Dornburg A, Smits P, MacGuigan DJ, Brent Hawkins M, Near TJ et al (2019). Historical contingency shapes adaptive radiation in Antarctic fishes. Nat Ecol Evol 3(7): 1102-1109.

Daane Jm, Detrich HW, III (2022). Adaptations and diversity of Antarctic fishes: A genomic perspective. Annu Rev Animal Biosci 10:1, 39-62.

Darriba D, Posada D, Kozlov AM, Stamatakis A, Morel B, Flouri T (2020). ModelTest-NG: A new and scalable tool for the selection of DNA and protein evolutionary models. Molecular biology and evolution 37(1): 291-294.

DeVries AL, Steffensen JF (2005). The Arctic and Antarctic polar marine environments fish physiology. Academic Press. Vol. 22, pp 1-24.

Dornburg A, Federman S, Lamb AD, Jones CD, Near TJ (2017). Cradles and museums of Antarctic teleost biodiversity. Nat Ecol Evol 1(9): 1379-1384.

Eastman JT (1993). Antarctic fish biology: evolution in a unique environment. Academic Press.

Eastman JT (2005). The nature of the diversity of Antarctic fishes. Polar Biology 28(2): 93-107.

Eastman JT (2020). The buoyancy-based biotope axis of the evolutionary radiation of Antarctic cryonotothenioid fishes. Polar Biology 43(9): 1217-1231.

Edgar RC (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5(1): 113.

Emms DM, Kelly S (2019). OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biology 20(1): 238.

Fu L, Niu B, Zhu Z, Wu S, Li W (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics (Oxford, England) 28(23): 3150-3152.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I et al (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29(7): 644-652.

Haas BJ. (2021). TransDecoder (Find Coding Regions Within Transcripts). https://github.com/TransDecoder/TransDecoder/

Kim B-M, Amores A, Kang S, Ahn D-H, Kim J-H, Kim I-C et al (2019). Antarctic blackfin icefish genome reveals adaptations to extreme environments. Nat Ecol Evol 3(3): 469-478.

Kocot KM, Citarella MR, Moroz LL, Halanych KM (2013). PhyloTreePruner: A phylogenetic tree-based approach for selection of orthologous sequences for phylogenomics. Evolutionary bioinformatics online 9: 429-435.

Kosakovsky PSL, Poon AFY, Velazquez R, Weaver S, Hepler NL, Murrell B (2020). HyPhy 2.5—A customizable platform for evolutionary hypothesis testing using phylogenies. Mol biol evol 37(1): 295-299.

Lau Y-T, Parker SK, Near TJ, Detrich HW, III (2012). Evolution and function of the globin intergenic regulatory regions of the Antarctic Dragonfishes (Notothenioidei: Bathydraconidae). Molecular biology and evolution 29(3): 1071-1080.

Longmuir IS (1957). Respiration rate of rat-liver cells at low oxygen concentrations. Biochem J. 65(2):378-82.

Löytynoja A (2014). Phylogeny-aware alignment with PRANK. Methods in molecular biology (Clifton, NJ) 1079: 155-170.

Masson-Delmotte V, Zhai P, Pirani A, Connors SL, Péan C, Berger S et al (2021). IPCC, 2021: Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press.

Mark FC, Lucassen M, Strobel A, Barrera-Oro E, Koschnick N, Zane L et al (2012). Mitochondrial function in Antarctic Nototheniids with ND6 translocation. PLOS ONE 7(2): e31860.

Matschiner M, Hanel R, Salzburger W. On the origin and trigger of the notothenioid adaptive radiation. PLoS One. 2011 Apr 18;6(4):e18911.

Merico D, Isserlin R, Stueker O, Emili A, Bader GD (2010). Enrichment Map: A Network-Based Method for Gene-Set Enrichment Visualization and Interpretation. PLOS ONE 5(11): e13984.

Meyerovich M, Mamou G, Ben-Yehuda S (2010). Visualizing high error levels during gene expression in living bacterial cells. Proceedings of the National Academy of Sciences 107(25): 11543.

Murrell B, Weaver S, Smith MD, Wertheim JO, Murrell S, Aylward A et al (2015). Gene-Wide Identification of Episodic Selection. Molecular biology and evolution 32(5): 1365-1371.

Near TJ, Dornburg A, Kuhn KL, Eastman JT, Pennington JN, Patarnello T et al (2012). Ancient climate change, antifreeze, and the evolutionary diversification of Antarctic fishes. Proceedings of the National Academy of Sciences 109(9): 3434.

Near TJ, MacGuigan DJ, Parker E, Struthers CD, Jones CD, Dornburg A (2018). Phylogenetic analysis of Antarctic notothenioids illuminates the utility of RADseq for resolving Cenozoic adaptive radiations. Molecular phylogenetics and evolution 129: 268-279.

Nowick K, Fields C, Gernat T, Caetano-Anolles D, Kholina N, Stubbs L (2011). Gain, loss and divergence in primate zinc-finger genes: a rich resource for evolution of gene regulatory differences between species. PLoS One 6:e21553.

Nowick K, Carneiro M, Faria R. (2013). A prominent role of KRAB-ZNF transcription factors in mammalian speciation? Trends Genet. 29, 130–139.

Pace DA, Manahan DT (2007). Cost of protein synthesis and energy allocation during development of Antarctic sea urchin embryos and larvae. Biological Bulletin, 212(2), 115–129.

Papetti C, Babbucci M, Dettai A, Basso A, Lucassen M, Harms L et al (2021). Not frozen in the ice: Large and dynamic rearrangements in the mitochondrial genomes of the Antarctic fish. Genome Biology and Evolution 13(3).

Peck LS (2016). A cold limit to adaptation in the sea. Trends in Ecology & Evolution 31(1): 13-26.

Place, SP, Hofmann GE (2005). Comparison of Hsc70 orthologs from polar and temperate notothenioid fishes: differences in prevention of aggregation and refolding of denatured proteins. Am J Physiol-Reg Int Comp Physiol 288(5): R1195-R1202.

Perdomo-Sabogal A, Kanton S, Walter M BC, Nowick K. (2014). The role of gene regulatory factors in the evolutionary history of humans. Curr. Opin. Genet. Dev. 29, 60–67

Price MN, Dehal PS, Arkin AP (2010). FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. PLOS ONE 5(3): e9490.

Rico F, Chu C, Abdulreda MH, Qin Y, Moy VT (2010). Temperature modulation of integrin-mediated cell adhesion. Biophys J. 99(5):1387-1396.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S et al (2012). MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Systematic Biology 61(3): 539-542.

Sela I, Ashkenazy H, Katoh K, Pupko T (2015). GUIDANCE2: accurate detection of unreliable alignment regions accounting for the uncertainty of multiple parameters. Nucleic Acids Res 43(W1): W7-14.

Shin SC, Ahn DH, Kim SJ, Pyo CW, Lee H, Kim M-K et al (2014). The genome sequence of the Antarctic bullhead notothen reveals evolutionary adaptations to a cold environment. Genome Biology 15(9): 468.

Shevenell AE, Kennett JP, Lea DW (2004). Middle miocene Southern Ocean cooling and Antarctic cryosphere expansion. Science 305(5691): 1766.

Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31(19): 3210-3212.

Sidell BD, O'Brien KM (2006). When bad things happen to good fish: the loss of hemoglobin and myoglobin expression in Antarctic icefishes. Journal of Experimental Biology 209(10): 1791-1802.

Smith MD, Wertheim JO, Weaver S, Murrell B, Scheffler K, Kosakovsky Pond SL (2015). Less is more: an adaptive branch-site random effects model for efficient detection of episodic diversifying selection. Molecular biology and evolution 32(5): 1342-1353.

Somero GN (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. Journal of Experimental Biology 213(6): 912-920.

Song W, Li L, Huang H, Jiang K, Zhang F, Wang L et al (2019). Tissue-based transcriptomics of *Chionodraco hamatus*: Sequencing, de novo assembly, annotation and marker discovery. J Fish Biol 94(2): 251-260.

Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, Diemer K, Muruganujan A, Narechania A (2003). PANTHER: a library of protein families and subfamilies indexed by function. Genome Res. Sep;13(9):2129-41.

Tirosh I, Reikhav S, Levy AA, Barkai N (2009). A yeast hybrid provides insight into the evolution of gene expression regulation. Science 324(5927):659–662.

Wagner GP, Lynch VJ (2008). The gene regulatory logic of transcription factor evolution. Trends Ecol. Evol. 23, 377–385.

Wertheim JO, Murrell B, Smith MD, Kosakovsky Pond SL, Scheffler K (2015). RELAX: Detecting Relaxed Selection in a Phylogenetic Framework. Molecular biology and evolution 32(3): 820-832.

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D et al (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 13(11): 2498-2504.

Wilson DF, Erecińska M, Drown C, Silver IA (1979). The oxygen dependence of cellular energy metabolism. Arch Biochem Biophys. 195(2):485-93.

Yates AD, Achuthan P, Akanni W, Allen J, Allen J, Alvarez-Jarreta J et al (2020). Ensembl 2020. Nucleic Acids Research 48(D1): D682-D688.

Zieger MAJ, Gupta MP, Wang M (2011). Proteomic analysis of endothelial cold-adaptation. BMC Genomics 12(1): 630.

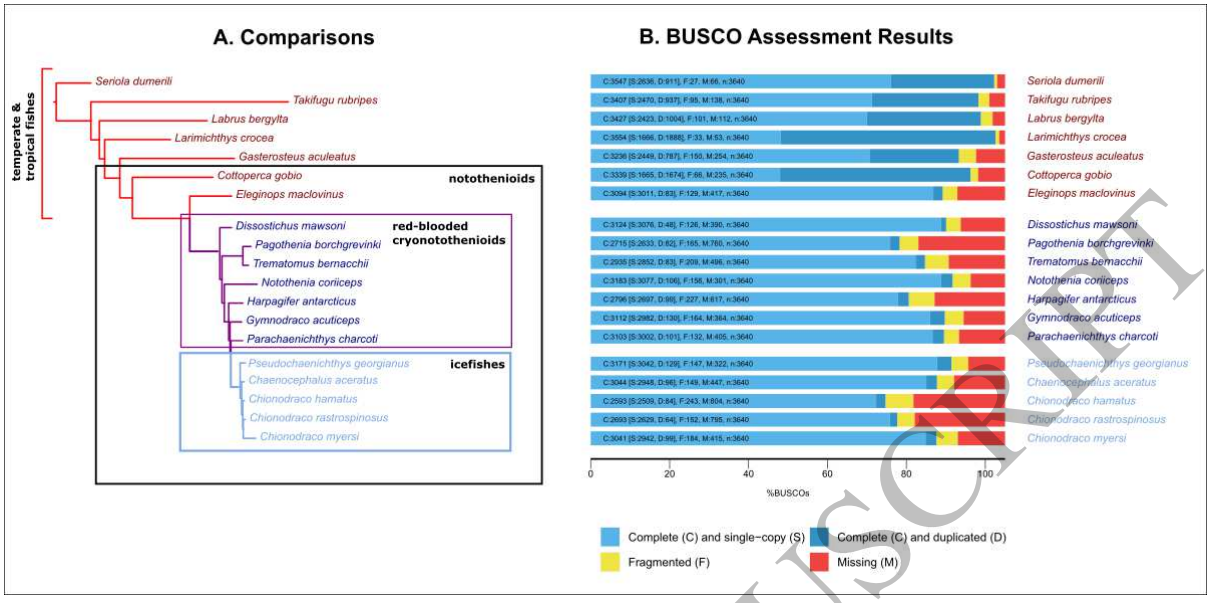

Figure 1
159x78 mm (x DPI)

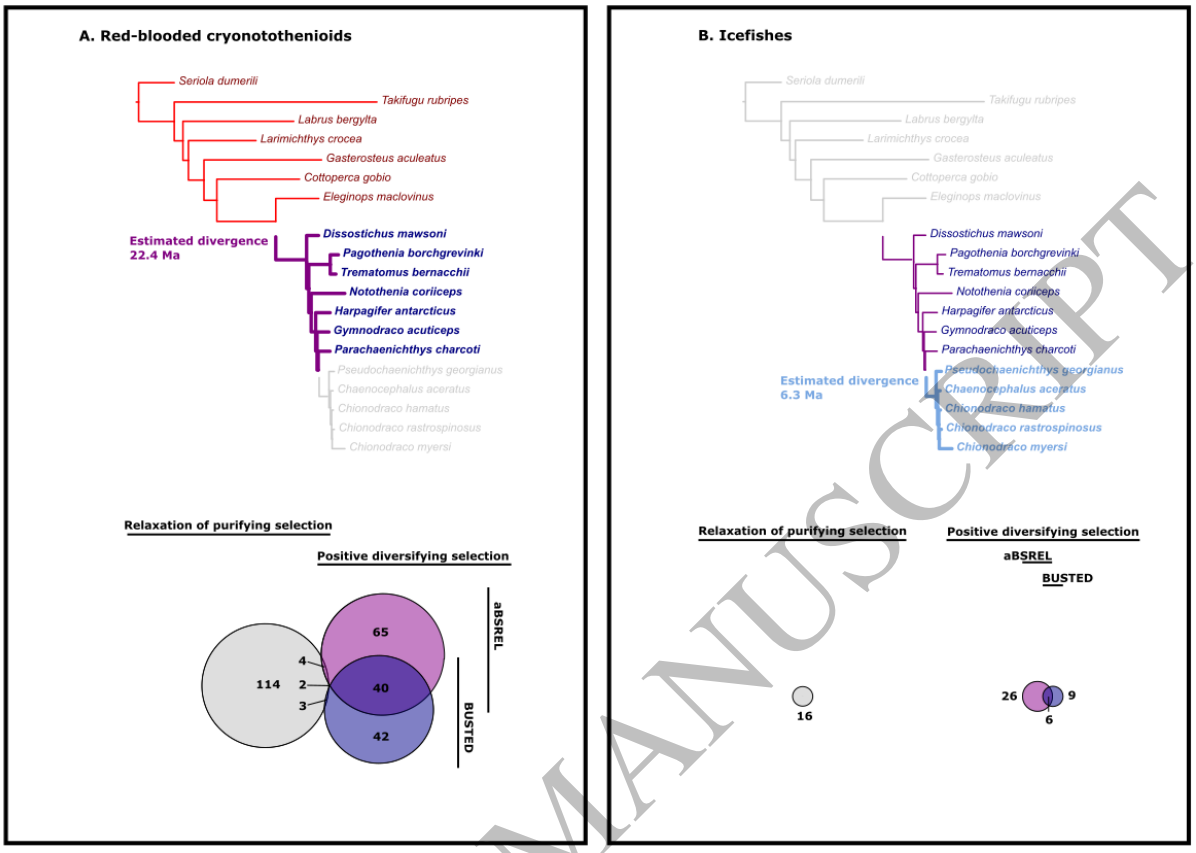

Figure 2
159x112 mm (x DPI)

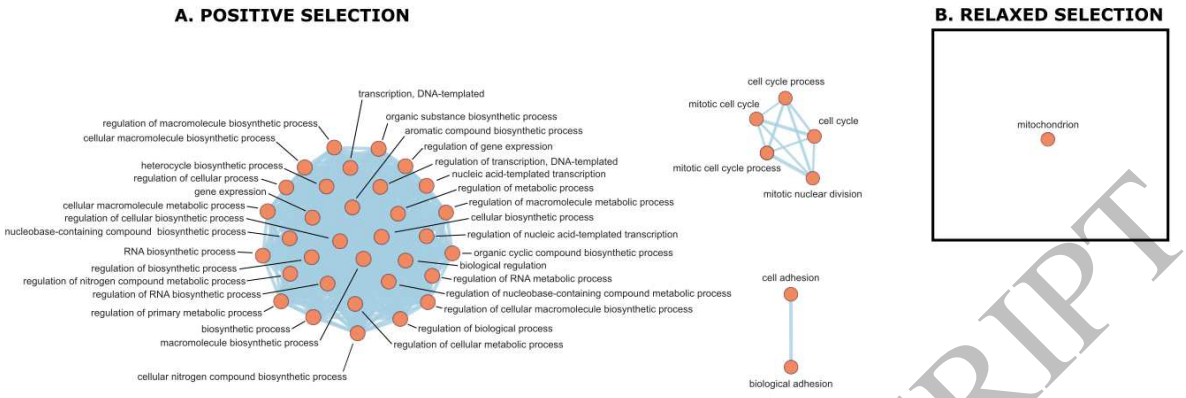

Figure 3
159x59 mm (x DPI)

Panther Protein Classification	aBSREL	BUSTED	RELAX (K<1)
aminoacyl-tRNA synthetase			*LARS2*
Apolipoprotein	***APOA1***	***APOA1***	
ATP-binding cassette (ABC) transporter	*ABCB8*		
ATP synthase			*ATP6V1F*
carbohydrate phosphatase		*FBP1*	
chromatin/chromatin-binding protein		*NIPBL*	
cysteine protease	*CAPN1*		
dehydrogenase			*DHRS12*
extracellular matrix structural protein	*SNRNP48*		
G-protein coupled receptor	*LPAR4*		
gap junction	*CX30.3*		
glycosyltransferase	*B3GNT3*		
heterotrimeric G-protein	*GNAO1B*, ***GNB1B***	***GNB1B***	
histone modifying enzyme	***SIRT5***	***SIRT5***	
homeodomain transcription factor	*IRX3A*		
ion channel			*KCNK5A*
ligand-gated ion channel	*GABRR1*		
MADS box transcription factor	*MEF2CB*		
membrane traffic protein	*MX1*		
Metalloprotease		*ACE*	
mRNA polyadenylation factor			*TOE1*
non-receptor serine/threonine protein kinase	*MAK*, *NEK3*		
primary active transporter			*DERlL*
protein modifying enzyme	***RPS6KB1***	*PRMT8*, ***RPS6KB1***	*JMJD7*, *ZDHHC23B*
protein phosphatase			*CDC25B*, *PLPP3*
pyrophosphatase		*PPA1B*	
scaffold/adaptor protein			*RB1CC1*
secondary carrier transporter	*SLC2A1B*		
Transferase	*CHST11*	*CHST15*, *GSTA1*	
translation factor	*PDCD4B*		
translation elongation factor	*TUFM*		
transmembrane signal receptor			*LEPROTL1*
transporter			*AQP7*
ubiquitin-protein ligase	*MOCS3*		
UNCHARACTERIZED	*CUNH2ORF69*, ***INAVAB***, ***PAGR1***, *PRCC*, *RHPN1*, *RNF146*, *TOMM6*, *TTC13*, *ZNF512B*	***INAVAB***, *FARP1*, ***PAGR1***, *RUNDC3AA*	*LRPPRC*, *MANEA*, *SSR4*

Table 1.
Genes identified under changed selection in the icefishes (family Channichthyidae).
This table shows the set of genes that were determined by aBSREL, BUSTED, or RELAX to have come under changed selective pressure in the icefishes compared to the red-blooded cryonotothenioids. Genes are characterized according to their Panther protein classification (Thomas et al. 2003), serving to highlight their diverse range of functions. Genes that were identified as experiencing positive selective pressure by both aBSREL and BUSTED are bolded in the table.