Coping with a warming world: genetic studies on thermal adaptation and links to membrane saturation in Antarctic fish

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of **Doctor of Philosophy**

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Stearoyl-CoA desaturase (SCD) plays a key role in thermal adaptation across organisms, allowing alteration of membranes in response to temperature change. Thermal adaptive signatures at the gene sequence level and in the primary structure of the SCD protein require investigation, especially in Antarctic fish. Owing to the stenothermal nature of Antarctic fish, it remains unclear whether they have retained capacity to modulate their membranes in response to warming temperatures. The hypothesis that membrane saturation, a major thermal adaptive mechanism, will occur at reduced capacities in Antarctic notothenioid fish in response to elevated temperatures was tested

SCD sequences were isolated from 21 fish species (Antarctic and non-Antarctic notothenioids, and non-notothenioid Antarctic fish species). Phylogenetic analyses supported the ancestral SCD1 duplication into SCD1a and SCD1b. Lineage-specific duplication and loss of gene duplicates were identified for specific orders of fish. Cold adapted notothenioids (Antarctic and non-Antarctic with Antarctic ancestry) SCD isoform sequences were evolutionarily distinct from non-Antarctic sequences. SCD1a and 1b from Antarctic fish displayed more diversity with amino acid composition varying across the isoforms. SCD1a isoforms had more sites under positive selection and a higher rate of molecular evolution in Antarctic fish than SCD1b, suggesting neofunctionalisation of SCD1a.

Ubiquitous tissue expression of SCD1b was seen in both the Antarctic species *Trematomus bernacchii* and *Pagothenia borchgrevinki,* while ubiquitous expression of SCD1a isoform was seen in *T. bernacchii* but not in *P.borchgrevinki*. Transcriptional response of the SCD isoforms, along with the biochemical response of membrane saturation, in *T. bernacchii* and *P. borchgrevinki* livers were determined in fish acclimated at 0 °C (control temperature), 4 °C and 6 °C. Overall, temperature had a significant effect on the expression of SCD1a and SCD1b

in *T. bernacchii*, while expression of SCD1a was low to be detected by qPCR in *P. borchgrevinki*.

Membrane lipid composition of Antarctic fish species varied significantly when compared to the New Zealand Perciforme species *Notolabrus celidotus*. Thermal acclimation at 4 °C did not result in any detectable change in membrane saturation state or membrane cholesterol in either Antarctic species. However, 6 °C thermal acclimation induced a homeoviscous adaptive (HVA) response in the benthic species *T. bernacchii,* as shown by the significant increase in membrane saturated fatty acids, and a significant decline in unsaturated fatty acids. HVA response was not observed in *P. borchgrevinki*.

This is the first study to determine thermal adaptive signatures in the SCD gene of Antarctic fish and provides evidence for asymmetric molecular evolution of SCD. This provides a framework for future functional work. This is also the first study to show a homeoviscous response to higher temperature in an Antarctic fish although only for one of the two species examined.

Keywords: Antarctic fish, Stearoyl-CoA desaturase, homeoviscous response, thermal adaptation, membranes

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1 Introduction

The predicted long term impacts of anthropogenic global warming (AGW) indicate that the marine ecosystem of Antarctica may be among the most vulnerable regions of our planet. Polar ecosystems are more sensitive to global warming due to the amplifying temperature effects exhibited at the poles and the past adaptation of the organisms resident in polar regions to extreme cold. Perceptible deleterious changes are already evident in polar ecosystems on a decadal scale (Aronson et al., 2011).

1.1 Marine Environment of Antarctica

The continent of Antarctica is surrounded by the Southern Ocean (SO). The Antarctic circumpolar current (ACC), a powerful ocean current, circulates in a clockwise direction and creates the northernmost boundary of the SO (Kennett, 1977). The Southern Ocean comprises 9.6 % of the surface area of the planet and is aproximately 34.8 million km^2. Nestled within the ACC is the Antarctic Polar Front or Antarctic Convergence, where cold Antarctic waters meet warmer subantarctic waters with a steep drop in temperature from across this Convergence. This marked climatic change is a major physiological barrier preventing any organism insufficiently adapted to polar temperatures from becoming resident in the high latitudes (Aronson et al., 2011).

1.1.1 Climatic history of Antarctica

Over the past 800,000 years there has been marked climatic variation in Antarctica. In general however, the temperature of the waters around Antarctica have been below 0 °C following the formation of the continental ice sheet approximately 15 million years ago (Mya) (Kennett, 1977). The average temperature on the continental shelf is -1.9 °C. Furthermore, the conditions of extremely stable and cold temperatures have been mantained over the last 15 million years (My) (Somero, 2010).

1.1.2 Principle effects of climate change in the Antarctic marine environment

Rising air and sea temperatures have direct impacts on the extent of sea ice and will impact the life history and biology of many species in the SO (Barnes & Peck, 2008).

Key changes predicted from rising temperature

Reduction by 24-33% in the Antarctic sea ice extent by the end of the 21st century (Arzel et al., 2006), that will decrease the amount of sea ice algae and have cascading effects down the food web (Smetacek & Nicol, 2005).

1. New habitats and hydrodynamic disturbance will result from decreased ice density leading to the disintegration of ice shelves (Ingels et al., 2012).

2. Sedimentation and iceberg scouring resulting from increased deglaciation on land and glacial discharge will have localised effects on benthic communities as will increased iceberg scouring from ice shelf disintegration (Ingels et al., 2012).

3. Higher seawater temperatures and decreased salinity will reduce the oxygen penetration in the water column with adverse biological effects and the creation of hypoxic zones (Hofmann & Schellnhuber, 2009; Ingels et al., 2012).

4. Direct temperature effects on the Antarctic biota relating to thermal specialisation will be widespread across all trophic levels (Pörtner et al., 2007).

1.1.3 IPCC Report on Current Impacts on Antarctic Ecosystems

The Intergovernmental Panel on Climate Change reported on impacts of climate change on Antarctic regions (IPCC, 2007). Observations of change in the Antarctic include:

- The thermally stable oceanic climate of the sub Antarctic Marion Islands appear to be changing with a rise in the annual mean surface temperature of 1.2 °C between 1969 and 1999.
- Annual temperatures of the Antarctic Peninsula have risen rapidly > 2.5 °C since measurements began fifty years ago at Vernadsky (formerly Faraday) Station. The west coast warming has been sufficient to raise the number of positive degree days by 74%.

Over the period of 2002 to 2011 the Antarctic ice sheets have been losing mass mainly from the northern Antarctic Peninsula and the Amundsen Sea sector of West Antarctica (IPCC, 2014).

1.1.4 Ectotherms of the Southern Ocean are highly vulnerable to the impacts of climate change

Global warming is most pronounced in higher latitudes (Turner et al., 2006). Within these regions, species that experience temperature near their thermal maxima will be the first species to experience

severe stress from global warming (Somero, 2010). The reasons for the prediction of vulnerability of these species to the impacts of AGW are listed below.

1.1.4.1 Stenothermal nature and genetic lesions

Ectotherms of the Antarctic are referred to as stenotherms as they experience negligible seasonal variation, resulting in limited ability to adapt to temperature variation (Somero, 2010). A narrow annual marine temperature range in Antarctica (-1.9 °C to approximately 1.8 °C) makes marine ectotherms most vulnerable to climate change effects (Aronson et al., 2011). In temperate and tropical latitudes, marine ectotherms experience seasonal variation and are correspondingly more thermally tolerant or eurythermal (Aronson et al., 2011). Evolution of stenotherms in a "stable ice bath" has involved many critical changes in the genome that facilitate life in the cold, such as losses of certain traits that are no longer required. These losses tend to be examples of the loss without penalty scenario (Pörtner et al., 2007) and are referred to as 'DNA decay'. Further lack of purifying selection leads to losses from the genetic "tool kit" (Hoffmann & Willi, 2008).

The genetic lesions that influence the response of the ectotherm to rising temperatures are of three types (Somero, 2010) and described below.

1.1.4.1.1 A protein coding gene that is partially or completely lost from the genome

This type of lesion is irreversible if an entire protein coding gene or major fraction of the gene is lost by mutation. One example is the lack of erythrocytes in the fishes of the notothenioid family Channichthyidae (icefish) (Ruud, 1954). The icefish have lost the gene that encodes beta-globin, a subunit of haemoglobin, either through deletion or through rapid mutation (Cocca et al., 1997).

1.1.4.1.2 A mutation that disrupts the open reading frame

Mutations may cause a loss of the corresponding protein from the organism's proteome (Somero, 2010). An example is the loss of intracellular oxygen binding protein myoglobin from the oxidative muscle tissue of the family Channichthyidae (Sidell & O'Brien, 2006). As a result of these genetic mutations, icefish have made compensatory changes that include unusual physiological and anatomical properties; such as an increase in size of their heart and gills and increased blood volume. Warmer waters with decreased levels of dissolved oxygen, however, pose a major challenge for icefish (Somero, 2010).

1.1.4.1.3 Loss of gene regulatory abilities

Lesions in the gene regulatory regions due to mutations have resulted in loss of heat shock response (HSR) in Antarctic fish (Somero, 2010). HSR generates heat shock proteins which play a crucial role in reducing and repairing the damage to cellular proteins from chemical and physical stress. Loss of HSR has been observed in *Trematomus bernacchii* and other species (Hofmann et al., 2000), resulting in extreme stenothermality of these fish, due to an incapability to minimise damage to their protein pool caused by elevated temperatures (Podrabsky, 2009).

1.1.4.2 Less time for adaptive evolution

Temperature-related adaptive changes in proteins typically occur in regions that influence conformational mobility (Hochachka, 2002). However, for adaptive evolution to be successful, in general, a number of substitutions in the protein need to occur. Whether such adaptive evolution can keep up with the pace of AGW is critical for organisms (Somero, 2010). For those ectotherms resident in polar regions however, where warming is occurring faster than other geographic areas, these species have the least time available for adaptive evolution.

1.1.4.3 Drop in functional or behavioural capacity

Loss of aerobic scope due to limited capacity of oxygen supply pathways results in a decrease in functional capabilities with even a small rise in temperature. The swimming speed in the stenothermal notothenioid *Pagothenia borchgrevinki* is at optimal levels (2.7 X body length/second) between -1.0 °C and at +2 °C. With further increases in temperature, a decline in performance is observed and the speed of swimming is halved at 7 °C (Seebacher et al., 2005).

1.1.4.4 Limitations of aerobic performance

The conceptual physiological model describes the response of thermal stress on the whole animal performance (Figure 1). The limitation of aerobic performance has led to the concept that animals are characterised by oxygen and capacity limited thermal tolerance, as has been observed in Antarctic eelpout (Pörtner et al., 2007). At either side of the optimal thermal range for a species there is a shift from aerobic to anaerobic metabolism involving mitochondrial pathways (Figure 1). As temperatures continue to move away from thermal optima near the low and high edges of the thermal envelope and approaches the pejus (getting worse) temperature (T_p), this signifies loss of aerobic scope with oxygen supply mismatched to tissue demand. Within the pejus window (T_p) there is acclimation of functional capacity (Figure 1). As warming (or cooling) continues beyond the pejus threshold, aerobic scope finally vanishes at the critical threshold temperature (T_C) and beyond this range the acclimation

that occurs is centred on protection and protective mechanisms are shown in Figure 1. Further warming leads to severe molecular damage such as protein denaturation and membrane disruption (T_d), with acclimation in this range focussed on repair. In Antarctic eelpout the upper critical temperature (T_C) is reached at 12 °C and at this stage the oxygen consumption switches off and there is an increase in succinate accumulation. Thus this model establishes the limitations associated with the aerobic metabolism in Antarctic fish when subjected to thermal stress.

Figure 1 Conceptual model of thermal limitation and functional optima (Pörtner et al., 2007).

1.1.5 Impact of global warming on marine ecosystems

Key impacts of environmental changes on Antarctic ecosystems are briefly listed below.

1. **Affected food webs**

The projected rise in average temperature by 2 °C over the next century will have varying impacts on different species of fish and invertebrates that occupy the various trophic levels of the food web, through differences in their thermal acclimatization capacities (Somero, 2010). In polar food webs, lower trophic species are dependent on sea ice for survival. Changes in the structure and dynamics of food webs associated with sea ice in the polar regions are predicted globally to be among the most pronounced ecosystem level changes that will occur under AGW (Carscallen & Romanuk, 2012).

2. **Extinctions in the Southern Ocean**

Some species may face extinction as sea temperatures rise. An extreme example is the dominant ophionoid in coastal waters around the Antarctic Peninsula, *Ophionotus victoriae.* This brittle star is found at depths from 5 to 126 m and is designated as the primary scavenger species. The brittle star

is not able to acclimate to more than 2 °C for any length of time, indicating that this species is very sensitive to long term temperature increases (Peck et al., 2009). The current rate at which AGW is occurring may lead to extinction of vulnerable species like *O. victoriae*, resulting in ecological imbalance (Somero, 2010).

3. **Altered ecosystem and ecological imbalance**

Animal size and activity level have a significant effect on thermal tolerance. Smaller individuals have higher tolerance than larger individuals. More active species have higher tolerance than less active or sessile species. Species decline or extinction based on size and activity can alter ecosystems and lead to imbalance (Peck et al., 2009). Any extinction events of stenothermal species resulting from rises in sea temperatures, could lead to entry of cold adapted temperate species, especially in the Peninsula region (Somero, 2010).

1.2 Adaptation of organisms to thermal stress

How then do organisms adapt to thermal change? There are a number of mechanisms involved in thermal adaptation. These are described below.

1.2.1 Adaptation at cellular level

When eurythermal and stenothermal ectotherms are subjected to thermal stress, considerable differences are observed in their cellular responses, as evidenced from microarray analyses (Buckley & Somero, 2009; Buckley, Gracey, & Somero, 2006).

1.2.1.1 Upregulation of heat shock proteins in eurythermal fish

Gillchthys mirabilis, a eurythermal fish, was exposed to higher temperatures in a thermal acclimation experiment and cDNA microarray and protein analyses performed to monitor gene expression in gill and white muscle tissue. Induction or repression of over 200 genes was observed in each tissue. Genes associated with maintenance of protein homeostasis, cell cycle control, cytoskeletal reorganization; metabolic reorganization and signal transduction were upregulated. The most strongly inducible genes in both gill and muscle were the molecular chaperones, including the heat shock proteins (Hsp). Every size class of Hsp was upregulated in this species, with Hsp 70 and Hsp 90 the most strongly upregulated throughout the heat shock and recovery period. Hsp 70 was more strongly induced in muscle than in gill (Buckley et al., 2006).

1.2.1.2 Inability to upregulate heat shock proteins under thermal stress in a stenothermal species

For Antarctic notothenioids, cDNA microarray analysis of transcriptional changes during heat stress in *Trematomus bernacchii* demonstrated that it retained the ability to alter gene expression, but lacked the ability to upregulate the Hsp genes required for mitigating thermal stress (Buckley & Somero, 2009). The micro chaperone Hsp 40 was the only gene upregulated (Buckley & Somero, 2009). Similar muted heat shock responses during thermal stress was observed in the Antarctic notothenioid, *D. mawsoni* (closely related to *T. bernacchii*), when compared to temperate or tropical fish (Chen et al., 2008). Upregulation of gene families which operate in protein biosynthesis, protein folding and degradation, lipid metabolism, anti-oxidation, anti-apoptosis, and chorion genesis were observed. All these gene families have functional importance in mitigating stress in freezing temperatures (Chen et al., 2008). Thus, Antarctic notothenioid fish gene expression enables survival in freezing waters coupled with a lost ability to upregulate the heat shock proteins.

1.2.1.3 Unfolded protein response

The unfolded protein response (UPR) is a cellular stress response related to endoplasmic reticulum (ER) function and is conserved from yeast to animal cells (Ron & Walter, 2007). It is activated in response to an accumulation of unfolded or mis-folded proteins in the lumen of the ER and seems to serve two functions: (1) to restore normal cell function by halting translation; and (2) activation of signalling pathways that lead to increasing the production of molecular chaperones involved in protein folding (Ron & Walter, 2007). However, the transcriptomic response in gills of the Antarctic fish, *T. bernacchii* exposed to high temperature and pCO_2 for 7 days, indicated an upregulation of a number of genes encoding disulphide isomerases. These are involved in correct protein folding and play a role in the unfolded protein response in the lumen of the ER (Huth & Place, 2016b). Thus, Antarctic fish display UPR when exposed to stress.

1.2.2 Adaptation at the molecular level

Another feature of thermal specialization involves temperature-related protein structural changes, changes associated with the positive selection of proteins, and changes in composition of macromolecules. Evidence for molecular thermal specialisation is given below:

1.2.2.1 Composition of genomes and proteomes influence thermal adaptation

Thermal adaptive signatures are reflected in the amino acid compositions of proteins and the nucleic acid contents of the genome. This can be seen in analyses (Kreil & Ouzounis, 2001) of complete genome sequences of six thermophilic archaea, 17 mesophilic bacteria, and 2 eukaryotes analyzed by principal component analysis and hierarchical clustering. The analyses grouped all organisms with high GC content together. PCA analysis clustered all the thermophilic species with an unusually high GC content (57-67%) and there was a clear distinction of all thermophilic species based on the amino acid composition, which was independent of GC content (Kreil & Ouzounis, 2001). In a study of 204 complete proteomes of archaea and bacteria, a universal set of amino acids (isoleucine, valine, tyrosine, tryptophan, arginine, glutamine and leucine) were identified to have a strong correlation (0.93) with the optimum growth temperature (Zeldovich et al., 2007). Specific thermal-adaptive signatures were detected in the composition of the macromolecules (DNA and protein) of zoarcid fish from different latitudinal zones (Antarctica: *Pachycara brachycephalum*, temperate zone: *Zoarces viviparus*) and these were influenced by the temperature of the habitat. Transcriptomic analysis shows a distinct codon usage pattern, with lower CG3 codon usage and specific amino acid substitutions (high amounts of serine and low contents of glutamic acid and asparagine) in Antarctic species. Although patterns of amino acid replacement do not completely support the theory that flexibility is important in cold adaptation (Windisch et al., 2012), these studies do demonstrate the idea that thermal adaptive signatures present in the genomes of cold-adapted species could help to unravel the patterns of thermal adaptation.

1.2.2.2 Positive selection and thermal adaptation of proteins

Thermal adaptive functions in proteins involve amino acid substitutions that are under purifying selection during evolution at cold temperatures. The relative rates of synonymous and non-synonymous substitutions can be used to determine the selection pressures on proteins (McDonald & Kreitman, 1991). The normalised ratio of synonymous to non-synonymous substitutions is denoted by ω. In the absence of selection (relaxed selection), where most mutations are neutral, i.e. they have little or no effect on fitness, ω=1; under positive selection, where at least some mutations confer an adaptive advantage, ω >1; and under negative (purifying) selection, where most mutations decrease fitness, ω<1 (Yang & Bielawski, 2000). Examples of positive selection in proteins from Antarctic fish conferring thermal adaptation include the Toll like receptor 2 in *T. bernacchii* and *C. hamatus*; this is a type 1 transmembrane glycoprotein. Six sites were found to be under positive selection in the

Antarctic fish. Four were found in the ligand binding site, two located in the external loop of LRR 11 (Leucine-Rich Repeat 11) and two in the loop of LRR12. The changes in these confer increased flexibility to the Antarctic Toll like receptor 2 when compared to their temperate orthologs (Varriale et al., 2012). Similarly, a complement system C3 gene in the Antarctic fish has sites under positive selection that contribute to more pronounced movements of the thioester and the carboxyl domains of the protein, conferring flexibility to the protein in the cold (Melillo et al., 2015). The above studies demonstrate that analyses of selection in proteins has become a valuable tool for understanding molecular evolution in changing environments, such as Antarctic waters.

1.2.2.3 Protein structure and adaptive function relationship

A change in adaptive function may require a change within the amino acid sequence, which is partly determined by the location of the change in the three-dimensional structure of amino acid. For example, a single substitution in A_4 lactate dehydrogenase is sufficient to shift the K_m^{PYR} from a high value found in species from cold environment to a lower value for temperate orthologues. Amino acid changes may also alter the activation energy and substrate affinity (Fields & Houseman, 2004). Other common changes are in the conformational stability within regions of the enzyme that undergo large displacement during substrate binding, and which determine the speed at which the enzyme works (Pörtner et al., 2007).

One of the other major mechanisms that proteins have evolved to ensure function in cold conditions is to increase their flexibility (Siddiqui & Cavicchioli, 2006). Pepsin sequences isolated from the gastric mucosa *of T. bernacchii* comprise three different forms. One of them, pepsin A2, has significantly reduced hydrophobicity in *T. bernacchii* and more flexibility in the substrate cleft region when compared with the protein from mesophilic fish (Carginale et al., 2004). Likewise, major substitutions in the α and β-tubulins predicted to increase flexibility and specific to Antarctic fish, are located in the interior part of the polypeptide within the M loops of and these confer cold adaptation to microtubule assembly (Detrich et al., 2000). Similarly, in Sec61alpha which forms a channel for the translocation of secretory proteins across the membranes of the endoplasmic reticulum, the amino acids specific to cold water fish cluster in the luminal loop between transmembrane 7 and 8 of the protein and are important for the translocation of proteins (Romisch et al., 2003). Other examples of proteins having flexible loops that confer cold-adaptive features include the cold-adapted esterase isolated from an intertidal metagenomics library (Fu et al., 2013). Bacterial esterases isolated from environmental samples at a range of temperatures (10 °C to 70 °C) had frequent amino acid exchanges in four loops

on the protein surface and flexibility of these loops is temperature dependent (Kovacic et al., 2016). Other mechanisms that confer cold-adaptation may include repeat motifs. The PEPT1 transporter protein isolated from Antarctic icefish (*Chionodraco hamatus*) has six unique repeats of a seven amino acid motif within the carboxyl domain and when this domain was transferred to warm-adapted PEPT1 protein it conferred a cold-adaptation function (Rizzello et al., 2013).

1.2.2.4 Gene duplication

Evolution of complexity in an organism is acquired primarily by genome duplication and is vital for the evolution of an organism (Ohta, 1989). Gene duplication has played a major role in evolution anti-freeze glycoproteins (AFGP) in Antarctic fish. Initial duplication of the repetitive AFGP sequence resulted in the large AFGP polypeptide existing in the present Antarctic notothenioid, (Chen et al., 1997a). Duplicated genes have been further identified and shown to have a role in cold adaptation. Eight species of Antarctic fish examined had two Metalathioneins isoforms (MT1 and MT2), a result of independent gene duplication and gene conversion that occurred only in the Antarctic notothenioid. These isoforms have differential expression patterns in the Antarctic species, while other teleosts have only one MT isoform (Bargelloni et al., 1999). Also seen is a duplication of the pepsin gene in Antarctic fish which allows digestion at low temperature due to the increased enzyme production to compensate for the low kinetic efficiency at cold temperatures (Carginale et al., 2004). Similarly, duplication and diversification has resulted in a type II, 4 cysteine variant of Hepcidin having only 2 disulphide bonds found only in the Antarctic notothenioid lineages (Xu et al., 2008). Furthermore, transcriptomic data from the skeletal muscle of ice fish, when compared with the whole genome data of the model species had significantly, higher gene duplicates involved in mitochondrial biogenesis and aerobic respiration which is advantageous for supply of energy and oxygen diffusion to the aerobic tissues (Coppe et al., 2013).

1.2.3 Membrane aspects of thermal specialization

Fatty acids are key components of membranes forming the hydrophobic section of phospholipid and glycolipid molecules. Saturated fatty acid (SFA) chains have only one single bond between adjacent carbon atoms and they allow rotation around the C-C bond (Hulbert, 2007). Polyunsaturated fatty acids (PUFA) have 2-6 such double bond units. The average chain length in vertebrates is 18 carbons long but with a range of 16 to 22 carbon atoms. Fatty acyl chains found in membranes are derived either from diet or de-novo synthesis (Hulbert, 2007).

Within the fatty acyl chains, rotation about the carbon-carbon single bonds freely propagates up and down the length of the chain to result in a relatively fluid, disordered liquid crystalline phase at physiological temperatures. At temperatures below the chain melting transition temperature the fatty acyls adopt a 'trans' conformation and pack efficiently to form a highly ordered gel phase (Figure 2). When the temperature exceeds the physiological range, the lipids, especially phosphatidylethanolamine (PE), a common component of membranes, form the inverted hexagonal H_{11} phase (Figure 2), and this results in the loss of bilayer integrity (Seddon, 1990). Consequently, at both reduced and higher temperatures the constraints on the physiological membranes lead to apparent loss of membrane function.

Figure 2 Thermal alterations of membrane structure (Hazel, 1995).

1.2.3.1 Membrane Remodeling

Poikilotherms have a system in place to remodel their membrane lipids of appropriate physical structure to suit changing environmental temperature and to offset the membrane constraints (as shown above) to restore their biological functions (Hazel, 1995). Temperature-induced remodelling of membrane lipid composition is referred to as homeoviscous adaptation. Optimal membrane function is restricted to a limited range of membrane fluidities. As the temperature increases there is an increase in the membrane fluidity beyond the optimal range and, conversely, a drop in

temperature decreases the fluidity of the membrane, with both of these situations affecting membrane functions (Hazel, 1995).

1.2.3.2 Evolutionary/ Acclimatory Homeoviscous Adaptation

Adaptational alterations in Antarctic fish (over evolutionary time) or acclimatory alterations in eurythermal fish (within the lifetime of the individual) in the lipid composition of membranes takes place following constant exposure to temperatures either above or below those required to maintain optimal fluidity (Hazel, 1995). Growth at lower temperatures leads to one or a combination of the following adjustments to membrane lipid composition (Hazel, 1995).

1.2.3.2.1 Increased proportions of cis unsaturated fatty acids

Unsaturated fatty acids (UFAs) pack less compactly which partially offsets the increase in membrane lipid order that results from a drop in temperature. Monoenoic fatty acids are superior to PUFA with respect to the magnitude of changes they confer and are produced at a lower associated metabolic cost (Hazel, 1995).

1.2.3.2.2 Increase in the proportions of phosphatidylethanolamine to phosphatidylcholine

Sphingolipids and glycerolipids, including glycerophospholipids, are the abundant lipids in animal membranes. Phospholipids with a glycerol backbone are categorised based on the polar head group, with phosphatidylcholine (PC) having a choline head and phosphatidylethanolamine (PE) having an ethanolamine head (Crockett, 2008). At lower temperatures the proportion of PE to PC increases. The ethanolamine group has reduced hydration and steric bulk compared to choline and there is also capacity for hydrogen bonding between the head groups of PE but not PC. Destabilizing lipids at low temperatures are conically shaped with this shape increasing the lateral pressure within the plane of the bilayer and the tendency of the bilayer to curve, which results in phase displacement of membranes (Hazel, 1995).

1.2.3.2.3 Reduced proportions of plasmalogens at low temperatures and increased proportions at high temperatures

At lower temperatures membrane remodelling includes a reduced proportion of plasmalogens, a subclass of membrane glycerophospholipids. Plasmalogens contain a vinyl–ether linkage in the Sn-1 position between the glycerol backbone and the non-polar head tails. They represent up to 30% of the PE in muscular tissue and are enriched in the nervous, immune and cardiovascular system but they are less well known lipids (Crockett, 2008). Plasmalogens serve as antioxidants although their

functional roles are not clearly defined (Nagan & Zoeller, 2001). At reduced temperatures, reduction in the plasmalogen levels have been observed in nervous tissue (Hazel, 1995). Conversely, at elevated acclimation temperatures there is an increase in plasmalogen. Such an increase was observed in the brain of the eurythermal fish *Crassius crassius* acclimated to 30 ˚C and may serve as membrane protection against oxidation (Kakela et al., 2008). Warm acclimation of the red muscle mitochondria of rainbow trout also caused an increase in the plasmalogen forms of PE and PC (Kraffe et al., 2007).

1.2.3.2.4 Effect of thermal acclimation on plasma membrane rafts

Rafts are cholesterol and sphingolipid enriched micro-domains of the plasma membrane that facilitate signal transduction pathways. Favourable interactions amongst cholesterol and saturated hydrocarbon chains of lipids, especially sphingomylein, create patches of liquid–ordered (L_o) phase membrane that is separated from the remaining liquid-disordered (L_d) phase membrane (Ahmed et al., 1997; Brown & London, 1998). Stripping cholesterol from the raft portion of the membrane can lead to losses of function of the proteins housed within the rafts (Pike & Miller, 1998). The raft structure is dependent on the temperature-induced changes in lipid phase behaviour and an increase in temperature can lead to dissolution of the raft structure, while decreases in temperature can lead to inappropriate assembly of membrane components (Zehmer & Hazel, 2003). Interestingly, the compositional changes that occur in response to thermal acclimation in the raft portions of the membrane differ from those in raft-depleted membrane regions. An elevation of the cholesterol: phospholipid ratio in rafts has been observed from warm to cold temperature (Zehmer & Hazel, 2003).

1.3 Notothenioid fish as a model system for thermal adaptation studies

The Antarctic notothenioids are a marine species flock that have evolved under extreme environmental conditions (Eastman, 2000). This Perciforme suborder, the Nototheniiodei, diversified into at least 130 species within eight families, encompassing over 100 Antarctic species (Eastman, 2005). Bovichtidae, Pseudaphritidae and Eleginopidae are the three ancestral families and comprise 11 primarily non-Antarctic species, distributed around southern South America, the Falkland Islands, southern New Zealand and south eastern Australia (Eastman, 1993). The remaining five families: Artedidraconidae, Bathydraconidae, Channichthyidae, Harpagiferidae and Nototheniidae are predominantly endemic to Antarctic waters and are referred to as the Antarctic clade (Eastman, 1993). Notothenioids account for approximately 46% of Antarctic fish species diversity and over 90% of the fish biomass, dominating the Antarctic continental shelf and upper slope (Eastman, 2005).

1.3.1 An example of adaptive radiation

The isolation and subsequent cooling of Antarctic waters to subzero temperatures following the formation of the Drake Passage (38 Mya) provided the key opportunity for speciation within the group as Notothenioidei were able to survive and radiate owing to their development of antifreeze glycoproteins (AFGP). Other fish species lacked antifreeze and were unable to cope with the cooling oceans. The absence of competition led to the diversification into unfilled niches at various depths in the water column. Notothenioids evolved, through alteration of buoyancy, to occupy these niches, including pelagic and epibenthic habitats (Eastman, 2005). The Southern Ocean is highly unusual in that a single suborder (the Notothenioidei) dominates the Antarctic shelf and the upper slope fauna and are confined to a depth less than 1000 m, but with varying depth range for individual species. Notothenioids typically feed on or near the substrate, are bottom spawners, although the larvae and juveniles are frequently pelagic and they display considerable ecological and morphological diversity. Such ecological diversification is a classic example of adaptive radiation (Eastman, 2005).

1.3.1.1 High level of endemism

High rates of endemism, three fold higher than fauna from other isolated marine habitats (Eastman, 2005), are a distinguishing feature of the Antarctic fish fauna with 88% endemism for species, 76% for genera if only notothenioids are considered (Andriashev, 1987). Such endemism is due to the large geographical area of the Antarctic region and its geographic, and thermal isolation (Clarke & Johnston, 2003).

1.3.1.2 The model fish species for this study

The fish species targeted in this study primarily comprise both Antarctic and non-Antarctic notothenioid fish species from two families (although other notothenioid and non-notothenioid species are also examined). The major features of these families which led to their selection for this study are listed below with a description of the specific fish species.

1.3.1.2.1 Bovichtidae

Bovichtidae is a basal notothenioid family and the species of this family tend to have a largely non–Antarctic distribution, being found around southern South America**,** south eastern Australia, New Zealand and a few isolated islands. There are 11 species within 3 genera. The Bovichtidae were never cold adapted and lack features such as AFGPs. The Bovichtid species used in this study is *Bovichtus variegatus*.

Bovichtus variegatus: This is the most basal Notothenioidei species as determined by systematic (Balushkin, 1984) and karyological research (Prirodina, 1986) as mentioned in (Eastman, 1993). It is an ideal subject for comparison studies of cold-adapted tissue and organs from Antarctic notothenioids due to its lack of features that are found in their Antarctic relatives-eg. AFGPs and aglomerular kidneys. As adults they are bottom dwellers and they are found in southern New Zealand and subantarctic waters.

1.3.1.2.2 Nototheniidae

This family comprises 49 species within 13 genera and are the most diverse family with respect to size, body form, habitat and distribution. They are distributed throughout the Antarctic as well as in coastal waters of New Zealand and South America. Fifteen species have a non-Antarctic distribution. There is an evolutionary departure from the ancestral benthic habitat in 50% of the Antarctic species of this family.

Pagothenia borchgrevinki is the principal Antarctic model fish species for this study. *P.borchgrevinki* are cryopelagic as they are associated with the under surface of the ice and this niche specialisation has led to them becoming morphologically and physiologically diverse from other notothenioids (Eastman & DeVries, 1985). They also exhibit a degree of ecological plasticity. Robust antifreeze protection is essential for their cryopelagic habitat due to their constant contact with ice in the upper 33 cm of the ice-water column. To achieve this, they have higher concentrations of AFGPs in their body fluids relative to other notothenioids and these lower the freezing point (DeVries et al., 1971). *P.borchgrevinki* are not neutrally buoyant but are lighter than the benthic trematomiids and in the austral summer they live in the upper 6m of the water column under the ice. They have a more streamlined body that is laterally compressed compared with benthic trematomiids. They are also characterised by higher levels of oxygen consumption, haemoglobin concentration and hematocrit than some of their close relatives. Primarily they are zooplanktivorous (Foster et al., 1987) but they also consume the notothenioid *Pleuragramma antarcticum.*

Trematomus bernacchii is a benthic Antarctic species found in both shallow areas and in water up to 550m deep. They feed on plankton to a greater extent than other benthic Trematomiids. As a benthic species they have a less buoyant body and a heavier skeleton and are also less streamlined than the cryopelagic *P. borchgrevinki*. Pelvic and anal fins are used for support and substrate contact and they have a diet described as predominantly polychaetes, small fishes, fish eggs and amphipods (Eastman, 1993).

Notothenia angustata is a benthic member of the Antarctic family Nototheniidae, but is distributed in the shallow coastal waters of southern New Zealand and is a secondarily temperate species originally present in the Antarctic up until approximately 11 Mya. Its dispersal from the Antarctic to the cool-temperate region of New Zealand is not clearly understood (Eastman & Eakin, 2000). *Notothenia angustata* retains a number of cold adaptive characteristics of the endemic Antarctic species despite its ice-free present habitat, including functional AFGP genes (Cheng., 2003). At their environmental water temperature they show intermediate levels of membrane saturation between cold temperate and endemic Antarctic species (Logue et al., 2000). Unlike their Antarctic sister species they possess haemoglobin multiplicity (Macdonald et al.,1988) and differ in other haematological properties as well as possessing glomerular kidneys, a temperate characteristic (Eastman & Devries, 1986). Overall, there is a mixture of anatomical and physiological similarities and differences between the endemic Antarctic notothenioids. As such they are an interesting comparative species, owing to the fact that they are now temperate but were previously cold-adapted.

1.3.1.2.3 Channichthyidae.

This family is one of the eight families of the suborder, Notothenioidei (Eastman, 2005). They live in the most cold stable and oxygen rich environment of the Southern Ocean and comprise 15 to 17 species (Kock, 2005). Channichthyidae species lack haemoglobin and this loss occurred 8.5 Mya when they diverged from the other Antarctic notothenioid fish (Near, 2004). Few species of this family do not express myoglobin (Borley & Sidell, 2011) *Chionodraco hamatus* is the most abundant among the icefish species found in the Ross Sea and the most studied for thermal adaptation and evolutionary studies. One of the Icefish species *Champsocephalus esox* have been distributed in the non-Antarctic water and are found north of the Antarctic polar frontal zone (APFZ) around the Falkland/Malvinas Islands, and in the Strait of Magellan (Kock, 2005).

1.4 Homeoviscous adaptation is a key molecular adaptation in notothenioid fish

Evolutionary gain of AFGP in notothenioid fish led to their adaptive radiation in the Antarctic. Maintenance of enhanced membrane fluidity at sub-zero temperatures was also an acquired evolutionary adaptation. The study by (Logue et al., 2000) showed that teleost fish from cold environments have higher disordered membranes accompanied by changes in membrane lipid composition; thus providing an ideal physical structure to function in the cold.

1.4.1 Alteration in membrane fluidity in response to temperature shifts involves Stearoyl-CoA–desaturase (SCD).

Saturated fatty acids are synthesized from acetate by fatty acid synthase in the cytoplasm of animal cells, yeast and fungi and are then converted to unsaturated fatty acids through introduction of a double bond(s) between two carbon atoms in a fatty acyl chain, in a reaction known as desaturation by enzymes such as desaturases. Poikilothermic organisms respond to decreases in temperature through homeoviscous adaptation, whereby enhancement of the desaturation of fatty acids within their membranes occurs (Cossins, 1977; Lee & Cossins, 1990). Unsaturation of fatty acids ensures optimum membrane function by reducing the temperature for transition from the gel to the liquid phase to provide fluidity and activation of membrane-bound enzymes (Hazel, 1995). Desaturases are universally found in organisms studied with the exception of *E coli* (Los & Murata, 1998).

There are three types of fatty acid desaturase.

1. Acyl-CoA desaturase: These are bound to the endoplasmic reticulum (ER) in animals, yeast and fungal cells and introduce double bonds to fatty acids bound to coenzyme A (Los & Murata, 1998).

2. Acyl-Lipid desaturase: These introduce double bonds to fatty acids that have been esterified by glycerolipids. They are bound to the ER, chloroplast membranes in plant cells, and thylakoid membranes in cyanobacteria. They are the most efficient regulators of membrane unsaturation in response to temperature (Murata & Wada, 1995).

3. Acyl-carrier protein desaturase: These are present in the stroma of plastids and introduce double bonds to fatty acids bound to the Acyl carrier protein (Los & Murata, 1998).

Molecular adaptation of the key genes (explained in section 1.2.2.3) involved in various physiological processes in Antarctic fish are attractive models for evolutionary studies because they evolved specific features to function in cold temperature. Similarly, the evolution of SCD in Antarctic fish is not understood. Presented below is the review of the SCD gene, evolutionary understanding and expression pattern in response to thermal stress.

1.4.1.1 Biochemistry of Stearoyl-CoA desaturase

Biosynthesis of unsaturated fatty acids by SCD is an aerobic process requiring molecular oxygen, NADH or $NADPH_2$-cytochrome *b*5 reductase, and the electron acceptor cytochrome *b*5. The flow of electrons takes place from NAD(P)H2 via cytochrome *b*5 reductase to cytochrome b5, to SCD and

finally to O_2 to form H_2O. Desaturation by SCD introduces a double bond between the C_9 and C_{10} position of the long acyl-CoA chain (Paton & Ntambi, 2009).

1.4.1.2 Central role of MUFA

The preferred substrates for SCD are palmitoyl and stearoyl-CoA and they are converted to palmitoleyl and oleyl–CoA. The mono unsaturated fatty acids (MUFA) serve as substrates for the synthesis of various kinds of lipids: phospholipids, triglycerides (TG), cholesterol esters (CE), wax esters and alkyldiacylglycerols (Miyazaki & Ntambi, 2003).

1.4.1.3 Physiological roles of SCD

SCD plays a central role in lipogenesis. Majority of the physiological roles of SCD have been determined using asebia mice which have a naturally occurring mutation in the SCD1 gene (Sundberg et al., 2000). Asebia mice are deficient of triglycerides, cholesterol, wax esters and alkyl diacyl glycerols. Reduced levels of MUFA, such as palmitoleate (16:1 n-9) and oleate (18:1 n-9), in both plasma and tissue lipid fractions of these mice occurs with a concomitant increase in the saturated fatty acid (SFA) palmitate and stearate (Miyazaki et al., 2001). The skin and eyelid of SCD1 deficient mice are deficient in TG, CE and wax esters and instead have more free cholesterol leading to the development of cutaneous abnormalities and narrow eye fissures as a result of atrophic sebaceous and meibomian glands. Both of these glands secrete a lipid-rich protective lubricant termed as sebum and mebum, which contain TG, CE and WE (Miyazaki et al., 2001).

1.4.2 The SCD gene in teleosts

Cyprinus caprio SCD of size ~2.7 kb, containing a single ORF and encoding a polypeptide 33.65 kDa in mass and 292 amino acids (aa) long, was cloned by screening a hepatic cDNA library with rat SCD cDNA (Tiku et al., 1996). Further re-probing of the carp cDNA library with the above clone led to identification of another clone *Cds2*, which contained a single open reading frame (ORF)-encoding protein of 325 amino acids with 93% identity to *Cds1* and 62% identity to rat SCD1. The clone had an additional 269 nucleotides in the 3'UTR (Polley et al., 2003). A full length SCD cDNA clone of 2240 bp with an ORF of 324 amino acids was obtained from grass carp (*Ctenopharyngodon idella*) using a combination of PCR techniques. The gene is homologous with mammalian desaturases, is highly expressed in liver and expressed at low levels in brain (Chang et al., 2001). SCD in milkfish (*Chanos chanos*) contains a 972 bp ORF encoding a 233 amino acid protein and is 79% and 75% identical to common carp and grass carp, respectively and is 63-64% identical to the SCD of other vertebrates

(Hsieh et al., 2002). The full length SCD cDNA from tilapia (*Orechromous mossambicus*) is 1172 bp encompassing a 1008 bp ORF encoding 336 amino acids. It shares 78-82% identity with other teleosts and 64-66% with mammals, with expression restricted to liver (Hsieh et al., 2004). Recently both SCD isoform sequences have been isolated from *Sparus aurata* (Benedito-Palos et al., 2012). The primary sequences and genomic organisation of SCD isoforms are phylogenetically conserved between fish and mammals, but regulation of the gene expression and patterns of tissue expression are divergent among species (described in more detail below) (Chang et al., 2001). Regulation of SCD is dependent on diet and temperature (Polley et al., 2003) and hormones (Hsieh et al., 2004) (and further outlined in subsequent sections). Summary of SCD gene organisation in teleosts has been provided in Appendix (B.2). All the SCDs cloned to date share a common characteristic of three histidine cluster motifs, which function as ligands for iron chelation and form the electron transfer centre for the catalytic reaction (Shanklin et al., 1994).

1.4.2.1 SCD structure

Based on the mouse topology model, SCD1 contains four transmembrane helix domains. The NH_2 terminus is oriented towards the cytosol. The cytoplasmic loop and the COOH terminus contain eight histidine residues over three motifs, which are collectively known as the histidine box. Desaturases from a wide range of organisms, including mammals, fungi, cyanobacteria, insects and plants, show conservation of the histidine box motifs (Man et al., 2006). With the recent purification and crystallization of SCD1, the structure was elucidated (Bai et al., 2015) at a resolution of 2.6 Å and this structure has been deposited in the protein databank (4YMK). The cytoplasmic domain provides the pathway for the entry of the substrate and exit of the desaturated product. The acyl chain of stearoyl-CoA is localized in a tunnel that is buried in the cytosolic domain. The geometry of the tunnel, and the conformation of the bound acyl chain, provide the basis for selectivity of the desaturase reaction between carbons 9 and 10 of the fatty acyl chain as well as the stereo-specificity. The structure has two Zn^{+2} ions that form the metal centre and coordinates with nine histidine residues; eight in the three histidine motifs and one in transmembrane domain 4. This forms the catalytic reaction centre of the enzyme for oxygen activation.

1.4.3 Evolutionary history of stearoyl-CoA desaturase

The SCD gene family has undergone evolutionary change, including loss and duplication events. In humans, the SCD gene localizes to the long arm of chromosome 10, which is the region of the homeobox gene paralogs. These genes comprise a series of unrelated but linked genes on one

chromosome with linked paralogues on three other chromosomes due to the two rounds of genome duplication that have occurred during vertebrate evolution. SCD1 and SCD5 orthologues evolved at the stem of vertebrate evolution with SCD1 retained in all lineages, but with losses of SCD5 in some branches (Figure 3). SCD1 and SCD5 are found in human and birds; however, SCD5 was lost in the mouse lineage. The presence of SCD5 in the bovine and chicken genomes suggests that the duplication event giving rise to SCD1 and SCD5 occurred before the bird/reptile-mammal divergence (Castro et al., 2011). Through tandem duplication events, SCD1 has increased its gene number so that in rodents and lagomorphs there are four SCD genes, SCD1-4 (Castro et al., 2011).

Figure 3 Evolutionary history of SCD gene (Castro et al., 2011).

1.4.3.1 Evolution of the SCD gene in teleosts:

The SCD1 gene in vertebrates experienced two rounds of genome duplication (2R) (Figure 3). The lancelet *Branchiostoma floridae* offers evolutionary information about the ancient chordate lineage (Putnam et al., 2008). Genome duplication (2R) occurred at the stem of the vertebrate lineage and shaped the vertebrate genome (Figure 3). Also 2R arose after the divergence of the urochordates and before the split between cartilaginous fish and bony vertebrates (Putnam et al., 2008). As established by the draft genome analysis of the pufferfish *Tetraodon nigroviridis* (Jaillon et al., 2004), teleosts experienced a third round of genome duplication (3R), this resulted in duplication of SCD1 into SCD1a and 1b, while SCD5 is absent (Figure 3) (Castro et al., 2011).

1.4.4 SCD tissue expression

In birds and mammals SCD1 is ubiquitously expressed in tissues, with high expression in liver and adipose tissue, whereas SCD5 expression is localized to the brain and pancreas (Lengi & Corl, 2008). This pattern also holds true for the elasmobranch *Sylirhinus canicula*, but with additional low expression levels of SCD5 in the salt and rectal glands, implying conservation of tissue expression patterns (Castro et al., 2011). In mice, there are four SCD isoforms SCD1, 2, 3, and 4, with SCD1 expressed in the liver, SCD2 restricted to brain, SCD3 expressed in the skin (Zheng et al., 2001) and SCD4 expressed in the heart (Miyazaki et al., 2003).

1.4.4.1 SCD tissue expression patterns in teleost

The expression profile of SCD1a/SCD1b (Table 1) shows ubiquitous expression in the stickleback (*G. aculeatus*) but this expression profile differs from that found in zebrafish (*Danio rerio*), where SCD1 expression is parallel to that of ELOV-2, the gene involved in elongation of long chain PUFA (LC-PUFA). This ELOV-2 enzyme is absent in sticklebacks as most stickleback species inhabit oceanic ecosystems, rich in LC-PUFA from phytoplankton sources and have no need for endogenous synthesis of LC-PUFA (Morais et al., 2009). Common carp (*Cyprinus carpio*) has only SCD1a comprising of two isoforms, *Cds1* and *Cds2* which are exclusively expressed in the liver. However, *Cds1* is regulated by diet, whilst *Cds2* is regulated independent of diet and instead its expression is entirely dependent on temperature (Polley et al., 2003). In grass carp (*Ctenopharyngodon idella*) however, SCD1a is present and SCD1b is absent (Chang et al., 2001). Milkfish (*Chanos chanos*) SCD is more widely expressed than in grass carp with expression in liver, muscle, kidney, brain and gill (Hsieh et al., 2002). The evolutionary history and the expression pattern of SCD reveals differential expression patterns for teleost fish (Table 1). Expression of SCD 1 isoforms needs further investigation across a panel of fish species to determine the expression pattern of the isoforms.

Table 1 Differential tissue expression of SCD in teleost fish (Castro et al., 2011).

SCD isoform	Species	Tissue expressed in
SCD1	*Scyliorhinus canicula*	Ubiquitous
SCD1a	Common carp (*Cyprinus carpio*)(*cds1*) Common carp (*Cyprinus carpio*)(*cds2*) Grass carp (*Ctenopharyngodon idella*) Stickleback (*Gasterosteus aculeatus)*	Liver Liver Liver and vestigial expression in brain Ubiquitous
SCD1b	Stickleback (*Gasterosteus aculeatus)* Common carp (*Cyprinus carpio*)(*cds1*) Common carp (*Cyprinus carpio*)(*cds2*) Grass carp (*Ctenopharyngodon idella*)	Ubiquitous Absent Absent Absent
SCD2/3/4	Absent	
SCD5	Absent in teleost fish and only present in elasmobranch *Scyliorhinus canicula*	Ubiquitous

1.4.5 Regulation of stearoyl-CoA desaturase gene expression

Many factors regulate the expression of SCD1, including developmental, dietary, hormonal and environmental factors (Ntambi & Miyazaki, 2004). A high carbohydrate diet, insulin, glucose and fructose, cholesterol, cold temperatures, light, drugs (for example, LXRα agonists) and retinoic acid all induce hepatic SCD1 expression, whilst inhibition occurs through PUFA, conjugated linoleic acid (CLA), tumor necrosis-factor-α (TNF-α) and thyroid hormone mediated actions. The promoter regions of mouse SCD1 have been extensively investigated and contain binding sites for several transcription factors.

SREBP 1c: SCD contains the sterol regulatory element (SRE) in its promoter for binding of sterol regulatory element binding protein (SREBP) (Paton & Ntambi, 2009). SCD1 knockout mice do not produce SREBP 1c and fail to induce lipogenesis.

LXRs: Liver X Receptors (LXRα and LXRβ) belong to the nuclear hormone receptor superfamily and form heterodimers with oxysterols. LXRβ in conjugation with SREBP1c promotes SCD1 expression in a wide range of tissues (Paton & Ntambi, 2009).

PRARs: Peroxisome proliferator activated receptors (PPARs) are nuclear receptor transcription factors. Deletion of PRARα has been shown to halt the expression of SCD. (Paton & Ntambi, 2009).

ER: Estrogen receptors are a class of nuclear receptors and exist in two forms: ERα and ERβ. Absence of either estrogen receptor has been shown to increase the expression of lipogenic genes, including SCD1 (Paton & Ntambi, 2009). In fish, 17 beta estradiol hormone and high doses of testosterone result in elevated SCD activity and expression levels (Hsieh et al., 2004).

1.4.6 SCD gene expression in response to thermal acclimation and diet

1.4.6.1 Expression of SCD in cold acclimation studies

Enhanced lipid storage under cold conditions was observed in Antarctic eelpout *Pachycara brachycephalum* and the temperate eelpout *Zoarces viviparus*, with a predominance of MUFA and PUFA over SFA (Brodte et al., 2008), invoking a role for SCD in thermal acclimation. Studies of low temperature acclimation in rainbow trout (*Salmo gairdneri*) show an involvement of SCD in the later stages (3-28 days) of acclimation. The proportion of MUFA correlated well with the desaturase enzyme activity (Hagar & Hazel, 1985). SCD correlation with MUFA and 2-3 fold increase in SCD activity increase by 2-3 fold was also observed during cold acclimation in the hepatic microsomes of milkfish (*Chanos chanos*) (Hsieh, Chen, & Kuo, 2003). The same authors subsequently compared cold acclimation at 15 °C in both the stenothermal milkfish and eurythermal grass carp and found both species increased the proportion of UFA, with an associated increase in the desaturase index, SCD activity and gene expression (Hsieh & Kuo, 2005). However, the adaptation capability differed significantly between these two fish species, with all milkfish comatose and dead at day 8, but all grass carp surviving at the termination of the experiment on day 21. This was due to the varying expression pattern of SCD across the two species over the acclimation period. cDNA microarray analysis of transcript levels in seven carp tissues as a function of time and under three different cold acclimation regimes revealed that SCD had the largest fold change values across tissues in response to thermal acclimation (Cossins et al., 2006).

In carp liver, the microsomal SCD activity increased initially after cold induction due to a likely post translational activation mechanism of latent enzyme, but this was followed by an 8-10 fold increase in SCD transcript level after 48-60 hours of cold induction (Tiku et al., 1996). Further research identified that activation of the latent desaturase occurs on cooling, whilst induction of desaturase gene transcription and an unidentified lipid compensatory mechanism occurs during extreme cooling (Trueman et al., 2000).

1.4.6.2 Differential tissue expression in response acute and chronic cold stress

Effects of acute and chronic cold stress on SCD transcription in muscle, gill, heart and liver of tilapia (*Orechromis niloticus*) showed no response in either of the tissues to acute cold stress. Chronic cold stress provided no response of SCD in liver and heart, but SCD expression increased with cooler temperatures in muscle and gill tissue. Muscle was the most responsive tissue with a 16-fold increase

following 7 days of cold exposure. Tissues with high metabolic demands may respond more dramatically to cold acclimation regimes, including changes in SCD expression (Zerai et al, 2010).

1.4.6.3 Differential expression in response to diet and temperature

Differential expression of SCD isoforms, namely *Cds1* and *Cds2,* occur in common carp. A saturated fatty acid rich diet led to a four-fold increase in SCD transcripts (*Cds1*), but this was blocked by cooling to 15 °C. *Cds2* expression, however, was largely unresponsive to the SFA rich diet but was transiently upregulated during the first days of cooling from 30 °C to 10 °C. This differential expression is possibly a result of a recent gene duplication which may be unique to this species (Polley et al., 2003).

1.4.6.4 Influence of dietary lipids on SCD expression

Dietary lipids strongly affect fatty acid composition, SCD activity and gene expression in tilapia under cold shock scenarios (Hsieh et al., 2007). Activity reached highest levels for fish fed with a mixed diet of fish and corn oil followed by a coconut oil diet and was lowest for either a palmitoleic or fish oil diet. SCD gene transcript was increased for all diets but there was a greater induction in the mixed fish diet.

1.4.7 Major targets of SCD

SCD plays a central role in lipid metabolism, as mentioned above, and a balance of SFAs (the major substrate of SCD), and their products (MUFAs), is critical for normal physiological functioning. These fatty acids are the major targets in the control of metabolic disorders and cancer (Igal, 2010), while in fish they are targeted for varied purposes.

1.4.7.1 SCD as a major nutritional biomarker in marine food production

In general, SCD1b is upregulated compared to SCD1a for the normal feeding condition, while feeding until 70% satiation resulted in a significant down regulation of both SCD isoforms and muscle-specific lipoprotein lipase genes (Benedito-Palos et al., 2012). A panel of biomarkers, including SCD1b were identified for marine production of the lean muscle phenotype (Benedito-Palos et al., 2012). In *Dicentrarchus labrax*, a commercially important species in Mediterranean aquaculture, feeding resulted in a relatively high SCD1b expression in liver, adipose and brain, while fasting resulted in significant downregulation of SCD1b in liver (33 fold) and adipose tissue, but expression was unaltered in brain and was upregulated in skeletal muscle. The expression trend was reversed after 10 days refeeding and SCD1b was shown to be an informative marker for lipogenesis in liver and adipose tissue under the nutritionally challenged regime (Rimoldi et al., 2016).

1.4.7.2 Potential biomarker of cold tolerance

Microarray data from carp exposed to low temperatures was assessed with 13,440 cDNA probes across seven tissues. A major set of cDNAs (≈3,400) were affected by cold, with upregulation of 252 genes involved in important biological and physiological functions (Gracey et al., 2004). Many genes showed tissue specific regulation and one of them was related to the modification of the structure of lipid membranes. SCD was one of the few genes expressed in all tissues with high fold induction and statistical significance, demonstrating it to be a consistent marker of cold exposure (Gracey et al., 2004). Warm-adapted tropical fish species, like tilapia, face extensive economic losses due to mortalities during winter. Attempts are being made to improve this situation by identifying traits associated with cold-tolerance with SCD being one of the major targets. Effect of rearing temperature on SCD expression and fatty acid composition in genetically improved farmed tilapia showed low temperatures (22 and 28 °C) had significantly increased SCD gene expression and activity compared to fish at a higher temperatures (34 °C) (Ma et al., 2015). Higher temperatures resulted in an increase in saturated fatty acids, while lower temperature resulted in an increase in unsaturated fatty acids, especially (n-3)PUFA (Ma et al., 2015).

1.4.8 Need for examination of response of fatty acid saturation levels in Antarctic fish to elevated temperatures

The involvement of unsaturation of membrane lipids as a major adaptive mechanism to cold acclimation has been outlined above. Studies have been conducted in Antarctic fish (Gonzalez-Cabrera et al., 1995), eurythermal fish (Skalli et al., 2006), plants and cyanobacteria (Nishida & Murata, 1996) to establish links between membrane saturation and temperature change. However, few studies have examined Antarctic fish upon warm acclimation.

1.4.8.1 Studies linking SCD and thermal adaptation have been examined in plants and cyanobacteria

Studies in plants have linked desaturase enzyme activity with cold adaptation (Nishida & Murata, 1996). Genetic manipulations of desaturases in transgenic tobacco and cyanobacteria showed that unsaturated fatty acids (UFA) are of crucial importance in protecting the photosynthetic machinery from photo inhibition on cold exposure (Nishida & Murata, 1996). Decrease in temperature exposure from 36 °C to 22 °C in cyanobacteria increased desaturase transcript levels by 10-fold within 1 hour, indicating desaturation of membrane lipids is regulated at the level of desaturase gene expression (Los et al., 1993).

1.4.8.2 Thermal adaptation by membrane saturation in non-Antarctic fish (carp)

When liver ER membranes from carp acclimated to 10 °C or 30 °C were compared using fluorescence polarization techniques with DPH as a probe, cold-acclimated fish membranes displayed lower polarizations (less membrane order) than warm-acclimated fish membranes (Wodtke & Cossins, 1991). Desaturase activity increased at lower temperatures, which coincided with the lower DPH polarization indicating homeoviscous adaptation as a direct result of the expression of the SCD gene in carp (Wodtke & Cossins, 1991). Lower temperature acclimation in eurythermal fish is associated with decreased levels of saturated fatty acids and increased levels of unsaturated fatty acids (Hazel & Williams, 1990). Conversely, fishes and ectotherms acclimated to warmer temperatures should alter their fatty acid composition and increase the cell membrane order due to an increase in saturated fatty acids (Hazel & Williams, 1990). Membrane remodelling at higher temperature acclimation in European sea bass juveniles have shown to alter their saturation states (Skalli et al., 2006). Fresh water alewives *(Alosa pseudoharrengus*) also exhibit such change in saturation states in response to warm acclimation; however, the response was tissue specific(Snyder et al., 2012)**.**

1.4.8.3 Evolutionary adaptive response of membrane saturation in Antarctic fish

A comparative study of teleost fish provided evidence of links between membrane saturation and cold adaptation as polar fish had more disordered brain synaptic membranes than fish from warmer climates (Logue et al., 2000). Membrane disorder was attributed to the large and linear increase in the proportion of unsaturated fatty acids (UFA) within the glycerophospholipids, from 35 to 60% in phosphatidylcholine (PC) and from 55 to 85% in phosphatidylethanolamine (Logue et al., 2000). For PC, the increase in unsaturation was associated almost entirely with increased proportions (7-40%) of PUFA, with MUFA levels largely unchanged in all species (Logue et al., 2000). Enhanced membrane fluidity is an evolutionary adaptive response which it acquired over evolutionary periods.

1.4.8.4 Acclimatory adaptive response of membrane saturation in Antarctic fish

Despite acquiring evolutionary adaptive response of homeoviscous adaptation, Antarctic fish have shown a lack of acclimatory adaptive responses of membrane remodelling as well as unchanged saturation states in response to warm acclimation.

Trematomus bernacchii and *Trematomus newnessi* acclimated at 4 °C for 5 weeks were compared with the control temperature acclimation of -1.5 °C. Membrane lipid composition determined after acclimation in four tissues of both the species viz*.,* kidney, liver, muscle tissue and gill tissue showed

unchanged saturation states (Gonzalez-Cabrera et al., 1995). A similar trend of unchanged saturation was observed when the Antarctic species, *Notothenia rossii*, and the subantarctic species, *Lepidonotothen squamifrons* were exposed to warming temperature of 9 °C and 7 °C, respectively under normocapnic or hypercapnic conditions for 4-6 weeks (Strobel et al., 2013). Liver mitochondrial membrane unsaturation was unaltered by acclimation to warm temperatures in both species, while the subantarctic species had less saturated fatty acids than the Antarctic species (Strobel et al., 2013). However, the mitochondrial capacities were ideal to function to warmer acclimation in the subantarctic species than in the Antarctic species, suggesting metabolic acclimation potential in the subantarctic species (Strobel et al., 2013).

1.5 Research gap and significance of the study

1.5.1 Evolutionary analysis of SCD in Antarctic fish

Evolutionary gain of homeoviscous adaptations in Antarctic fish and SCD enzymes plays a major role allowing alterations of membranes in response to shifts in environmental temperature. Furthermore, temperature is also known to influence the composition of macromolecules, such as nucleic acids and proteins. The evolutionary history of SCD has been elucidated in general for vertebrates, but the history for Antarctic fish still needs to be understood. Evolutionary analysis, involving phylogenetic analysis and identification of thermal adaptive signatures by amino acid composition analysis and selection analysis, in the SCD gene of Antarctic fish in comparison with non-Antarctic fish has not been studied.

1.5.2 Response of SCD isoforms in Antarctic fish

Genome duplication has resulted in loss of SCD isoforms and functional swapping of roles between the isoforms. Study of desaturase isoforms are needed in a larger panel of teleosts to determine the expression pattern. Examining the response of SCD isoforms to temperature change in Antarctic as well as other fish has not been studied. Also, it is not known whether SCD isoforms are regulated independent of external stimuli or regulated by temperature.

1.5.3 Unchanged saturation states upon thermal acclimation in Antartcic fish

Eurythermal species exhibit a response of membrane saturation to elevated temperature (Skalli et al., 2006; Snyder et al., 2012). Studies as discussed above have demonstrated unchanged saturation on thermal acclimation in Antarctic fish (Gonzalez-Cabrera et al., 1995; Strobel et al., 2013). These

studies have not investigated pelagic species like *Pagothenia borchgrevinki.* Also, these studies show that the membrane-associated functions are not compromised as Antarctic fish have positive compensation, shown by hyper-osmoregulation even at this higher temperature. Mechanisms other than lipid saturation could contribute to this effect. The question of whether the usual saturation states seen at sub-zero temperatures are sufficient for normal functioning of cell membranes at temperatures as high as 4 °C, still needs to be investigated.

1.5.4 Prognosis of climate change

With amplification of anthropogenic global warming and its effects on polar habitats, there is a need for a system to be put in place to assess the impacts of rises in temperature at the molecular level. Chemical changes associated with membrane remodelling and SCD gene expression level changes in response to temperature elevation are ideal research targets for the assessment of AGW impacts on Antarctic fish. This is especially relevant for Antarctic notothenioid fish as their adaptation to such a stable, cold environment and their associated stenothermal nature may see them optimally adjusted to maintain membrane saturation states for sub-zero temperatures, but unable to adjust their saturation states (through altering the level of transcription of desaturase and its activity) to higher temperatures.

1.6 Hypothesis and research questions

The hypothesis of the current study is that lower temperature leaves thermal adaptive signatures in biological macromolecules, such as the DNA and the primary protein structure of the enzyme SCD. I hypothesise that membrane saturation, a major thermal adaptive mechanism, will occur at reduced capacities in Antarctic notothenioid fish in response to elevated temperatures and make them vulnerable to the effects of anthropogenic global warming (AGW). The research presented here is intended to answer questions related to the adaptive capacity of the SCD gene acquired over an evolutionary timescale, as well as the acclimatory capacity of the SCD gene and membrane saturation over a short exposure to thermal stress. **In this context, the major research questions for the current study are as follows.**

1. What is the evolutionary pattern of SCD in the Antarctic fish?
2. What are the key differences between the SCD of Antarctic fish and non-Antarctic fish?
3. Are there any temperature-specific SCD isoforms in the Antarctic fish and are they tissue-specific?

4. What is the transcriptional response of SCD in a thermally challenged environment?

5. What is the biochemical response of membrane saturation in a thermally challenged Antarctic fish?

The overall aim for this research is to study the SCD gene evolution in the Antarctic and non-Antarctic fish from the notothenioid suborder and to investigate membrane remodelling, and expression of the desaturase (SCD) enzyme in thermally challenged Antarctic fish.

To accomplish the above aims of the current study the objectives are as follows.

1) Isolation and identification of partial SCD isoform mRNA sequences from a range of Antarctic and non-Antarctic notothenioid fish species.

2) Evolutionary analysis of the SCD gene in Antarctic fish by comparative approaches of phylogenetic analysis, amino acid composition analysis, identifying signatures of selection and mapping of adaptive signatures on the SCD structure.

3) Determination of tissue expression through end point PCR from stenothermal notothenioid Antarctic fish.

4) Ascertain the transcriptional response to temperature in the Antarctic stenotherm through qRT-PCR (target gene: SCD and antioxidant markers) of RNA isolated from liver tissues harvested from fish from a range of temperatures and time-points in a pilot thermal acclimation experiment.

5) Ascertain the biochemical response to temperature in the Antarctic stenotherm through analysis of membrane lipids to determine the phospholipid profile and desaturation indices.

1.7 Thesis structure

This thesis is divided into five chapters.

Chapter 1	Describes the review of literature as well as the introduction to the research topic
Chapter 2	This chapter addresses objective 1 and 2 of this research and describes evolutionary analysis of the SCD gene in the Antarctic fish.
Chapter 3	This chapter addresses objective 3 and 4 of the research and comprises transcriptional response study of SCD isoforms to thermal acclimation in two Antarctic fish species.
Chapter 4	This chapter addresses objective 5 of the research and comprises the establishment of phospholipid fatty acid profiles in liver of Antarctic fish in comparison with the non-Antarctic New Zealand fish species and secondly studies changes in phospholipid fatty acid profile in response to thermal acclimation in two Antarctic fish species
Chapter 5	This chapter summarizes the key findings of the study and presents the future line of work.

2 Thermal adaptive signatures in an Antarctic fish enzyme

2.1 Introduction

The geographic restriction of most fish species within the notothenioid suborder to the stable, cold environment of Antarctica for 22 My, make these fish ideal models for evolutionary studies of biochemical and physiological adaptations to extreme environments. Evolutionary analysis at the molecular level demonstrate how these molecules have adapted to low temperatures. Major evolutionary adaptive mechanisms that drive such changes in proteins primarily involve natural selection and various other contributing mechanisms, one of them being gene duplication. Selection alters proteins at specific sites enabling low temperature adaptation, while gene duplication has enabled Antarctic fish to acquire key traits to function in the cold, such as AFGP syntheses. Gene duplication formed one of the major contributing factor in the evolution of anti-freeze glyco protein (AFGP), resulting in initial expansion of AFGP in Antarctic fish (Chen et al., 1997b; Cheng, 1998).

The evolution of Stearoyl-CoA desaturase (SCD) in teleosts follows a complex history of duplication and loss of isoforms arising from both ancient and recent genome duplication, including the loss of SCD2 from teleosts and the duplication of SCD1 into two genes, SCD1a and 1b (Castro et al., 2011). Although Castro's work on vertebrates inferred the evolutionary history of SCD in teleosts, as represented by fish species whose genome sequences were available, the evolutionary history of the SCD in a larger sample of fish lineages, and its role in cold tolerance in Antarctic fish, is not known.

Environmental temperature also has an influence on the composition of DNA sequences, such as the GC content (Kreil & Ouzounis, 2001), amino acid substitutions and amino acid usage (Kreil & Ouzounis, 2001), resulting in structural changes of proteins. The study by Windisch on Antarctic fish (Windisch et al., 2012) examined patterns of thermal adaptation in nucleic acids of Antarctic fish. High AT content in synonymous codon position in an Antarctic fish contrasted to the high GC content found in hyperthermophiles and a distinct pattern of codon usage compared to its temperate counterpart. However, the Windisch et al. (2012) study compared all of the orthologous sequence pairs in the transcriptomes of just two confamiliar non-notothenioid fish species (Antarctic and Non-Antarctic). While an investigation of thermal adaptive signatures at genomic level such as SCD sequences across wider range of Antarctic (mainly notothenioid) and non- Antarctic fish species still needs to be conducted.

Protein structure adaptation in Antarctic fish in relation to temperature has been documented for a few cytoplasmic proteins, and the protein lactate dehydrogenase has been particularly well studied (Coppes & Somero, 2007). In addition, membrane proteins the Sec61 translocation channel protein (Romisch et al., 2003) and PEPT1 peptide transporter protein from *Chionodraco hamatus* (Rizzello et al., 2013) have been investigated for signatures relating to cold adaptation. Thermal adaptive signatures of orthologous protein sequences have also been elucidated using cDNA libraries from two confamiliar fish species from different thermal habitats (Windisch et al., 2012). These studies indicated common principles that regulate thermal adaptation. At lower temperatures, due to low energy states, protein flexibility is enhanced, especially in the active sites. Flexibility achieved by specific amino acid replacement in the regions involved in the mobility of the enzyme during the catalytic cycle is the predominant structural cold adaptation (Feller, 2013).

In this chapter major Antarctic and non-Antarctic fish representatives of five of the eight families in the notothenioid suborder were analysed using partial SCD sequences. The rationale for the use of partial sequences was to obtain SCD sequences from a large data set including Antarctic fish species. SCD sequences were PCR amplified and sequenced partial SCD sequences from a range of Antarctic fish species samples as well as published sequences of notothenioid, and non notothenioid species of different thermal habitats from databases (GenBank : https://www.ncbi.nlm.nih.gov/genbank/ and ENSEMBL : (http://asia.ensembl.org/index.html), were used in a comparative study of nucleotide sequences and inferred primary protein sequences. The purpose of this study was to reconstruct the phylogenetic history of SCD in Antarctic fish and to determine the signatures of thermal adaptation in the two SCD isoforms. The term 'signature' is used as a proxy for specific changes in the nucleotide and amino acid sequences of the SCD isoforms of Antarctic fish when compared to non-Antarctic fish, primarily processes such as temperature reduction have left evidence of such changes. Amino acid composition and codon usage of both the SCD isoforms were examined from Antarctic fish in comparison to non-Antarctic fish. The sites of positive selection were determined. Locations of amino acid substitutions specific to Antarctic fish, and amino acids under positive selection were mapped onto the primary amino acid sequence of SCD. With the integration of structural data and sequence data, I have attempted to establish its relationship to temperature.

2.2 Methods

2.2.1 Fish species

Fish species of the suborder Notothenioidei were obtained from a number of sources. Notothenioids from Antarctic and non-Antarctic origin were sampled by Bill Detrich (Northeastern University) and his team during the ICEFISH 2004 cruise of Research Vessel Ice Breaker (RVIB) *Nathaniel B. Palmer* (Table 2). The cruise track consisted largely of a traverse of the transitional zone linking the South Atlantic to the Southern Ocean (Detrich et al., 2012). Fish liver tissues collected were gifted to Dr Victoria Metcalf and used for genomic investigation in this study. Fish samples from McMurdo Sound and Terra Nova Bay within the Ross Sea were collected by Dr. Victoria Metcalf. Sampling and tissue harvesting procedures are provided in the Appendix (A.1.1).

Table 2: Fish species sampled for the isolation of SCD sequences.

Species	Family	Sequences obtained	Location
Antarctic-notothenioid fish			
Chaenoocephalus aceratus	Channichthyidae	SCD1b& SCD1a	Palmer Station, Antarctic Peninsula
Champsocephalus gunnari	Channichthyidae	SCD1b& SCD1a	Shag Rocks, South Georgia
Chionodraco hamatus	Channichthyidae	SCD1b& SCD1a	Terra Nova Bay, Antarctica
Gobionothen gibberifrons	Nototheniidae	SCD1b	South Georgia
Gobionothen marionensis	Nototheniidae	SCD1b	South Sandwich Island
Gymnodraco acuticeps	Bathydraconidae	SCD1b	Cape Evans, McMurdo Sound, Antarctica
Lepidonotothen squamifrons	Nototheniidae	SCD1b	South Sandwich Island
Notothenia rossii	Nototheniidae	SCD1b& SCD1a	Ross Sea, Antarctica
Notothenia corciceps	Nototheniidae	SCD1b& SCD1a	Obtained from databases
Pagothenia borchgrevinki	Nototheniidae	SCD1b &SCD1a	McMurdo Sound, Antarctica
Pseudochaenicthys georgianus	Channichthyidae	SCD1b	Shag Rocks, South Georgia
Trematomus hansoni	Nototheniidae	SCD1b	Cape Evans, McMurdo Sound, Antarctica
Trematomus nicolai	Nototheniidae	SCD1b	Cape Evans, McMurdo Sound, Antarctica
Trematomus pennellii	Nototheniidae	SCD1b	Cape Evans, McMurdo Sound, Antarctica
Trematomus bernacchii	Nototheniidae	SCD1a	Obtained from databases
Non-notothenioid-Antarctic fish			
Pachycara brachycephalum	Zoarcidae	SCD1b	McMurdo Sound, Antarctica
Non-Antarctic-notothenioid fish			
*Pagothenia tessellata**	Nototheniidae	SCD1b	Falkland Islands/Islas Malvinas
*Champsocephalus esox**	Channichthyidae	SCD1b & SCD1a	Falkland Islands/ Islas Malvinas
*Dissostichus eleginoides**	Nototheniidae	SCD1b	Burwood banks, north of Antarctic Polar Front (APF)
Bovichtus variegatus	Bovichtidae	SCD1b & SCD1a	Otago, New Zealand
Cottoperca gobio	Bovichtidae	SCD1b	Burwood banks, north of APF
Eliginops maclovinus	Eleginopidae	SCD1b	Falkland Islands/ Islas Malvinas
*Notothenia angustata**	Nototheniidae	SCD1b& SCD1a	Otago, New Zealand

* Fish species with an Antarctic ancestry and that have dispersed to non-Antarctic waters.

2.2.2 Nucleic acid isolation

RNA extracted from liver samples was subjected to DNase treatment. Purified RNA quality was checked and converted into cDNA. RNA extraction procedure, DNase treatment, quality checks are explained in (2.2.3 and 2.2.4).

2.2.3 Total RNA isolation

Total RNA was extracted from <100 mg of snap frozen tissue using TRIzol® reagent (Invitrogen Co, Grand Island, NY, USA) as per the manufacturer's protocols. The tissues were homogenised using liquid nitrogen in an autoclaved and pre-chilled mortar and pestle. One ml of TRIzol® was added per 100 mg of homogenised liver tissue and this mixture was further homogenised. The homogenous

solution was transferred to a 1.7 ml tube and 200 µL of chloroform was added, vortexed for 15 seconds and incubated for 5 min at 22 °C. After incubation, the sample was centrifuged at 12,000 g for 15 min at 2-8 °C. Following centrifugation, the aqueous phase was transferred to a fresh tube and the RNA was precipitated by the addition of 0.5 ml of 100% isopropanol and incubated at room temperature for 10 min. The sample was centrifuged at 12,000 g for 10 min at 4 °C and the supernatant was discarded. The RNA pellet was washed with 1 ml of 75% ethanol by brief vortexing and then centrifuged at 7500 g for 5 min at 4 °C**.** The supernatant was discarded and the pellet was air dried for 5-10 min and then re-suspended in 100 µL of β diethylpyrocarbonate (DEPC)-treated water and concentration of the total RNA was determined using a Qubit Fluorometer (Life Technologies). RNA purity was checked by determining the A_{260}/A_{280} ratio on a Nanodrop spectrophotometer (DeNovix), and RNA integrity was checked by denaturing agarose gel electrophoresis as described in section (2.2.10). RNA samples were treated with Ambion® TURBO DNA-free™ DNase Treatment (Life Technologies) as per the manufacturer's protocol to remove any contaminating genomic DNA. Following the DNase treatment, the concentration was checked using a Nanodrop spectrophotometer and Invitrogen Qubit® fluorometer with Qubit® RNA buffer and dye (Invitrogen, Carlsbad, CA, USA) and calibrated with the supplied standards. The RNA samples were stored at -80 °C.

2.2.4 Quality check for RNA

DNase treated RNA was analysed by electrophoresis on an agarose gel to check for the integrity of the RNA as described in section (2.2.10). DNA contamination was checked by amplification of the tubulin reference gene using 1 µl of RNA sample as a template. The PCR and the optimum cycling conditions required for beta-tubulin amplification are described in section (2.2.8). The presence of tubulin amplification in any sample indicated DNA contamination and that sample was not used for cDNA conversion.

2.2.5 cDNA conversion

DNase-treated total RNA (300 ng) quantified using a Qubit® fluorometer was reverse transcribed using the PrimeScript™ RT reagent kit (Perfect Real Time, Takara Bio). The protocol for reverse transcription for the SYBR Green qPCR assay was followed as described in the kit manual. A 10 µl reaction mixture comprising 1X of Prime Script buffer (5X), 0.5 µl Prime Script RT Enzyme Mix I, 25 pmol Oligo dT primer, 50 pmol random hexamers and 6.5 µl total RNA (300 ng) and RNase free

nanopure water. The reaction mixture was incubated at 37 °C for 15 min followed by inactivation of the reverse transcriptase enzyme by incubation at 85 °C for 5 seconds and then 4 °C. The cDNA obtained was diluted 20-fold to a total volume of 200 µl using double RNase free nanopure water and the cDNA was stored at -20°C until required for qRT-PCR.

2.2.6 cDNA synthesis

DNase treated RNA was reverse transcribed (Table 3) using the Primescript 1st strand cDNA synthesis kit (Takara, Cat# 6110A). Synthesized cDNA was used for the amplification of gene products required for phylogenetic and tissue expression analysis by end point PCR (Chapter 3).

Table 3 Details of the reverse transcription reaction.

Reagents	Reaction volume
Reaction 1	
Random 6mers (50 uM)	1.5 µl
dNTP(10 mM)	1.0 µl
Template RNA (500 ng)	2.5 µl
RNAase free dH_2o	5.0 µl
Above reaction (1) was incubated at 65 °C for 5 min followed by reaction 2 as given below	
Above reaction mix	10.0 µl
5X Primescript buffer	4.0 µl
RNAase inhibitor(40U/ µl)	0.5 µl
Prime script RTase (200U/µl)	1.0 µl
RNAase free dH_2o	4.5 µl
Total	20.0 µl

Reaction 1 and 2 was mixed gently and incubated at 42 °C for 40 min followed by an inactivation step at 95 °C for 5 min. Samples were stored at -20 °C.

Dilution of cDNA: For the tissue expression by endpoint PCR and isolation of partial gene sequences (SCD1a/SCD1b/SOD/β-Actin), cDNA was diluted 1:4.

2.2.7 Primer design

Primers were specifically designed for this study using the following approaches.

2.2.7.1 Identification of published coding sequences

The target genes in this study included two isoforms of the SCD gene, SCD1a and SCD1b (see review Chapter 1 for an explanation of these isoforms). Published SCD sequences from fish, human, mouse, and chicken were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) based on a BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) search using the carp temperature-specific SCD isoform sequence and known SCD sequences from other fish. Further sequences were obtained from the ENSEMBL database from fish species whose genomes had been sequenced.

2.2.7.2 Sequence alignment and primer design for SCD

The SCD sequences identified were used to generate amino acid and nucleotide alignments using the program ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/). SCD1a and SCD1b amino acid sequences were aligned and stretches of sequences unique to the each isoform and the nucleotides encoding these amino acids conserved across many fish species were used for primer design, this was primarily the reason for using both the alignments (nucleotide and amino acid). The SCD1a reverse primer was degenerate whereas the forward primer was specific primer. SCD1b primers were both specific. Primer sequences were checked using IDT (https://sg.idtdna.com/calc/analyzer) for the ideal parameters required for primer design and these were synthesized from Integrated DNA technologies (IDT), Singapore. Primer details for the partial SCD sequences is given in Table 4.

Table 4 Primers designed for isoform-specific amplification of SCD from fish.

Isoform	Forward primer(5'-3')	Reverse primer(5'-3')	Product size (bp)
SCD1a	CACACATATAAAGAGAAAGAAGG	GTRTGRTGRTARTTRTGRAA	769
SCD1b	TCGACGGTGGAGGATGTTTTTG	GGAACGTGTGATGGTAGTTGTG	768

2.2.8 General PCR protocols

The general PCR protocol required for various analyses (Table 5) is provided in Table 6 and the cycling parameters are provided in Table 7. The PCR protocol were carried out using the kit (HOT FIREPol® DNA polymerase, 01-02-000S, Solis Biodyne). The template varied with cDNA used for phylogenetic analysis and end point RT-PCR for tissue expression, plasmid DNA for clone confirmation, and DNase-treated RNA for analysis of DNA contamination.

Table 5 List of gene products and the specific requirement for the various analyses along with the research chapter.

Gene product	Purpose
SCD1a	Phylogenetic analysis (Chapter 2)
SCD1b	Phylogenetic analysis (Chapter 2)
SCD1a	Cloning and tissue expression (Chapter 3)
SCD1b	Cloning and tissue expression (Chapter 3)
β-actin	Cloning (Chapter 3)
Cu/Zn SOD	Cloning (Chapter 3)
Tubulin	For checking DNase contamination in RNA (Chapter 3)

Table 6 General PCR protocol.

Reagents	Stock concentration	Final concentration	Reaction Volume for (10 µl)
Taq Buffer	10X	1X	1.0 µl
dNTP	10 mM	200 µM	0.2 µl
Taq polymerase	5 U/µl	2 units	0.1 µl
25 mM Mg	25 mM	1.5 mM	0.6 µl
Forward Primer	10 µM	0.4 µM*	0.4 µl
Reverse Primer	10 µM	0.4 µM*	0.4 µl
Template(DNA)	Varies	0.5-1 ng/ µl	1.0 µl
PCR grade Water	------	-------	6.3 µl

*** SCD1a amplification for phylogenetic analysis had forward primer (0.8 µM) and reverse primer (0.8 µM)**

Table 7 Cycling conditions for PCR.

Temperature	95	95	51-61 °C *	72	72
Time	12 min	1 min	1 min	2 min	5 min
# Cycles	1	35**			1

* Annealing temperature varies with the gene product (SCD1a-52 °C, SCD1b-56 °C, β-actin -59 °C, β- tubulin-56 °C, Cu/Zn SOD-61 °C)

** Tissue expression by endpoint PCR (chapter 3) comprised only 30 cycles, while this was extended to 35 cycles for other purposes

2.2.9 Agarose gel electrophoresis

Electrophoresis samples were run on 1% agarose gels, which were prepared using 1.0 g of (Agarose Molecular grade, Bioline, London) dissolved by boiling in 100 ml of 1X TBE (89 mM Tris/ 89mM Boric acid / 2 mM EDTA). Molten agarose was cooled to 60 ˚C and 2% ethidium bromide (EtBr) was added

before pouring into a casting tray. A well-forming comb was inserted and the gel was allowed to set for 30 min and then placed in an electrophoresis tank containing 1X TBE buffer. The sample to be analysed consisted of 5 µl of amplified product and 3 µl of loading dye (0.2% w/v bromophenol blue, 0.25% Xylene cyanol, 40% w/v sucrose). Samples were separated at a constant 10 V/cm for 45 min, visualized on a UV trans-illuminator, and photographed under UV light using the VersaDoc™ Imaging System (Model 3000, Bio-Rad Laboratories Inc, CA, USA).

2.2.10 RNA gel electrophoresis

The RNA gel electrophoresis procedure is a slight modification of the denaturing agarose electrophoresis. Gels were prepared (2.2.9) without the addition of EtBr. A RNA sample (5 µl) was taken and 15 µl of loading buffer (Table 8) was added, and the samples were heated at 65 ˚C for 10 min, followed by a snap chill of 1-5 min on ice. One micro litre of loading dye (Table 9) was added and samples were loaded onto a 1 % agarose gel and run at 10 V/cm for one hour. The samples were visualized on a UV trans-illuminator and photographed under UV light using the VersaDoc™ Imaging System (Model 3000, Bio-Rad Laboratories Inc, CA, USA).

Table 8 Denaturing gel loading buffer composition.

Formaldehyde	37.5 µl
Formamide	125 µl
10 X MOPS	125 µl
Ethidium bromide	0.5 µl

Table 9 Loading dye composition.

Formamide	8 ml
Sterile water	1.8 ml
0.5 M EDTA	0.2 ml
Xylene Cyanol	10 mg
Bromophenol blue	10 mg

2.2.11 Amplification and sequencing of partial SCD sequences

The optimised cycling condition for the amplification of SCD1a and 1b is provided above (2.2.8) in general PCR protocols. Amplification was performed in an iCycler (Bio-Rad). Negative controls, in which the DNA template was substituted with sterile water, were included in each set of reactions to ensure that they were free of contamination. PCR products (5 µl) were mixed with 3 µl loading dye and separated on a 1% agarose gel by electrophoresis as described in (2.2.9) and successful amplicons

were sequenced at the Lincoln University Sequencing Facility within the Bio-Protection Research Centre at Lincoln University (http://www.lincoln.ac.nz/Lincoln-Home/Research/Research-and-testing-service/Sequencing-and-genotyping/) using an ABI PRISM® 310 Genetic analyser (Applied Biosystem, Foster City, California).

2.2.12 Quality checks of the partial SCD sequences

Sequences was checked with Sequence Scanner software of Applied Biosystem version 1 and the sequences compared with sequences in the GenBank EST database using BLASTn searches (http://www.ncbi.nlm.nih.gov/BLAST/).

2.2.13 Data mining

Sequences of SCD obtained from NCBI GenBank and ENSEMBL databases. The lists of sequences and accession numbers are provided in the Appendix (B.1). The nomenclature used in these databases is not the same as Castro's (Castro et al., 2011); hence the sequences obtained were categorised into SCD1a or SCD1b based on the clustering of the sequences in a phylogenetic analysis using the Neighbour Joining method in MEGA 5 (Saitou & Nei , 1987) .

2.2.14 Bioinformatic analysis

2.2.14.1 Phylogenetic analysis

Partial nucleotide sequences (2.2.12) and the published sequences obtained from data mining (see 2.2.13 above), were aligned using Web PRANK (Löytynoja & Goldman, 2010) using the default parameters. Web PRANK is a phylogeny-aware algorithm that generates high quality alignments for downstream evolutionary analysis as it considers the phylogeny in the placement of gaps that arise due to insertion-deletion events (Löytynoja & Goldman, 2010). The dataset comprised 71 sequences, including both published sequences and new (partial) sequences and are provided as a WebPRANK-aligned file in Fasta format in the temporary link and details of the files is in the Appendix [B.4 (1)].

2.2.14.1.1 Bayesian Phylogenetic analysis

Bayesian phylogenetic analyses were run for 10 million generations in BEAST v1.8.3 (Drummond & Rambaut, 2007) assuming a GTR+I+Γ substitution model and two different clock models: an uncorrelated lognormal relaxed clock (Drummond et al., 2006) and a strict molecular clock. For the uncorrelated lognormal clock, the mean value of the ucld.stdev parameter for 9,000 sampled trees (after removal of the first 10% as burnin) was 0.48 (ESS = 189), which is less than 1, the threshold for

statistical rejection of the hypothesis of a strict molecular clock. This result indicates that these data fit the assumptions of a strict molecular clock, and therefore the results of the strict molecular clock analysis are presented. For the strict molecular clock, a maximum clade credibility (MCC) tree was constructed from 9000 sampled trees (after removal of the first 10% as burnin) using TreeAnnotator v1.8.3.

2.2.14.2 Amino acid composition analysis

The nucleotide sequences of the SCD1a and SCD1b isoforms were translated into amino acid sequences using the Expert Protein Analysis System (ExPASy) (http://www.ca.expasy.org). Amino acid sequences were trimmed to maintain uniformity of sequence length across sequences from different sources.

2.2.14.3 Composition of the partial SCD amino acid sequence:

The partial sequences for the amino acid composition analysis comprised the four transmembrane domains and the three conserved histidine motifs required for the catalytic activity of the SCD enzyme. These sequences lack the N terminal 50 amino acids and C terminal 75 amino acids. The trimmed sequence of 208 amino acid residues was 77% of the complete amino acid sequences of SCDs shown in Table 10. To ensure homogeneity across all the amino acid sequences used for comparison, sequences of the species taken for the study were trimmed to the length of the shortest partial sequences

Table 10: Partial trimmed sequences of SCD1a and SCD1b isoforms for *Pagothenia borchgrevinki*. All of the trimmed SCD1a and SCD1b isoform sequences taken for the amino acid composition analysis started and ended with the motifs highlighted in green.

>*Pagothenia_borchgrevinki*_SCD1a

NVFLLTLLHIGAAYGICLVPSASTLTLLWSVLCFVLSALGVTAGAHRLWSHRSYKASLPLKIFLGVANSMAFQNDIYEWARD
HRVHHKYSETDADPHNAVRGFFFSHIGWLMVRKHPDVLEKGRKLEVHDLLADKVVMFQKKYYKASVLLMCFFVPMIVP
WYLWGESLWVAYFVPALLRYTLVLNATWLVNSAAHMWGNRPYDHHINP

>*Pagothenia_borchgrevinki*_ SCD1b

NIILMTLLHLGALYGLVLLPSASVSTVAWTAVCYLISALGVTAGAHRLWSHRSYKASFPLRVFLALGNSMAFQNDIYEWAR
DHRVHHKYSETDADPHNATRGFFFSHVGWLMVRKHPDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVMLCFVVPMM
VPWYFWGESLVLAYFVPGLLRYAVMLNATWLVNSAAHLYGNRPYDKSINP

The Antarctic fish species used for amino acid composition analysis belonged to the suborder Notothenioidei, while some non-Antarctic fish species belong to the suborder Notothenioidei and majority of the fish species belonged to the other major orders of fish (Appendix B.1). The details of the fish species under each group are provided in the Appendix (B.3). The dataset comprised nine SCD1a and seventeen SCD1b sequences from Antarctic fish species and seventeen SCD1a and twelve SCD 1b sequences from non-Antarctic fish species.

2.2.14.4 Determination of amino acid composition

Trimmed sequences were used to determine amino acid composition using MEGA 5 (Saitou & Nei , 1987) . The amino acid frequencies were determined for each sequence as well as an overall mean for any specified group such as SCD isoforms (1a and 1b) and thermal habitat (Antarctic and non-Antarctic). Amino acid compositions were compared (1) between Antarctic and non-Antarctic fish, (2) between non-Antarctic fish from different thermal habitats, and (3) between isoforms.

2.2.14.5 Principal component analysis

Principal components analysis (PCA; Minitab v17 software) was used to compare the amino acid compositions of the different species, and used to examine if any differences in composition were related to thermal habitat and enzyme isoforms. The PCA used the relative frequencies of twenty amino acids that occurred in each fish species as obtained from the MEGA 5 (Saitou & Nei , 1987) procedure. The data were not transformed prior to analysis and the PCA used a correlation matrix to standardize the variance among the different amino acids. Pearson's correlation coefficient (Stigler

(1989) was then used to assess which amino acids were 'influencing' the distribution of fish samples along PCA axes 1 and 2.

2.2.14.6 Codon usage analysis

Codon usage of SCD1a and SCD1b isoform sequences from Antarctic and non-Antarctic fish species were evaluated using the sequence manipulation suite at http://www.bioinformatics.org., which generates the number and frequency of each codon for each amino acid. The proportions of codons with an adenine or thymine in the third position (AT3) and the proportion of codons with a guanine or cytosine in the third position (GC3 = 1 - AT3) were computed for each amino acid.

For each amino acid, a chi-square test (χ^2) (Snedecor & Cochran, 1989) based on a 2 x 2 contingency table was used to examine whether there was an association between the frequency of codon type (AT v CG) and habitat (Antarctic v non-Antarctic). Cochran's test of Heterogeneity among the 2 x 2 contingency tables produced for the different amino acids was assessed using the method given by Zar (1999).

2.2.14.7 Test of positive selection

Signatures of positive and purifying selection acting on the SCD isoforms were observed. Tests compare the number of non-synonymous substitutions per non-synonymous site (dN) with the number of synonymous substitutions per synonymous site (dS). The ratio dN/dS is denoted by ω. Sites undergoing positive selection have ω values greater than one (Yang & Bielawski, 2000). Datamonkey webserver (http://www.datamonkey.org/) (Delport et al., 2010) was used to calculate ω and to identify sites under positive selection. Methods used for their identification of positive sites from the webserver include single likelihood ancestor counting (SLAC), random effects likelihood (REL), fixed effect likelihood (FEL) and internal branch fixed effect likelihood (IFEL) (Pond & Frost, 2005). Genetic algorithm (GA) branch analysis (Pond & Frost, 2005) was used to identify branches in the phylogeny where positive selection is inferred to have occurred. This analysis partitions the branches into different regimes based on selection classes and assigns each branch to a particular class.

2.3 Results

2.3.1 Identification SCD 1a and 1b Isoforms

Isoform-specific primers were used to amplify SCD cDNA obtained from mRNA isolated from liver tissues of Antarctic and non-Antarctic fish. SCD1b was isolated from 21 species and SCD1a was

isolated from ten species (Table 11). The Antarctic and non-Antarctic notothenioid species are mentioned in Table 2. Non-Antarctic notothenioid are species which have an Antarctic ancestry and have dispersed to non-Antarctic waters. While some notothenioid species which do not have an Antarctic ancestry are mentioned in Table 2. The amplified products were sequenced and then confirmed using BLASTn (http://www.ncbi.nlm.nih.gov) to confirm their identity as sequences from the gene. SCD1a sequences obtained from Antarctic species had 98% sequence similarity to SCD1a from the emerald rock cod *Trematomus bernacchii* (FJ177513.1), the black rockcod *Notothenia coriiceps (*XM_010780865), and 91% sequence similarity to SCD1a from the gilthead seabream *Sparus aurata (*JQ277703). The SCD1b sequences had 99% sequence similarity with SCD1b of *N. coriiceps* (XM_010769852.1) and 88% sequence similarity with the European seabass *Dicentrarchus labrax (*XM_010769852.1). This dataset was expanded using SCD sequences obtained from NCBI and ENSEMBL and provided in the Appendix (B.1)

Table 11 Number of sequences isolated from the notothenioid and non-notothenioid fish from Antarctic and non-Antarctic thermal habitats using isoform-specific primers.

	SCD1b	**SCD1a**
Antarctic notothenioid	13	7
Non-Antarctic notothenioid	7	3
Antarctic non-notothenioid	1	0
Total	**21**	**10**

2.3.2 Phylogenetic analysis

Phylogenetic analysis of the SCD gene in teleost species was achieved using Bayesian analysis and provides support for the duplication of the SCD1 gene into SCD1a and SCD1b (Figure 4). The SCD1 clade, comprising the basal ray-finned fish (*Polypterus senegalus*), a representative of Chondrichythyes (*Scyliorhinus canicula*), the coelacanth Latimeria *chalmunae,* the amphibian *Xenopus laevis* along with mammalian and avian species, is sister taxon to a clade consisting of the SCD1a and SCD1b clades. SCD1a and SCD1b duplicates are observed in fish species of the orders Perciformes, Gasterosteiformes and Tetraodontiformes, within the class Actinopterygii. Fish species belonging to the order Cypriniformes (*Danio rerio*), Characiformes (*Astyanax mexicanus*) and Gonorynchiformes (*Chanos chanos*) are found in the SCD1a clade only as they lack duplicates in the SCD1b clade.

Lineage-specific duplications of SCD1a are observed in *Gasterosteus* aculeatus (Gasterosteioformes) and *Cyprinus carprio* (Cypriniformes) belonging to the SCD1a clade. Lineage-specific duplicates of SCD1a for *G. aculeatus* are localized in contig 6293, while the genome duplicate of SCD1b is localized in contig 5012 (Figure 5, Figure 4-blue horizontal arrows). Lineage-specific duplications of SCD1b are observed only within *Oreochromis niloticus* (Figure 4-blue horizontal arrows). Cold adapted notothenioid, comprising Antarctic and non-Antarctic species belonging to the suborder Notothenioidei, formed separate clades within both the SCD1a and SCD1b clades (Figure 4 - enclosed box bracket "] "), indicating that they had inherited and retained both copies resulting from the ancestral duplication. For SCD1a *Bovichtus variegatus* and *Sebastiscus marmoratus* (posterior probability = 0.74) were the sister taxa to cold adapted Notothenioid clade (Figure 4 - vertical blue arrow). For SCD1b, *Eliginops maclovinus* (posterior probability = 1.0) was the sister taxon to the cold adapted notothenioids, and the sister taxon to this clade was a clade consisting of *B. variegatus* and *Cottoperca gobio* (posterior probability=1.0). The secondarily temperate species *Notothenia angustata* and the species with an Antarctic ancestry, *Dissostichus eleginoides*, *Pagothenia tesselata* and *Champsocephalus esox*, that have dispersed in the non-Antarctic waters *were* also part of the SCD1b clades. *N. angustata* and *C. esox,* the non-Antarctic fish with an Antarctic ancestry, were part of the SCD1a cold adapted notothenioid clade. The Antarctic non-Notothenioid, *Pachycara brachycephalum*, clustered with *G. aculeatus* in the SCD1b clade.

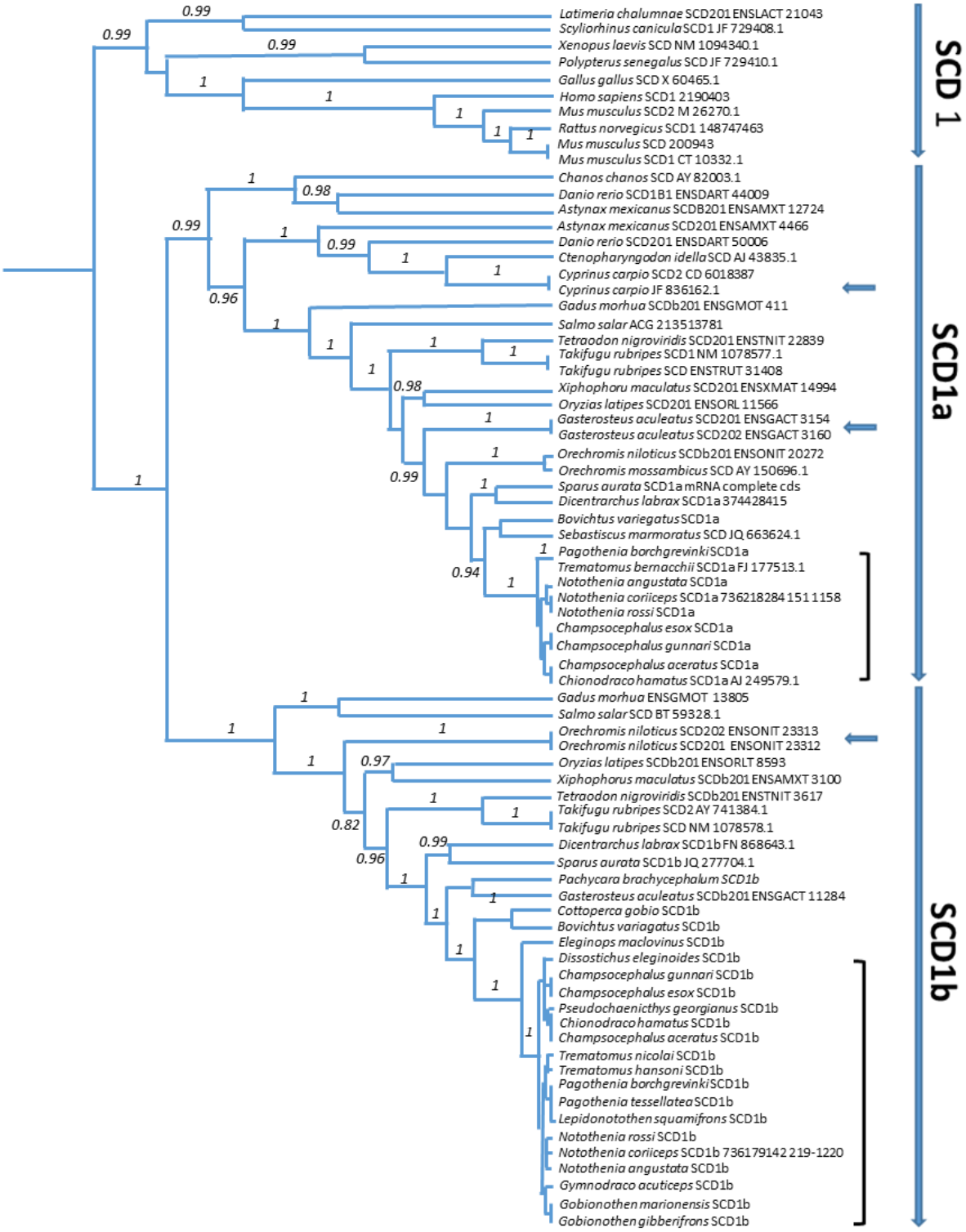


Figure 4 Bayesian phylogenetic tree for partial SCD cDNA sequences obtained from mRNA isolated from the livers of Antarctic and non-Antarctic fish along with sequences obtained from NCBI and ENSEMBL, with accession numbers. SCD1a and SCD1b clade (vertical blue arrow), Cold adapted notothenioid SCD1a and SCD1b clade are shown by "] ", lineage-specific duplicates (horizontal blue arrows), Numbers on branches are posterior probabilities.

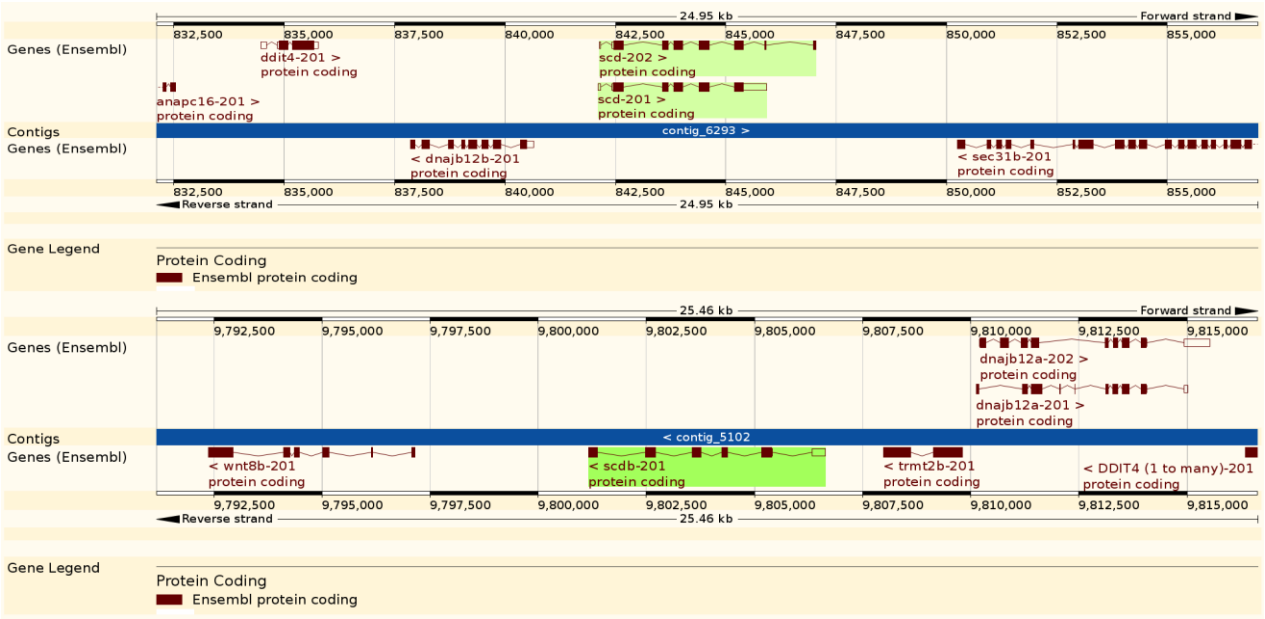


Figure 5: Lineage-specific duplicates and genome duplicates for *G. aculeatus.* Genome duplicates SCD1a present in contig 6293 (above) and SCD1b present in contig 5012 (below) are highlighted in green. Lineage-specific duplicates of SCD1a (SCD-202 and SCD-201) are localized in contig 6293.

2.3.3 Comparative mapping and gene organization of the SCD isoforms

The gene organization of the sequences was obtained from the ENSEMBL database (Figure 6). The basic organisation and number of exons varied among both paralogs and orthologs. Variation in the number of exons was higher in SCD1a sequences having a maximum of nine and a minimum of five exons. SCD1b sequences had a maximum of seven and a minimum of five exons. Most of the species had five exons across both paralogs as well as the Antarctic species. *Gadus morhua* had the maximum number of exons in the case of both SCD1a and SCD1b. The gene structure was similar across all the paralogs indicated by the conserved length of the exons. Three exon lengths of 131 (except group 5 in SCD1a), 206 and 233 are conserved across all the species for both the isoforms. Most of the variation in exon length is observed at the terminal regions of the genes across both paralogs, with the central portion being highly conserved. In general, the intron lengths were higher in SCD1b than in SCD1a isoforms [See in the Appendix (B.2) for details].

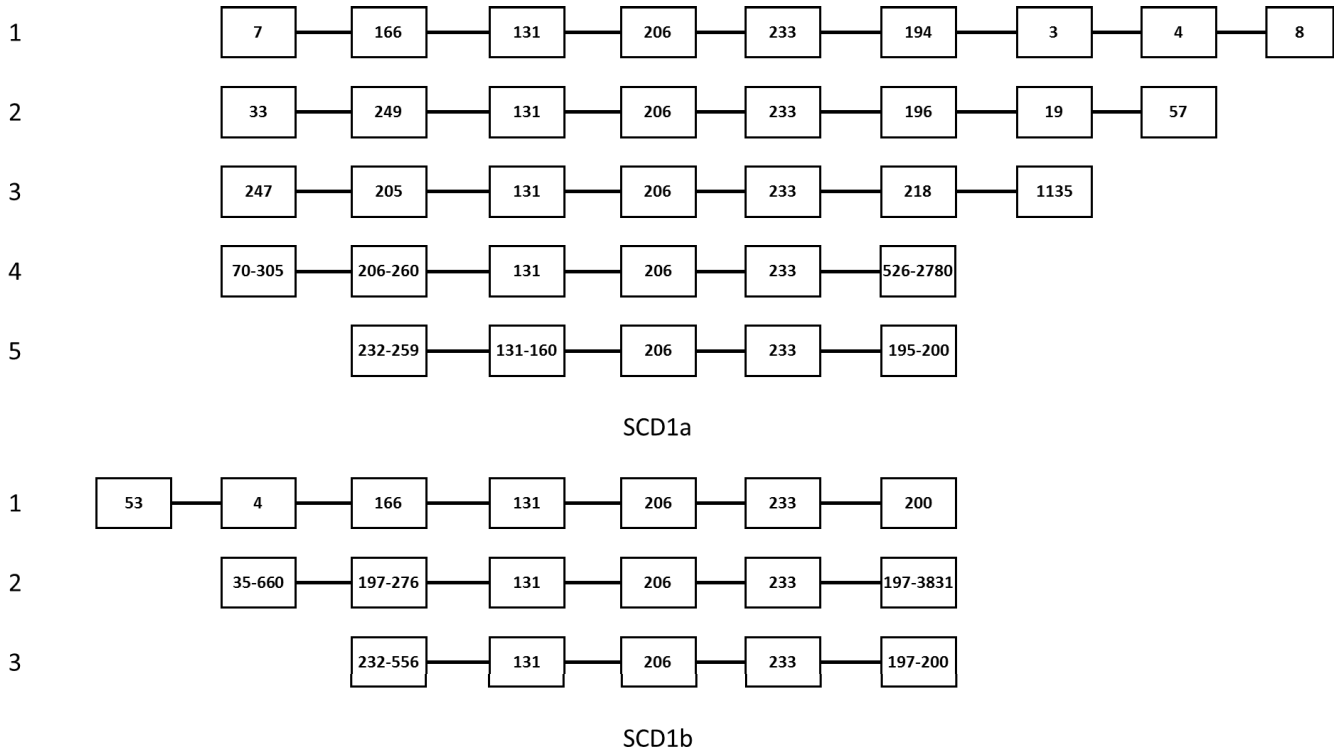


Figure 6 Comparative mapping and gene organization of SCD isoforms of teleost fish species. All of these species are non-Antarctic except for *Chionodraco hamatus.* The genome structure and organization of the SCD sequences (5' to 3') in the teleost species is shown. Exons are represented by boxes. The numbers inside these boxes represent the lengths of the exons in base pairs. Introns are represented by lines between these boxes. The numbers on the left represent different groups of fish as follows:
SCD1a, **1:** *Gadus morhua*; **2:** *Gasterosteus aculeatus* (SCD202)*;* **3:** *Astyanax mexicanus*; **4:** *Danio rerio* (SCDa201)*, Danio rerio* (SCDa001)*, Oryzias latipes, Xiphophoru maculatus,* and *Gasterosteus aculeatus* (SCDa201)*,* **5**: *Chionodraco hamatus* (Antarctic species)*, Takifugu rubripes, Tetraodon nigroviridis,* and *Orechromis niloticus* (SCDa201)
SCD1b, **1:** *Gadus morhua*; **2:** *Astyanax mexicanus, Oreochromis niloticus* (SCDb201) and *Gasterosteus aculeatus*; **3:** *Orechromis niloticus* (SCDb202)*, Oryzias latipes, Xiphophoru maculatus, Tetraodon nigroviridis* and *Takifugu rubripes.*

2.3.4 Amino acid substitutions in the SCD isoforms of Antarctic fish

Amino acids that could have a role in cold adaptation were identified by the analyses of complete cDNA sequences from databases compared with partial sequences obtained from Antarctic fish. Sequences were subjected to phylogenetic analysis (Figure 4) and those identified as SCD1a or SCD1b were translated into amino acids and aligned using ClustalOmega, alignments are provided as in the Appendix[(B.5) & (B.6)]. Comparison of the SCD1a and 1b isoforms indicated that they are

highly conserved across the vertebrates. Fish-specific amino acid replacements were observed, and most variation was at the N-terminal end of the protein sequence.

Specific amino acid substitutions were observed in the SCD1a and 1b sequences of Antarctic fish when compared to non-Antarctic fish (shown in Table 12 and 13). In SCD1a (Table 12), there are 11 amino acid substitutions specific for Antarctic fish and out of which five substitutions were for Antarctic as well as the cold adapted non-Antarctic fish (*Notothenia angustata* and *Champsocephalus esox*). Codon 245 were specific to cold adapted notothenioid fish (Antarctic and non-Antarctic fish) as well one non-Antarctic warm adapted species *Danio rerio*. Seven substitutions were localised in the loop regions, two in the transmembrane regions, one in the helix and one in the amphipathic helix (Figure 9). Majority of hydrophobic residues and polar residues were replaced with polar residues in Antarctic fish species (Table 12), this was observed in amino acid positions 36 (serine), 65 (threonine), 170, 245 (histidine). Some replacements involved replacement of hydrophobic amino acids by other hydrophobic amino acids seen in amino acid position 44 (phenylalanine), 169 (valine), 284 (tryptophan). Replacements of hydrophobic and polar amino acids by hydrophobic amino acids was observed in amino acid position 17 (phenylalanine and proline) and 282 (isoleucine). Replacements of polar uncharged by polar charged amino acids was observed in position 246 (histidine) and replacement of hydrophobic amino acids by polar amino acids was found only at position 293 (threonine).

Table 12 Amino acid replacements in the SCD1a isoform of cold adapted notothenioid fish compared with non-Antarctic fish.

Cold adapted Notothenioid fish	F,P	S	F	T	V	H	H	H	I	W	T
Position**	17*	36*	44*	65	169	170	245	246	282*	284*	293*
Amino acid in non-Antarctic fish	A,I,T	T,I,M ,K,R, V	L,I,V	V,F,P ,R,	L,M, F	S,T, N,S, R,G	S,A, N,G, K	S,T,N	C,W,S	L,M	L,F,A

****** Refers to the amino acid numbering of *Danio rerio* in alignment [Appendix**:** (B.5)]

***** Replacements observed only in Antarctic fish species. Hydrophobic amino acids are in red.

The SCD1b isoform had six amino acid substitutions that were specific for the cold-adapted notothenioid fish comprising of Antarctic and non-Antarctic fish species which has a thermal range from 5 °C to 11°C (*Notothenia angustata*, *Dissostichus eleginoides, Champsocephalus esox, Pagothenia tessellata)* and in *Eliginops maclovinus* (Table 13). The substitutions were localized in the transmembrane and loop regions (three in each, Figure 9). At positions 27 (Table 13) there is

substitution of acidic, basic, hydrophobic and polar uncharged with hydrophobic residues in cold adapted notothenioid species and less hydrophobic residues (leucine) (Position 66 and 193) with more hydrophobic residues (methionine and valine). Furthermore, at positions 209, 235 there is a drop in hydrophobicity caused by substitution of more hydrophobic residues with less hydrophobic residues (leucine) at position 209 and 235, while a hydrophobic residue is replaced by an uncharged polar residues (tyrosine) at position 236 (Table 13).

Table 13 Amino acid replacements in the SCD1b isoform of cold adapted notothenioid fish with non-Antarctic fish.

Cold adapted Notothenioid fish	M	V	V	L	L	Y
Position*	27	66	193	209	235	236
Amino acid in the non-Antarctic fish	E,K,A,Q	L	L	V,M	M,I	W

*Refers to the amino acid numbering of *Orechromis niloticus* SCD201 in the alignment shown in the Appendix (B.6)**.** Hydrophobic amino acids are in red.

2.3.5 Principal component analysis

Amino acids valine, tyrosine and lysine were positively correlated with PCA axis 1 and isoleucine, phenylalanine and tryptophan were negatively correlated with PCA axis 1 (Table 14). For PCA axis 2, glutamine and lysine were positively correlated and arginine and glutamic acid were negatively correlated. Only three amino acids having the strongest correlation were selected.

Based on the pattern of positioning along the PCA axis 1, the plot could generally distinguish between cold adapted notothenioid fish species, comprising a cluster of species from Antarctic habitat as well as non-Antarctic habitat, but having an Antarctic ancestry such as *N.angusata, D.eleginoides* and *C.esox* (Figure 7, cluster A) with the cluster of non-Antarctic fish species (Figure 7, cluster 2) that have no Antarctic ancestry, such as *Bovichtus variegatus*, *Eliginops maclovinus* and *Cottoperca gobio.* Clustering of SCD1b fish species from the Antarctic and non-Antarctic species with Antarctic ancestry indicated that they were high in valine, tyrosine and lysine (Figure 7). Furthermore, SCD1b isoforms from fish species from cold non-polar habitats (*Bovichtus variegatus, Eliginops maclovinus* and *Cottoperca gobio*) also had high proportions of these three amino acids, but they were separated from the SCD1b sequences for Antarctic fish (Figure 7).

SCD1a isoforms from Antarctic fish also formed a cluster characterized by high glutamine and lysine. This cluster comprised Antarctic species, cold adapted non-Antarctic notothenioid species (*C.esox*) and cold adapted non notothenioid species, such as *Gadus morhua* of SCD1a isoform as well as *Gasterosteus aculeatus* and *Takifugu rubripes* of SCD1b isoform (Figure 7, cluster B). SCD1a from *C. hamatus* had high proportions of glutamine acid and lysine compared to other Antarctic fish and hence was separated from the main cluster of SCD1a Antarctic species. The SCD1a and SCD1b sequences from non-Antarctic fish were characterized by high proportions of isoleucine, phenylalanine and tryptophan.

The non-Antarctic warm-adapted fish species *Oreochromis niloticus* had high proportions of glutamine and lysine (PCA axis 2) and also isoleucine, phenylalanine and tryptophan (PCA axis 1) when compared to other fish species. In general, SCD1b from Antarctic species had high proportions of valine, tyrosine and lysine, while the SCD1a from Antarctic species had high proportions of glutamine and lysine.

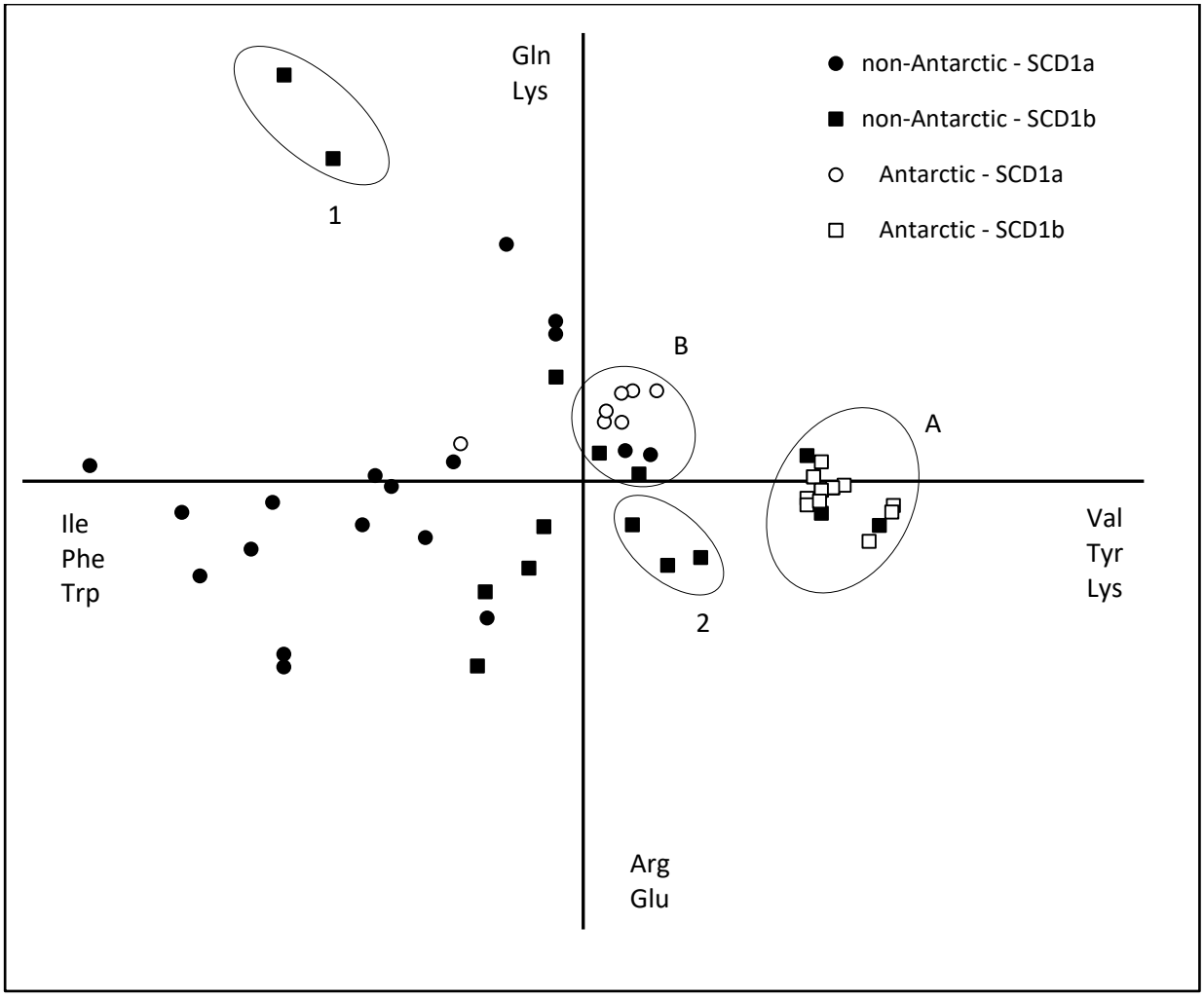


Figure 7 Principal Component Analysis of partial SCD1a and SCD1b sequences from Antarctic and non-Antarctic fish. Each point on the graph represents a single fish. PC1 = 26.4 %, PC2 = 13.4%. Amino acids significantly correlated with PC1 and PC2 are indicated on the axes. Warm-adapted fish species *Orechromis niloticus* are enclosed (1), non-Antarctic notothenioid fish species are enclosed (2), Antarctic fish species and non-Antarctic fish species with Antarctic ancestry are enclosed (A) and Antarctic and cold-adapted fish species are enclosed (B). Species in each enclosure are as follows:
1; *Orechromis niloticus* _SCD1b(1), *Orechromis niloticus* _SCD1b(2)
2; *Bovichtus variegatus*_ SCD1b, *Eliginops maclovinus*_SCD1b and *Cottoperca gobio*_SCD1b
A; All notothenioid Antarctic fish species with SCD1b sequences as given in Table 2
B; All Notothenioid Antarctic fish species with SCD1a sequences as given in table 2 (except *Chionodraco hamatus*) along with non-Antarctic fish species *C.esox*_SCD1a , *Gadus morhua*_SCD1a, *Gasterosteus aculeatus*_SCD1b and *Takifugu rubripes*_SCD1b.

Table 14 Correlations between the first two principal components and the amino acids. Correlation values strongest 3 (positive/negative) colour coded yellow for the PCA1, while strongest 2 (positive/negative) colour coded orange for PCA2 are taken for the analysis.

	PCA 1	PCA 2
Ile	-0.81024	-0.41206
Phe	-0.76056	0.236001
Trp	-0.71873	0.376603
Asn	-0.66516	0.271456
Thr	-0.53893	-0.45217
Pro	-0.46016	0.406286
Gln	-0.4582	0.582388
Cys	-0.32433	0.304829
Arg	-0.07819	-0.84001
Leu	0.002454	-0.24951
Glu	0.015323	-0.45965
Asp	0.142539	0.2302
His	0.160055	0.301528
Gly	0.253719	-0.11307
Ala	0.31603	-0.24188
Met	0.373938	-0.09381
Ser	0.414523	0.229667
Lys	0.653156	0.422134
Tyr	0.781634	0.101393
Val	0.836387	0.187449

2.3.6 Codon usage analysis

To test codon usage bias in Antarctic and non-Antarctic SCD sequences, complete SCD1a sequences of three Antarctic species were compared with 13 non-Antarctic species and partial SCD1b sequences of 10 Antarctic (including three non-Antarctic sequences with Antarctic ancestry) and 10 non-Antarctic species. In SCD1a, except for glycine and arginine, the proportions of A or T over G or C in the third position of all codons were not significantly different when codon triplets of non-Antarctic and Antarctic species were compared. Glycine had significantly higher proportions of AT3 codons, while arginine had lower proportions of AT3 codons when compared to non-Antarctic sequences. In SCD1b, seven amino acids had significant differences in the proportions of AT3 and GC3 codons in Antarctic and non-Antarctic fish, while the other amino acids did not show any significant differences (Figure 8). Aspartic acid, glycine, proline and tyrosine had significantly higher usage of A or T in the third codon position for Antarctic SCD1b sequences compared to non-Antarctic sequences ($P<0.05$, Table 16, Figure. 8). Glutamine, isoleucine and phenylalanine had significantly lower AT3 usage when compared with non-Antarctic sequences ($P<0.05$, Table 16, Figure 8). A Cochran's test of

heterogeneity and the chi-square total between the amino acids and the AT3 vs GC3 distribution across the Antarctic/non-Antarctic habitat was significant for SCD1b, but not for SCD1a (Table 15).

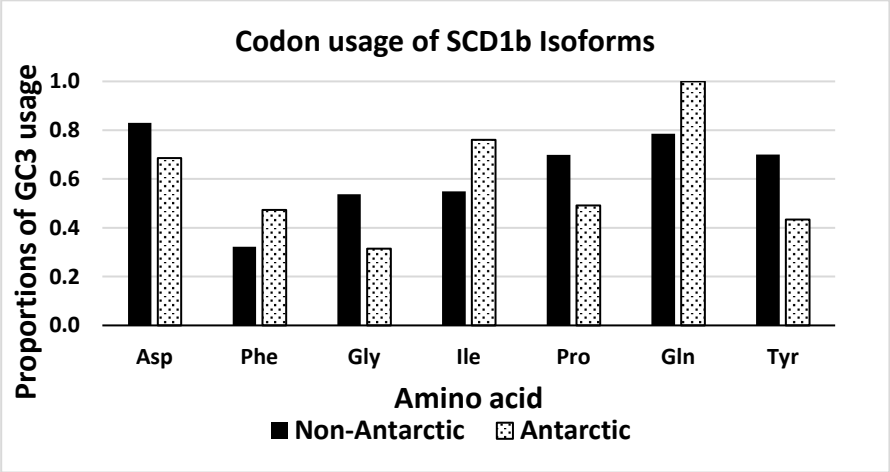


Figure 8 Proportions of GC3 codons in partial sequences of SCD1b from Antarctic and non-Antarctic fish. All the amino acids presented are significant at P<0.05 (Table 16).

Table 15 Cochran's test of heterogeneity for all amino acids in SCD isoforms.

	Chi.sq	Amino acids	P -value
SCD1a isoforms			
Total chi square	21.19	18	0.270
Heterogeneity	20.6	17	0.245
chi square total	0.584	1	0.445
SCD1b isoforms			
Total chi square	79.63	18	0.000
Heterogeneity	71.9849	17	0.000
chi square total	7.641	1	0.005

Table 16 Chi-square test for the amino acids of SCD1b isoforms and the P-value for the corresponding amino acid. Significant P-values at the <0.05 level are indicated in orange.

Amino acid	chi-sq	P value
Alanine	0.009	0.926
Cysteine	2.683	0.101
Asparagine	5.677	0.017
Glutamine	0.128	0.721
Phenylalanine	5.592	0.018
Glycine	12.322	0.000
Histidine	0.296	0.587
Isoleucine	5.937	0.015
Lysine	3.646	0.056
Leucine	2.434	0.119
Methionine	NA	NA
Asparagine	3.055	0.080
Proline	10.368	0.001
Glutamine	4.898	0.027
Arginine	2.278	0.131
Serine	2.071	0.150
Threonine	1.788	0.181
Valine	0.743	0.389
Tryptophan	NA	NA
Tyrosine	15.702	0.000

2.3.7 Test of positive selection

SCD sequences included in the phylogenetic analysis and were identified as either SCD1a or SCD1b (Figure 4) were taken for analyses. SCD1a [22 sequences; 3 Antarctic + 19 non-Antarctic, input sequence alignments are provided as a temporary link, link details in Appendix B.4 (2)] and SCD1b [35 sequences: 18 Antarctic +17 non-Antarctic, input sequence alignments are provided as a temporary link, link details in Appendix B.4 (3)] from Antarctic and non-Antarctic fish species were analysed separately for evidence of positive selection (Tables 17 and 18). The analysis was set at a threshold P=0.05 for SLAC, FEL and IFEL analyses, while an empirical Bayes factor of >100 was set for the REL analysis. In SCD1a, seven codons were identified to be under positive selection (Table 17). Four of these codons (203, 688, 769, and 875) were identified by all three methods (FEL, IFEL and REL), while codons 714 and 770 were identified by two methods (FEL and IFEL) and codon 477 was identified only by one method (REL). Four codons (203, 769, 770 and 875) were detected in Antarctic species (shown in **bold** in Table 17 and by an arrow in Figure 9). The first three codon sites under positive selection are localized in loop regions whereas codon 875 is localized in the amphipathic helix (Figure 9). At

codons 769 and 770, a histidine pair is present in all nine of the Antarctic species but is absent from non-Antarctic species (except for *C. esox*) (Figure 9 and 10).

Four codons in SCD1b, are under positive selection 223, 373, 428, and 464 (Table 18). Codons 373 and 464 were identified by all three methods (FEL, IFEL and REL), while codons 223 and 428 were identified by only one. Codons 223, 428 and 464 are in loop regions while codon 373 is part of the third transmembrane region of the protein. Of these four codons, 373 and 464 were detected in Antarctic and cold adapted non-Antarctic notothenioid species. Codon 373 was determined to have been under positive selection in the non-notothenioid Antarctic species *Pachycara brachycephalum*; a few non-Antarctic species*, P. tessellata and N. angustata*, Antarctic species, *N. rossii, N. coriiceps* and *G.gibbberifrons,* while other Antarctic species were not under positive selection at this site. Codon 464, which is part of the loop region appears to have undergone positive selection in the Antarctic species *N. coriiceps* but it is unclear whether this codon is under selection in other species as partial sequences of the Antarctic species are lacking.

Table 17 Amino acid sites under positive selection identified in SCD 1a sequences of Antarctic and non-Antarctic fish using four different methods in Datamonkey. FEL: Fixed effect likelihood, REL: Random effect likelihood, IFEL: Internal branch fixed effect likelihood, nd: not detected.

Amino acid	Amino acid change (From-To)	Protein domain	FEL P-value	REL Empirical Bayes factor	IFEL P-value
203	Ala-Thr **Ala-Pro** Ala-Ile **Pro-Phe** Ala-Thr Ala-Ala	Loop	0.0890057	95.2405	0.0478734
477	Arg-Arg Arg-Thr Arg-Ser Arg-Lys Arg-Met Met-Ile Ile-Thr Met-Ile Arg-lys Arg-Met Ile-Val Ile-Thr	Loop	nd	111.755	nd
688	Arg-Lys Arg-Arg Arg-Gly Arg-Lys Arg-Ser Arg-Ala	Loop	0.00429614	274.893	0.0339293
714	Leu-Val		0.0460927	nd	0.0422443
769	**Lys-His** Lys-Asn Lys-Gln Lys-Asn Asn-Ser Asn-Thr Ser-Ala Ser-Gly Asn-His	Loop	0.0323879	103.746	0.0389304
770	**Asn-His** Asn-Ser Asn-Thr Asn-Ser Asn-Thr Asn-Ser	Loop	0.0667923	nd	0.0252515
875	Leu-Phe Leu-Pro **Pro-Thr** Pro-Ala Leu-leu Leu-Leu Leu-Phe Leu-Leu Leu-Phe Leu-Thr	Amphipathic helix	0.0238863	105.481	0.0110167

Amino acid change shown with bold and by an arrow in Figure 9 are the amino acid changes that have occurred in the Antarctic species.

Table 18 Amino acid sites under positive selection identified in SCD1b sequences of Antarctic and non-Antarctic fish using four different methods in Datamonkey. FEL: Fixed effect likelihood, REL: Random effect likelihood, IFEL: Internal branch fixed effect likelihood, nd: Not detected, TM3: transmembrane region

Amino acid	Amino acid change (From-To)	Protein domain	FEL p-value	REL Empirical Bayes factor	IFEL P-value
223	Val- Ala Ala- Pro Ala- Gly Ala- Pro Ala- Val Ala- Thr Ala- Trp Ala- Pro Ala- Val Ala- Ala	Loop	nd	118.868	nd
373	Met- Ile Ile- Leu Leu- Leu Leu- Val Leu- Val Ile- Val **Met- Ile** **Met- Leu** **Met- Leu** **Ile- Leu** Val- Thr Leu- Leu Leu- Leu	TM3	0.010929	180.06	0.0117088
428	Ser- Thr Thr- Asn Thr- Ser Thr- Gly Thr- Asn	Loop	0.0207856	nd	nd
464	Val- Phe Phe- Cys Cys- Leu Leu- Leu Cys- Ser **Phe- Val** Phe- Ile Cys- Asn Leu- Val	Loop	0.0258428	105.642	0.0104854

Amino acid change shown in bold are the amino acid changes that have occurred only in the Antarctic species.

2.3.8 Mapping of amino acids onto the mouse SCD toplogical model

A topological model recently published for the mouse SCD protein based on an X-ray structure (Bai et al., 2015) was used to visualise the locations of important amino acids in the fish sequences. In Figure 9, the amino acids replaced in Antarctic fish (see Tables 12 and 13) are shown by blue circles. In SCD1a, of the 11 amino acid replacements specific for Antarctic fish, seven are localized in the loop regions while two are in the transmembrane regions, one in the helix and one in the amphipathic helix. The amino acid replacement and closest to the N terminus, which is also under positive selection, is phenylalanine in *C. hamatus* and proline in the other Antarctic species. A histidine pair

specific to Antarctic fish is also under positive selection and is localized near the catalytic centre (indicated by an arrow pointing to two adjacent blue circles in Figure 9a). There are four amino acids under positive selection in SCD1a (indicated by arrows in Figure 9a, with one arrow identifying a histidine pair) but no amino acids are under positive selection in SCD1b.

In SCD1b the amino acids that are replaced in the Antarctic species are spread evenly among the transmembrane and loop regions of the protein (Figure 9b), whereas in SCD1a they are concentrated in the loop regions.

(a) **(b)**

Figure 9 Mapping of amino acid replacements specific to Antarctic fish onto a mouse topological model along with those under positive selection. (a) SCD1a; (b) SCD1b. Red circles represent conserved histidine residues involved in coordination of the dimetal centre. Blue circles represent residues substituted in Antarctic fish. Arrows indicate residues under positive selection. The arrow showing two blue circles is the histidine pair present only in Antarctic SCD1a sequences. Source of topological model diagram Bai et al., (2015).

2.3.9 Detection of a Histidine pair in SCD1a isoform of Antarctic fish species

SCD1a amino acid sequences inferred from the nucleotide sequences, along with amino acid sequences from databases were aligned. Part of the alignment showing the location of the histidine pair specific to Antarctic fish is shown in Figure 10. Of the 18 species in this alignment, all the Antarctic species have this histidine pair. Except for *C.esox*, no non-Antarctic species possessed the histidine pair, including the secondarily temperate species *N. angustata*.

Figure 10 Alignment of partial SCD1a sequences from Antarctic and non-Antarctic fish. Presence of a histidine pair confined to Antarctic species enclosed in a box (the secondarily temperate species *N. angustata* is lacking one of the histidine).

2.3.10 Change in the residues of critical determinants of substrate chain length observed in SCD1a isoform

In the SCD1a isoform (Figure 11), tyrosine is replaced by phenylalanine across all the fish species used in the current analysis (except for *Salmo salar* and *Gadus morhua* species), while alanine is conserved in SCD1a (except for *Danio rerio)* and in SCD1b (except for *Oreochromis niloticus* species) (Figure 12). The SCD1b isoform residues tyrosine and alanine (boxed below in Figure 12) are located in transmembrane 2 region of the protein. These residues have a role in determining the substrate length of the fatty acyl chain to be desaturated. Both the tyrosine and alanine are highly conserved across the SCD of animals and the SCD1b isoform of fish (Figure 11). There was no distinction between Antarctic and non-Antarctic fish species with respect to these conserved residues either isoforms.

Figure 11 Alignment of partial SCD1a sequences from Antarctic and non-Antarctic fish species. Residues in SCD1a isoform which are critical determinants of the substrate length of fatty acyl chain are boxed (phenylalanine and alanine). Blue horizontal boxes represent the transmembrane regions.

Figure 12 Alignment of partial SCD1b sequences from Antarctic and non-Antarctic fish species Residues in SCD1b isoform which are critical determinants of the substrate length of fatty acyl chain are boxed (tyrosine and alanine). Blue horizontal boxes represent the transmembrane regions (TM1 and TM2).

2.3.11 Phylogenetic analysis of positive selection using Genetic Algorithm (GA) branch analysis

Figure 13 shows the results of a phylogenetic analysis of positive selection using a genetic algorithm (GA) branch analysis as implemented in Datamonkey [input sequence alignments are provided as a temporary link, link details in the Appendix [A.4(4)]. This shows the dN/dS (in six discrete classes) ratio on each branch of the tree. For computational reasons this tree contains only a subset of the sequences included in the original phylogeny. Additionally, this tree should be interpreted as unrooted as the analysis has forced the root of the tree to be incorrectly placed within SCD1a rather than between SCD1 and a clade consisting of SCD1a and SCD1b (Figure 13). This analysis shows that positive selection is concentrated around the split between SCD1a and SCD1b and on branches leading to Antarctic fish for SCD1a, but not SCD1b. The branch with the highest dN/dS ratio is node 4, with 93.2% support. This branch is close to the split between SCD1a and SCD1b, although the position of the salmon SCD1a sequence is problematic. Nevertheless, the split between this and the SCD1b clade also has a high dN/dS ratio. Within SCD1a the branch leading to the Antarctic fish *T. bernacchii* and *P. borchgrevinki* (node 41; 98.0% support), and the branch leading to the icefish species *Chionodraco hamatus* and *Chaenocephalus aceratus* (node 45; 96.2% support) both have high dN/dS ratios. While the branch leading to the other icefish species, *Chaenocephalus aceratus*, has a much lower dN/dS ratio, despite this species has the highest dN/dS ratio within the SCD1b clade. In general, Antarctic fish show elevated dN/dS ratios for SCD1a (indicated by a red arrow in Figure 13), but a relatively lower level of purifying selection for SCD1b, which shows a very different pattern of selection. Curiously, this is reversed in the icefish species *Chaenocephalus aceratus*, but not *Chionodraco hamatus* - the other icefish species represented here.

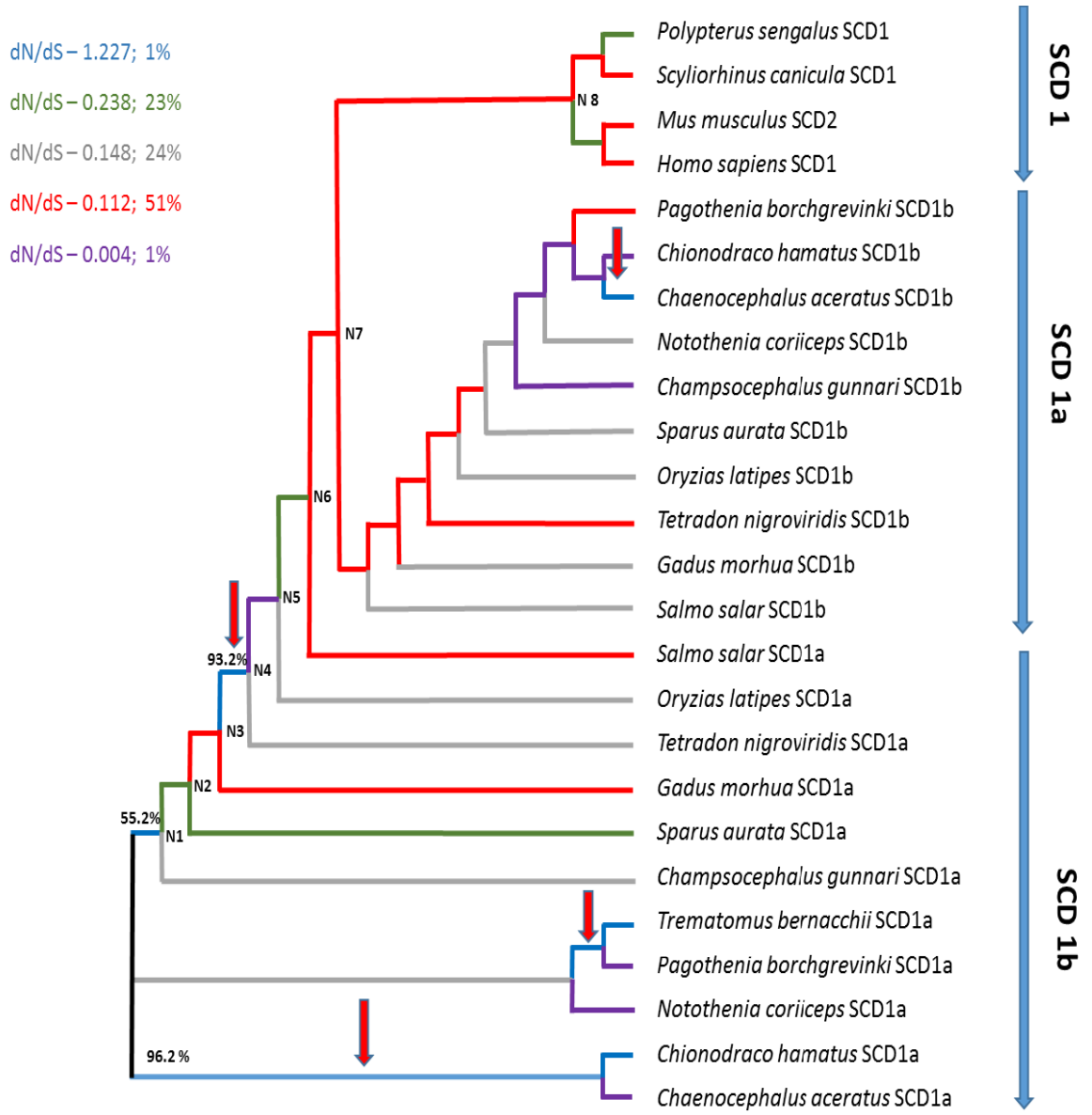


Figure 13 dN/dS ratios inferred using Genetic Algorithm as implemented in Datamonkey, mapped onto branches across a phylogeny of SCD1a and SCD1b sequences. dN/dS ratios are grouped into six discrete classes as shown on the right. Each dN/dS class is represented by a particular colour with the percentage of branches assigned to each class is listed alongside. Red arrows indicate the branches with the highest dN/dS ratios.

2.4 Discussion

This study investigated the evolution of the SCD gene in teleost fish, particularly Antarctic species, using a comparative genomics approach of new partial sequences isolated from Antarctic and non-Antarctic fish species, and existing sequences obtained from databases, comprising a total of 71 sequences. The phylogenetic analysis supports the ancestral duplication of SCD1 into two distinct isoforms; SCD1a and SCD1b. The sequences of each of these isoforms were separately taken for further analysis to determine patterns of thermal adaptation across isoforms and thermal habitat. Amino acid compositions were examined using principal component analysis (PCA) to assess their correlation with thermal habitat. The relationship between codon usage and thermal habitat was also investigated for each isoform. Amino acid replacements specific to Antarctic species, and sites and lineages under positive selection were also identified.

SCD1 gene duplication in teleost fish

In the present study, the SCD1 clade which comprises sequences from humans, mice, coelacanth, amphibians and birds also includes the basal ray-finned fish *Polypterus senegalus* and the cartilaginous fish *Scyliorhinus canicula*, which diverged prior to the third round of genome duplication (3R); therefore these species do not have duplicates (Castro et al., 2011), and these species has a sister-relationship to the other clade, consisting of the SCD1a and SCD1b clades. SCD 1 gene duplication appears to have occurred after the teleost divergence and the SCD1 gene has been expanded into SCD1a and SCD1b duplicates. These duplicates are the result of the third round of whole genome duplication (3R) which took place in teleosts. The analysis of the genome sequence of *Tetraodon nigroviridis* and its comparison with the human genome showed the duplication (3R) occurred after the emergence from mammals (Jaillon et al., 2004). The emergence of these duplicates (3R) occurred after the two rounds of whole genome duplication (1R and 2R) which occurred at the stem of the vertebrate lineage, as shown by the genome sequence of *Branchiostoma floridae,* (Putnam et al., 2008). Further, the syntenic analysis and the phylogenetic analysis by Castro et al agrees with a third round of duplication in teleosts (Castro et al., 2011). A similar phylogenetic analysis supporting the duplication was found by Bendito-Palos et al. (Benedito-Palos et al., 2012). With the limited data set of fish species supporting the duplication of SCD gene (Castro et al., 2011), the present study, with a larger data set including the Antarctic fish species, confirms the SCD gene duplication (Fig 4).

Variation in paralog retention between the orders of fish

In the present study, phylogenetic analysis (Fig. 4) indicates SCD gene duplicates occurring in all orders of fish used in this study except for Cypriniformes, Characiformes and Gonorynchiformes. Fish species, namely *Danio rerio*, *Cyprinus carprio* belonging to order Cypriniformes, *Chanos chanos* (Gonorynchiformes) and *Astyanax mexicanus* (Characiformes), do not have SCD1b paralogs but only have the SCD1a paralog suggesting the loss of SCD1b and retention of only SCD1a isoform in these orders. Also the loss of SCD1b might be due to a disabled pseudogene and not a deletion, which demands further investigation. One of the variations after gene duplication, involve retention of some paralogs and also loss of paralogs. Studies on the retention of paralogs in two superorders of teleosts, Ostariophysi and Acanthopterygii, show that 1.3 % of the teleost-specific gene duplicates (TSGD) gene paralogs are present in genomes of the superorder Acanthopterygii, whereas a single copy of the paralogs was retained in the genomes of the Ostariophysi species *Danio rerio* (Cypriniformes) and *Astyanax mexicanus* (Characiformes) (Garcia de la serrana et al., 2014). In general, the study showed, there was systematic retention of 1-2% of the paralogs across both superorders and these could be functionally significant for the evolution and diversification of particular lineages (Garcia de la serrana et al., 2014). Similarly, one of the duplicate genes of the evolutionarily conserved cortisol stress response genes in zebrafish is lost (Alsop & Vijayan, 2009). Such patterns of retention and loss of duplicate paralogs conforms to the finding for SCD reported here in the present study.

The relevance of the loss of SCD1b and the retention of SCD1a in some orders is further complicated by the observation that *D.rerio* and *A. mexicanus* have duplicates of SCD1a that are localized on different chromosomes. The reason for the loss of duplicates is unclear but it could have occurred after the sub-functionalization of the genes, when the need for the modified function of one duplicate was lost (Garcia de la serrana et al., 2014). This highlights the need for a better understanding of the differing functions of gene duplicates in general, and SCD1a and 1b in particular.

Diversity of SCD gene duplicates

The present phylogenetic analysis (Fig. 4) supports existence of lineage-specific duplicates (LSD) of SCD1a in *Gasterosteus aculeatus* and *Cyprinus carprio*, and SCD1b in *Oreochromis niloticus*. SCD1b appears to be lost from *Cyprinus carprio,* but in *G aculeatus* and *O.niloticus* both duplicates (SCD1a and 1b) are retained and a third copy of SCD1b arising from LSD. The apparent absence of SCD1b in *C. carprio* cannot be confirmed as presently the genome has not been sequenced and additional

sequences do not exist in the databases. However, supporting studies indicate losses of gene duplicates in other cypriniform fish, conforming with our observations (Garcia de la serrana et al., 2014). Other than whole genome duplicates, in which duplicates are localized on separate chromosomes, the lineage-specific duplicates identified in the present study occur on the same chromosome (Fig. 5) and could perhaps be the result of alternate splicing or gene duplication. The specific functions of these SCD duplicates in these species are unknown but in *C. carprio* one duplicate of the SCD1a gene is regulated by temperature while the other isoform is regulated by diet (Polley et al., 2003). Studies are needed to examine whether such regulatory patterns as seen in *Cyprinus carprio* exist across the isoforms of the genome duplicates in other species in order to elucidate their functions.

Presence of SCD1 gene duplicates SCD1a and SCD1b in Antarctic fish

This study is the first to show the presence of both the duplicates SCD1a and SCD1b across the Antarctic notothenioid fish. Previous studies of SCD (Porta et al., 2012) from Antarctic fish (*T. bernacchii and C. hamatus*) found the SCD1a isoform, as both these previously identified sequences occupy the SCD1a clade in the phylogenetic tree (Fig. 4) In addition, the genome sequence of the Antarctic notothenioid *Notothenia coriiceps* possesses both SCD1a and SCD1b duplicates (Shin et al., 2014). In general, gene duplication has played a major role in Antarctic fish influencing their successful establishment in cold conditions. Transcriptome analysis of three Antarctic species revealed the presence of Antarctic-specific duplications in 118 protein gene families and many of the duplicated proteins were upregulated in cold conditions (Chen et al., 2008). SCD1b sequences were successfully obtained from nineteen Antarctic species targeted in the study, SCD1a sequences could only be obtained from six species, mostly in the families Channichthyidae and Nototheniidae. The reasons for this are unclear, but may be due to low expression or loss of this isoform in some species, and requires further investigation in a larger panel of Antarctic fish species. Investigation involving genome sequencing of the fish species and also probing using cDNA libraries could perhaps confirm the presence of SCD1a.

Cold adapted notothenioid fish species are evolutionary distinct with respect to SCD

Phylogenetic analysis reveals that the cold adapted Antarctic and non-Antarctic notothenioid with an Antarctic ancestry (Table 2) form a separate clade for each isoform (Fig. 4), as the clade does not contain any non- notothenioid fish species. This indicates that the isoforms from these two groups are evolutionarily distinct with respect to SCD and also that they have inherited and retained both copies from the ancestral duplication. Non-Antarctic notothenioids which do not have Antarctic ancestry *B. variegatus* and *C.gobio* (family Bovichtidae) are together form the sister taxa of the Notothenidae (Near et al., 2004). This relationship is strongly supported, also more so for SCD1b than SCD1a. This suggests that the gene trees reflect the phylogenetic relationships of these species. Although, the secondarily temperate species *Notothenia angustata* has an Antarctic ancestry and dispersed to temperate waters around 11 Mya (Cheng et al., 2003), and its position in the phylogeny does not match its current biogeography, as it is part of the Antarctic clade for both SCD1a and SCD1b suggesting that the SCD gene tree may reflect phylogeny more than biogeography or habitat, as the genus *Nothothenia* is monophyletic for both isoforms. Similarly, phylogenetic position does not match biogeography occurs in other species of Antarctic ancestry but now dispersed to the non-Antarctic water such as *C. esox* for (SCD1a and SCD1b clade), and *D. elegenoides and P. tesselata* (SCD1b clade only)

Increased basic amino acids in Antarctic SCD1a isoforms and reduction in the hydrophobic amino acids in SCD1b

Whether amino acid usage and thermal habitat are correlated was examined in a previous comparative study of the confamiliar teleost fish species *Pachychara brachycephalum* (Antarctic) and *Zoarces viviparous* (temperate). The authors used transcriptome sequence information to establish general patterns of amino acid usage. Their comparison of the amino acid compositions of the orthologous sequence pairs demonstrated a reduction in the frequency of acidic residues (e.g. glutamic acid) and increase in the frequency of basic residues (e.g. lysine, arginine and histidine), causing reduction in the charges required for stabilization of salt bridges in the proteins (Windisch et al., 2012). The aligns with the trend as SCD1a from Antarctic fish in this study and cold-adapted fish was positively correlated with glutamine and negatively correlated with glutamic acid (Fig. 7). Perhaps the high frequency of basic amino acids, like lysine and the low frequency of acidic amino acids, like glutamic acid, could result in the reduction of salt bridges in SCD1a and confer flexibility. However,

contrary to this, the clustering of the warm-adapted tilapia for SCD1b in the PCA analysis of our present study are correlated with the presence of high glutamine and lysine.

In the present study a strong correlation of SCD1b from Antarctic species and cold adapted non-Antarctic fish species, with basic residues like lysine and histidine and a corresponding inverse correlation with hydrophobic residues, which could result in enhancing flexibility (Fig. 7). Cold-adapted proteins are characterised by the reduction in the core hydrophobicity, as the amino acids deeper in the structure tend to be smaller and less hydrophobic, when compared to mesophilic proteins or warm adapted proteins. Reduced hydrophobicity results in reduction of Van der Waals interactions. This contributes to destabilization of the protein core and also better solvent accessibility (Siddiqui & Cavicchioli, 2006). Overall, this suggests that the composition of the SCD1b isoforms of Antarctic fish could favour reduction in the hydrophobicity, although the localization of these changes in the protein needs investigation through structural modelling.

Amino acid replacements favouring increase in the protein lipid interaction

A positive correlation within SCD1b for valine in the Antarctic fish species and cold adapted non-Antarctic fish species was found (Fig. 7). This is in alignment with a previous study where in the transcriptome of non-notothenioid Antarctic fish were compared to confamiliar temperate fish (Windisch et al., 2012). That study showed that Antarctic fish have increased replacements of valine over alanine when compared to confamiliar temperate fish (Windisch et al., 2012). Such replacements have been suggested to increase protein-lipid interactions due to valine having larger, nonpolar residues than alanine. The present study showed valine is selectively replaced over leucine, within the transmembrane 2 and 3 regions in SCD1b of the Antarctic fish species (Fig. 9 and Table 13). Transmembrane regions are typically rich in hydrophobic residues, but in Antarctic fish species SCD1b there is an amino acid exchange of phenylalanine with valine in transmembrane 3, located besides another existing valine residue, forming valine pairs in the SCD1b of Antarctic fish. The membranes of Antarctic fish have a high proportion of unsaturated fatty acids (Logue et al., 2000) and valine pairs could be an adaptation to allow interaction with unsaturated membrane lipids of the endoplasmic reticulum (Windisch et al., 2012).

AT3 codon usage varies across amino acids in the SCD1b isoform of Antarctic fish when compared to non-Antarctic AT3 codon usage

A strong correlation exists between codon usage and the expression of proteins and this is due to mutation and selection shape codon usage. An analysis of RNA–seq data from two species of arthropods has shown that highly expressed genes have a greater proportion of AT3 codons than less highly expressed genes (Whittle & Extavour, 2015). This has been attributed to these having greater accuracy and efficiency of translation (Whittle & Extavour, 2015). A similar trend of correlation of AT3 codons with high expression has been found in yeast (Lin et al., 2006). There is little information on the correlation of codon usage with temperature in fish. One study has shown increased AT3 codon usage in the Antarctic fish *P. brachycephalum* in comparison to a confamiliar temperate species (Windisch et al., 2012) was attributed to a reduction of proof reading costs, which may be advantageous in energy-constrained cold conditions. Higher preference for AT3 would favour less stabilized transition enzymatic states during translation, preventing kinetic repression of translation. Lowering the GC content in the cold environment also helps to prevent the formation of stabilized mRNA secondary structure ; such secondary structures can inhibit translation (Windisch et al., 2012). This study show a similar trend to Windish's study, with some amino acids in SCD1b (but not in SCD1a) showing a bias for AT3 codons (Figure 8). While for SCD1a, the overall trend across all amino acids is not statistically significant. While asparagine, glycine, proline and tyrosine all show statistically significant AT3 bias in SCD1b, phenylalanine, Isoleucine, and glutamine all show significant GC3 bias in this same isoform, and therefore there is no clear pattern of codon usage bias. AT3 usage is high for tyrosine, which is more common in SCD1b, while AT3 usage is low for less used amino acids like phenylalanine, isoleucine and glutamine. The reason for high AT3 usage for less used amino acids like proline, asparagine and glycine is unclear. Perhaps more proteins other than SCD should be compared among a larger range of notothenioid fish to examine whether this fits with the findings of higher AT3 use seen in non-notothenioid Antarctic fish (Windisch et al., 2012).

Signatures of thermal adaptation in SCD isoforms of Antarctic fish

Amino acid replacements in the loop regions of Antarctic SCD confer flexibility

The present study identified that the majority of the amino replacements in Antarctic fish have occurred in the loop regions (seven out of eleven replacements for SCD1a and three out of six for SCD1b). In general, at low temperatures proteins have evolved structural features which introduce

flexibility (Siddiqui & Cavicchioli, 2006). Proteins of cold-adapted organisms are often modified in regions that are involved in intramolecular flexibility, especially those involved in catalytic activity. These changes change in loop or hinge regions to enhance flexibility. Loops generally connect the secondary structures of the enzymes and they surround the active sites or catalytic sites of the enzymes and add to flexibility (Siddiqui & Cavicchioli, 2006). Other studies investigating adaptive changes in relation to protein structure have identified localization of amino acid replacements in the loop regions, as shown in Sec 61 protein, a transmembrane protein localised in the endoplasmic reticulum that forms a channel in the ER (Romisch et al., 2003). All the secretory proteins enter the secretory pathway through the channel formed by Sec 61 and certain amino acids in the luminal loop are important for translocation of proteins. Amino acids replacements in Sec 61 specific to cold fish were localized in the luminal loop of between transmembrane regions 7 and 8. Furthermore changes in loop regions appear to facilitate the conformational change required for the translocation of proteins at low temperatures (Romisch et al., 2003).

Just as the location of the amino acid replacements are important for thermal adaptation, so too are the types of amino acids that are replaced. The majority of the changes occur at the surface of the proteins where they are exposed to the environment. Such amino acid replacements are associated with the change from the non-polar to polar residues, which interact with the environment (Fields et al., 2015; Siddiqui & Cavicchioli, 2006). For example, lactate dehydrogenase has amino acid substitutions E233M and Q317V (substitutions at codon 233 and 317 are written from cold- to warm-adapted) in Antarctic fish compared to warm adapted fish. Methionine (M) and valine (V) in the warm adapted species were replaced by the polar amino acids glutamic acid (E) and glutamine (Q) in cold-adapted fish (Fields et al., 2015). However, the reverse is true in the mussel species *Mytilus trossulus*, which had a V114N amino replacement with the polar amino acid arginine (N) in the warm-adapted species replaced by non-polar valine (V) in the cold-adapted species. Thus we do not see a common pattern and other factors could also contribute to the type of amino acid replacement. In this study few replacements changed from hydrophobic states in non-Antarctic species to polar amino acids in the cold adapted Nototheniod species and these replacements were seen in SCD1a than in SCD1b (Table 12 and 13). A trend observed in cold-adapted proteins involves increase in polar residues which is attributed to better solvent interaction with the protein by increasing the polarity (Feller & Gerday, 1997; Windisch et al., 2012). However, in the present study changes were observed in both ways and no clear pattern emerges. However, a pattern in the location of the amino acid replacements, especially replacements found in the loop does reflect patterns of thermal adaptation.

Fate of SCD1 gene duplication in Antarctic fish:

SCD1a of Antarctic fish is under positive selection and SCD1b is under purifying selection

The present study identified seven codons in the SCD1a isoform found across the fish species examined in the present study, among these four sites under positive selection and were detected only in Antarctic fish (Table 17, Fig.9), and these sites were replaced in all Antarctic fish, suggesting a role in thermal adaptation. However, the timing of the positive selection is still needed to be determined, and also functional studies are needed to ascertain whether temperature or any other factors associated with narrow temperatures contribute to positive selection. At Codon 203 for the SCD1a, proline is changed to phenylalanine in the icefish, C*. hamatus* (family Channichthyidae) and alanine to proline in other Antarctic fish. Although these changes are localised in the loop regions and are the result of positive selection, proline is a less preferred amino acid in the loop regions of cold adapted proteins when compared to mesophilic ones as introduction of a proline introduces a kink in a protein away from the α helical structures towards the β pleated sheets and reduces conformational flexibility (Siddiqui & Cavicchioli, 2006). It is unclear why proline is present at codon 203 across the Antarctic species (excluding *C. hamatus*), as this contradicts the flexibility hypotheses. In the icefish, with phenylalanine instead of proline, this favours flexibility. Also, it must be noted that this family has further diversified and evolved with additional, more extreme, physiological changes, as they completely lack haemoglobin and myoglobin (Sidell & O'Brien, 2006).

The other sites under positive selection for the SCD1a specific to Antarctic fish were codon 769 and 770, comprising the histidine pair, and present in all eight of the Antarctic species examined in the present study (Fig. 9). Histidine may increase the strength of interactions with water molecules and add to hydrophilicity. Histidine, is a basic amino acid with an imidazole ring and lower temperatures enhance its protonation, thereby strengthening its interaction with the water molecule. Water at low temperatures has a high dielectric constant and more energy is required for release of hydrogen. This is overcome by charged residues like histidine, which help in the solvation of the water molecule and contribute to flexibility (Siddiqui & Cavicchioli, 2006). Added to that, histidine is less substituted in proteins and there are three existing motifs that comprise histidine residues in SCD of both the isoform, shown by red circles in Fig. 9. These residues are conserved across all the taxonomic groups and are the catalytic residues of the enzyme. They form ligands with Zn^{+2} at the centre of activation of O_2 required for the oxidation-reduction reaction (Bai et al., 2015). This additional histidine pair detected in the SCD1a isoform from the present study could perhaps perform additional functional

roles and this could be investigated by examining structure-function relationships to attribute whether it offers catalytic or structural advantage to SCD1a. The other site (codon 875 in SCD1a) under positive selection is localised in the amphipathic helix region and could aid in the reduction of steric hindrance as proline has been replaced by threonine.

Among the four sites detected for positive selection within the SCD1b isoform for all the fish species taken for the analysis, only one site was under positive selection in Antarctic species. The codon change site in the Antarctic species was detected across few Antarctic species, suggesting that these changes could be associated with specific taxa and less to do with thermal adaptation. Following gene duplication, some genes evolve at a rapid rate by various mechanisms and often one of the copies undergoes positive selection (Steinke et al., 2006).This implies that Antarctic fish SCD1a could perhaps be evolving at a rapid rate due to more sites under positive selection than the SCD1b isoform.

Signals of asymmetric rates of SCD evolution

The fates of genome duplications can be divided into (1) sub-functionalization, where the ancestral functions are shared across the duplicates, and (2) neo-functionalization, where one of the duplicates retains the ancestral functions and the other evolves at a rapid rate to take up a new function (asymmetric evolution). This can be represented by the DDC (duplication, degeneration, and complementation) model describing the fate of gene duplications in vertebrates (Force et al., 1999). In general, in Antarctic fish, few sites are under positive selection in SCD1b compared with SCD1a. This could perhaps result from asymmetric rates of molecular evolution. A similar fate for duplicated genes was found in a study examining 2466 genes in four fish species (Steinke et al., 2006). This previous study identified 49 new paralogous pairs in which one was under positive selection and had significantly higher rate of molecular evolution than the other, which retained the ancestral functions and did not have adaptive changes. The results here indicate that SCD1a in Antarctic fish is under positive selection and could perhaps have a higher rate of molecular evolution than SCD1b, shown by the GA analysis (Fig. 13). This analysis showed that dN/dS ratio was higher for SCD1a in the Antarctic species (*P. borchgreviniki* and *C. hamatus)* than for the SCD1b isoform in the same species. Another outcome of faster evolution is an increased probability of the evolution of new functions (Steinke et al., 2006). A previous study of the sodium channel gene, comprising two paralogs, Scn4aa and Scn4ab, (Thompson et al., 2014) showed that there was down-regulation of Scn4aa paralogs in muscle tissue in ancestral lineages of the electric fish and high expression of Scn4ab. Muscle tissues have lost expression of Scn4aa and obtained a new function in the muscle-derived electric tissues of the fish

(Thompson et al., 2014). The expression levels of SCD1a are low, and there is also supporting evidence from other studies for the low expression levels of SCD1a in muscle, brain, liver and white muscle of *Sparus aurata* (Benedito-Palos et al., 2014; Benedito-Palos et al., 2012). Low level expression of SCD1a suggests that the SCD1a paralog is rapidly evolving, indicated by the higher number of sites under positive selection and lower expression of this gene, which could indicate that this paralog is undergoing neo-functionalization and acquiring a new function. A study similar to that conducted on the sodium channel gene needs to be conducted in different species of the Antarctic fish to determine the fate of the SCD1a duplicate.

Another feature displaying diversity among the SCD isoforms was the structural evolution in the SCD1a isoform which did not occur in the SCD1b isoform. The conserved tyrosine residue located in transmembrane 2 of SCD1b isoform is highly conserved across animal SCD1's (Fig. 12). This residue is the critical determinant of the length of the fatty acyl chain which the enzyme desaturates. Presently, the alignments of SCD1a show that the tyrosine is replaced by phenylalanine in the all the fish species taken for the analysis, suggestive that the fatty acyl chain length on which the enzyme acts could change (Fig. 11). A change from the conserved tyrosine to methionine in *Drosophila melanogaster* resulted in the specificity of SCD to fatty acyl chain substrates containing 14 carbons only, whereas the conserved tyrosine residue present in fish SCD1b isoform and the animal SCD1, can only act on (C18:0) fatty acyl chains (Bai et al., 2015).

Conclusion

This study has established a SCD gene duplication has occurred in teleost fish, and that both duplicates are retained in Antarctic fish. Patterns of duplication (ancestral genome-wide duplication and lineage-specific duplication) have been identified across the SCD phylogeny, along with probable losses of duplicates in certain orders of fish. This is the first study to identify SCD gene duplicates in a group of Antarctic fish and that the notothenioid Antarctic fish are evolutionary distinct with respect to SCD. Thermal adaptive signatures were found in the Antarctic fish SCD isoforms. Although, molecular dynamic simulations and functional analysis is still needed to establish the precise nature of the structure-function relationships in Antarctic fish SCD, comparative analysis of patterns of structural changes which confer molecular flexibility in cold temperatures across cold-adapted proteins, reveal that SCD isoforms also exhibit such patterns. SCD has specific amino acid replacements in Antarctic fish and localization of these replacements in the protein, as determined by comparison with the structure of mouse SCD, suggests they also have signals of thermal adaptation

conferring flexibility to function in the constant cold. Sites that are under positive selection in both of the SCD isoforms, with more sites under positive selection in SCD1a than SCD1b specific to Antarctic fish species and hence a higher rate of evolution in SCD1a, suggesting the evolution of additional functions associated with cold tolerance in SCD1a. SCD1a could indeed be evolving new functions, or losing ancestral functions, while SCD1b has likely retained ancestral functions following gene duplication. Sites under positive selection could be potential targets for future mutational studies to establish their functions with respect to cold tolerance. The expression levels of SCD1a are low compared to SCD1b suggesting a signal of asymmetric evolution in the SCD gene, where one paralog evolves at faster rate and has lower expression than the other, which retains the ancestral functions and has higher expression levels. A study of a larger panel of fish species would further confirm such a pattern of evolution.

3 Transcriptional response of SCD to temperature in Antarctic notothenioid fish

3.1 Introduction

Homeoviscous adaptation by membrane remodelling, through changes in lipid saturation levels catalysed by SCD, have enabled Antarctic fish to adapt the frigid conditions of the Southern Ocean (Logue et al., 2000). The role of SCD in thermal tolerance by membrane saturation has been studied in ectotherms, which have a wide thermal adaptable range. Expression of SCD increase with temperature reduction has been observed in temperate fish species (Hsieh & Kuo, 2005 ; Zerai et al., 2010). Currently only one study in common carp has indicated that SCD isoforms are differentially expressed and expression of one isoform is dependent on temperature while the other isoform is regulated by diet (Polley et al., 2003). However, this change involved a lineage specific duplication of the SCD1a isoforms that was unique to the common carp species, shown by SCD gene phylogeny in the present study (Chapter 2, Figure 4) as well as the SCD phylogeny by Castro (Castro et al., 2011) and it is unclear whether the two isoforms of Antarctic fish would behave similarly.

Other than the lineage specific SCD gene duplication in common carp, teleost species have generally lost SCD2 with the duplication of SCD1 into SCD1a and 1b occurring after the teleost divergence (Castro et al., 2011). Gene duplication can lead to partitioning of gene functions and, as they evolve further, there may be a loss or gain of function through changes in gene structure, promoter activity and/or transcript processing (He & Zhang, 2005). The fate of SCD gene duplicates and the consequence(s) for SCD gene function, have yet to be fully studied. There are few studies (Benedito-Palos et al., 2014; Castro et al., 2011) on tissue expression of SCD isoforms in fish species, thus, determination of expression patterns in a large group of fish species is needed to establish the function of the SCD isoforms post duplication.

Expression of SCD isoforms in response to thermal challenge in fish, and especially in Antarctic fish, still need to be understood. In the context of climate change, whereby ocean temperatures rise, adaptation to the narrow ocean temperature range by Antarctic species presents a major challenge which could have resulted in limited capacity to alter SCD expression and membrane lipids in response to changes in temperature. This is in contrast to temperate species which exhibit

temperature- induced modulation of membrane lipids (Cossins et al., 2006; Hsieh et al., 2003; Wang et al., 2014).

Furthermore, SCD is the main target gene in cold tolerance studies where gene expression and cold tolerance across species are studied (Hsieh & Kuo, 2005). These studies aim to improve SCD expression by diet modification and examine SCD expression in genetically improved fish species, mainly to understand SCD gene role in cold tolerance (Hsieh et al., 2007; Ma et al., 2015; Zerai et al., 2010). The differential expression of the two isoforms, SCD1a and 1b, in this context requires investigation. Currently research is in progress to breed fish with specific cold-tolerance traits. A knowledge of the expression of these isoforms in a thermally challenged Antarctic fish would provide new insights for such research.

Higher polyunsaturated fatty acids (PUFAs) within Antarctic fish, concomitant with the high dissolved oxygen content in the frigid waters of the Southern Ocean, makes them more prone than temperate species to oxidative damage and lipid peroxidation resulting from an increased production of reactive oxygen species (ROS) (Crockett, 2008). There is a suite of antioxidant defence mechanisms in place to offset oxidative damage and superoxide dismutase (SOD) is one of the enzymatic antioxidants that scavenge oxygen (Lesser, 2006). Although, upregulation of SOD in the transcriptome of thermally challenged *T. bernacchii* (Huth & Place, 2013) was observed, little information is available on changes in gene expression of SOD at different time points during a thermal challenge.

The research hypothesis in the present study is that the stable cold environment of the Antarctic could have resulted in species with limited adaptive capacity to modulate SCD gene expression in response to temperature change. This research examined SCD and SOD expression in thermally challenged Antarctic fish species to provide information about the acclimatory capacity of Antarctic fish. The tissue expression pattern of SCD isoforms was analysed by end point PCR in Antarctic fish species (*Pagothenia borchgrevinki* and *Trematomus bernacchii*). In addition, the transcriptional response of SCD isoforms (SCD1a and SCD1b) and Cu/Zn SOD 1 in two Antarctic fish species (*T. bernacchii* and *P. borchgrevinki*) subjected to a thermal challenge of 4 °C over a time period of 14 and 28 days, respectively, at various time points was determined. An additional experiment examining challenge at 6 °C over seven days was determined for both species.

3.2 Methods

3.2.1 Experimental fish

The stenothermal fish species used in this study were *Trematomus bernacchii*, collected in the bay off Cape Evans, Antarctica, and *Pagothenia borchgrevinki*, collected from McMurdo Sound, also in Antarctica. Details of methods employed for capture of these fish are described in the Appendix (A.1.1).

3.2.2 Thermal acclimation experimental design and sampling

Pagothenia borchgrevinki and *T. bernacchii* were subjected to a thermal acclimation experiment. Experimental details and sampling methods are described in the Appendix (A.1.2). Sampling comprised isolation of 10 different tissues from the experimental fish. Tissue expression analysis used all the 10 tissues and were the pre-acclimation controls, while transcriptional analysis for thermal acclimation experiment used only liver tissue. Details of the samples taken for the transcriptional response study are given in Table 19. Liver tissue was chosen for the transcriptional as well as the biochemical analysis as this tissue is known to have strong homeoviscous response than other tissues such as brain and muscle (Cossins, 1977). Liver is the major organ for lipogenesis and the temperature specific SCD isoform was isolated from liver (Polley et al., 2003), also liver was more responsive in warm acclimation studies of *Alosa pseudoharengus* resulting in increase of saturated fatty acids and a decline in unsaturated fatty acids (Snyder et al., 2012).

Table 19 Details of the samples taken for the tissue expression analysis at various time points after acclimation at different acclimation temperatures. D: Days after acclimation, T: temperature (0 °C, 4 °C and 6 °C) PB: *Pagothenia borchgrevinki,* TB: *Trematomus bernacchii*

Days after acclimation		Fish species sampled at each acclimation temperature (number of fish)		
		0 °C	4 °C	6 °C
DO0*(Pre-acclimation)	TB/PB(N=2)			
D1		TB/PB(N=3)	TB/PB(N=3)	
D2		TB/PB(N=3)	TB/PB(N=3)	
D3		TB/PB(N=3)	TB/PB(N=3)	
D7		TB/PB(N=3)	TB/PB(N=3)	TB/PB(N=3)
D14		TB/PB(N=3)	TB/PB(N=3)	
D28		PB(N=3)	PB(N=3)	

***DO0 is pre-acclimation control samples which were used for the tissue expression analysis by endpoint PCR.**

3.2.3 RNA isolation and quality check

Total RNA was extracted from snap-frozen tissues harvested during the thermal acclimation experiment as described in section (2.2.3) and quality check of RNA isolated was done as described in section (2.2.4).

3.2.4 cDNA conversion

DNase-treated total RNA (300 ng) quantified using a Qubit® fluorometer was reverse transcribed using the PrimeScript™ RT reagent kit (Perfect Real Time, Takara Bio). The protocol for reverse transcription for the SYBR Green qPCR assay was followed as described in the kit manual. A 10 µl reaction mixture comprising 1X of Prime Script buffer (5X), 0.5 µl Prime Script RT Enzyme Mix I, 25 pmol Oligo dT primer, 50 pmol random hexamers and 6.5 µl total RNA (300 ng) and RNase free nanopure water. The reaction mixture was incubated at 37 °C for 15 min followed by inactivation of the reverse transcriptase enzyme by incubation at 85 °C for 5 seconds and then 4 °C. The cDNA obtained was diluted 20-fold to a total volume of 200 µl using double RNase free nanopure water and the cDNA was stored at -20°C until required for qRT-PCR.

3.2.5 Primer design for the Gene of Interest (GOI)

Design of primers included the following approaches.

3.2.5.1.1 Identification of published coding sequences

The target genes in the study included SCD1a, SCD1b, and SOD. The reference gene was β-actin. Published SCD sequences from fish, human, mouse and chicken with similarity to fish SCD were identified by GenBank BLASTx searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using carp temperature-specific SCD isoform sequences or known SCD sequences from other fish species. Further sequences were also obtained from the ENSEMBL database (http://asia.ensembl.org/index.html) from fish species whose genomes had been sequenced.

3.2.5.1.2 Identification of SCD 1a and 1b Isoforms

A phylogenetic analysis was performed using sequences from NCBI and ENSEMBL databases using the neighbour joining method [MEGA version 5 (Saitou & Nei , 1987)]. Aligned sequences were assigned to either SCD 1a or SCD 1b based on the phylogeny and these sequences were used for primer design.

3.2.5.1.3 Sequence alignment and primer design for SCD

The SCD1a and 1b sequences identified by phylogenetic analysis were used to generate amino acid and nucleotide alignments using ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Conserved regions, especially between fish species, within these alignments were used to design primers for the respective SCD isoforms. SCD1a and SCD1b amino acid sequences were aligned and stretches of sequences unique to the particular isoform were identified. The nucleotides encoding these amino acids were used as sites for primer design. Primer sequences were checked by IDT (https://sg.idtdna.com/calc/analyzer) for the ideal parameters required for primer design and the primers were synthesised from Integrated DNA Technologies (IDT), Singapore. Primers had previously already been designed for β-actin and β-tubulin by Anthony Van Rooyen and Dr Victoria Metcalf. Primers were designed for SOD by using the partial coding sequence of *Trematomus bernacchii* Cu/Zn superoxide dismutase (SOD1) mRNA, Sequence ID: **AY736280.1.** Primers details are given in Table 20.

Table 20 Details of the primers used for the generation of the specific gene products and the construction of gene-specific plasmids.

Gene	Forward primer(5'-3')	Reverse primer(5'-3')	Product size (bp)
SCD1a	CGGAGGCGTTGGAGAAGAAG	AGTCACAGATCCTACAAGGCCT	309
SCD1b	TCGACGGTGGAGGATGTTTTTG	GGAACGTGTGATGGTAGTTGTG	768
SOD	TAGCGGGACTGTCTTCTTCG	TCCACCAGCATTGCCTGTC	384
β-actin	CCACTGGTATYGTCATGGACTCC	CCTTCTGCATCCTGTCRGCGATG	504
β-tubulin	ATCATGAACACYTTCAGCGT	TCCTGGTACTGCTGGTACTC	794

3.2.6 Cloning

All PCR products were generated using the primers listed in Table 20, following the procedures given in (2.2.5). The cDNA template required for the amplification of the gene products was isolated from *T. bernacchii* liver tissue. The amplified gene products were gel purified using the Axygen® AxyPrep™ DNA Gel Extraction Kit according to the manufacturer's instructions and then cloned into the TOPO TA vector using the TOPO®-TA Cloning Kit, Thermo Fisher Scientific. The cloning reaction comprised 0.5 - 4 µl of gel purified PCR product, 1 µl salt solution, 1 µl TOP vector, and nano pure water added to make the final volume up to 6 µl. The components were mixed gently and incubated at room temperature for 5 min before being taken for transformation. Two µl of the reaction mix were added to One Shot TOP 10 chemical competent cells supplied with the kit. The reaction was incubated on ice for 5 min followed by a heat shock at 42 °C, without shaking, for 30 seconds. The reaction mixture was immediately transferred to ice and 250 µl of super optimal broth with catabolite repression (SOC) Medium, provided in the kit, were added and incubated at 37 °C for 1 hour with horizontal shaking at 200 rpm. Following transformation, 50 µl of transformed cells were spread onto LB agar + 50 µg/ml of Kanamycin plates. The plates were incubated at 37 °C overnight and the colonies formed were taken for screening.

3.2.7 Clone confirmation by colony PCR and sequencing

Individual colonies obtained after cloning were inoculated in 100 µl of LB broth and incubated in a shaker incubator at 37 °C for four hours. Four colonies were inoculated and these were confirmed positive for cloning by colony PCR. Colony PCR comprised PCR with gene-specific primers and 2 µl of the colony culture, which was added as a template. The PCR and the cycling parameters are described in section (2.2.5). Colony PCR products were analysed on 1.0% agarose gels and confirmed by the presence of the expected product size. Positive colonies were further inoculated for plasmid isolation in 5 ml of LB broth + 50 ug/ml of kanamycin and kept overnight in a shaking incubator at 37 °C. Plasmids were isolated from the overnight colony culture, using the Axygen® AxyPrep™ Plasmid Miniprep Kit, as per the protocol provided in the kit. Plasmids were sequenced using M13 Primers on an ABI Prism 3130 sequencer. Complete clone insert sequences were identified using the Blastn search algorithm on the NCBI GenBank website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their identity.

3.2.8 Tissue expression of SCD1a and SCD1b by end point PCR

Analysis of the expression of the SCD isoforms and the reference gene β-actin in the tissues of *P. borchgrevinki* and *T. bernacchii* was performed by end-point PCR. Two biological replicates of each species were taken for the analysis. Details of the samples used are provided in Table 19. RNA was extracted from the tissues as described in section 2.2.3 and then treated with DNase. A quality check was then performed as described in section 2.2.4 and the RNA was converted into cDNA as described in section 2.2.5.

After cDNA conversion, expression of SCD1a and SCD1b isoforms in the respective tissues was checked by end-point RT-PCR after 30 cycles to prevent plateau formation and to determine the detectable limits. The details of the PCR and the cycling parameters are provided in section (2.2.5). The details of the primers used are given in Table 20. PCR products were run on 1.5% agarose gels stained with ethidium bromide.

3.2.9 Quantitative real-time PCR

3.2.9.1 RT-qPCR primer design

The amplification products obtained for the target and reference genes were used to design qPCR primers. The primer sequences and product sizes generated by each qPCR primer pair are given in Table 21. In case of SCD1a and 1b, and SOD, the forward primers used for obtaining the partial sequence were retained as forward primers for qPCR, as they were specific for the isoform. Reverse primers were designed to ensure the minimum size of the gene product. DNAMAN Version 7.0 and IDT (https://sg.idtdna.com/calc/analyzer) were used to assess the suitability of the primer pairs. For the reference genes, primers were designed using primer 3 plus (http://biotools.umassmed.edu/cgi-bin/primer3plus/primer3plus.cgi).

Table 21 RT-qPCR primers for target and reference gene used for the transcriptional response study.

Gene	Forward primer(5'-3')	Reverse primer(5'-3')	Product size (bp)
SCD1a	CGGAGGCGTTGGAGAAGAAG	CGGCTCCTATATGCAACAGAGTCA	178
SCD1b	TCGACGGTGGAGGATGTTTTTG	GAGCACTGATGAGATAGCACACT	195
SOD	TAGCGGGACTGTCTTCTTCG	TGTTGTGGGGATTGAAGTGA	164
β-actin	ATCACCATCGGAACGAGAG	ATTCCGCAAGACTCCATACC	98

Primers were obtained from Integrated DNA technologies (IDT), Singapore.

3.2.9.2 Template for standard curve generation in RT-qPCR

Plasmids containing the target and reference gene inserts were linearized by restriction digestion with the restriction endonuclease ScaI (New England Biolabs). The reaction mixture comprised 10 U of *Sca*I and 1.5 µg of plasmid in a reaction mixture of 75 µl incubated at 37 °C for 6 hours. Following digestion, the samples were analysed for linearization by electrophoresis on a 1% agarose gel and comparing with the undigested plasmid. Linearized plasmids were gel purified using the Axygen® AxyPrep™ DNA Gel Extraction kit as per the manufacturer's protocol. Following gel purification, the linearized plasmids were quantified using a DeNovix Ds-11+ Nanodrop spectrophotometer and run on a 1.0 % agarose gel to compare with known quantities present in a standard marker (Hyper ladder 1Kb, Bioline). Linearized plasmid stocks of 1 ng/µLwere prepared and stored in -20 °C.

3.2.9.3 RT-qPCR primer efficiency test

Primer pair efficiency for the target and reference genes was determined by preparing standard curves for each primer pair. The templates were prepared as given in section 3.2.9.2 and diluted to 1 ng/µL. Tenfold serial dilutions of each diluted template were prepared using the Eppendorf epMotion 5070 robot, Eppendorf epBlue software and molecular grade water. A five point standard curve was generated using the dilutions 1 x 10^{-2} ng/µL, 1 x 10^{-4} ng/µL, 1 x 10^{-5} ng/µL, 1 x 10^{-6} ng/µL and 1 x 10^{-7} ng/µL. Standards were loaded onto a 48 well plate using the Eppendorf epMotion 5070 robot. Standards were run in triplicate with a positive control and a non-template control to detect the presence of any contamination. Each RT-qPCR mixture comprised 5 µL SYBR® *Premix Ex Taq* ™ II (Til RNaseH Plus), TaKaRa, RT-qPCR primers (0.2 µM), 4 µL of each serial dilution template and molecular grade water to make up the volume (10 µL). qRT-PCR was performed using an Illumina® Eco™ Real Time thermal cycler with RT-qPCR conditions of 95°C for 5 seconds, (95°C for 30 seconds, 60 °C for 30 seconds) x 40 cycles, 95°C for 15 seconds and 30°C for 15 seconds. The data were collected using Eco Real Time Version 5.0 and analysed using Eco study software Version 5.0. For SCD1a and 1b the annealing temperature was raised to 62 °C to increase the efficiency of the amplification, while for β-action and SOD annealing temperature was 60 °C. Eco study software Version 5.0 generated data on the efficiencies of the primer pairs and the melt curves. Comparisons were made with the melt curve of the standards to determine whether the correct product had been amplified. To ensure the specificity of the products, gene products obtained from the qPCR primers were analysed on a 3.5% agarose gel with Hyper ladder V (Bioline) and the qPCR reaction mix of the amplified gene product

was subjected to PCR clean-up and confirmed by sequencing using an ABI Prism 3130 sequencer. The identities of sequences obtained were confirmed by BLASTn.

3.2.9.4 Reference gene for the RT-qPCR

The reference gene for this study was β-actin and this gene has previously been validated and used for gene expression studies in marine species (Benedito-Palos et al., 2012) and in Antarctic fish (Ghosh et al., 2013) for studying gene expression related to environmental toxicology. To determine the homogeneity of variation between the thermal acclimation treatments for β -actin, Levene's test was performed on the variability within the data-set. The Cq values obtained for β -actin across all the treatments were converted to copy numbers and the variation across the dataset was determined at $P<0.05$.

3.2.9.5 RT-qPCR of Thermal acclimation experiments

An Illumina® Eco™ Real Time thermal cycler was used to study the relative expression of SCD1 isoforms and SOD in thermally acclimated liver tissue of two Antarctic fish species, *Trematomus bernacchii* and *Pagothenia borchgrevinki*. The data were collected using Eco Real Time Version 5.0 and analysed using Eco study software Version 5.0. The analysis comprised 3 biological replicates of cDNA samples diluted to 1.5 ng/µL. Each biological replicate was run in triplicate for each gene of interest (GOI) and the reference gene. Standards were prepared for the analysis of each GOI and the reference gene. These were run in triplicate and were prepared using dilutions 1×10^{-2} ng/µL, 1×10^{-4} ng/µL, 1×10^{-5} ng/µL, 1×10^{-6} ng/µL and 1×10^{-7} ng/µL. Plate controls in triplicate comprised linearized plasmid as a template of that respective gene and the corresponding primer pair without a template as a non-template control. Plate controls were a quality check across the plates, and a large variation in the cycle threshold (Ct) values across the plates would have indicated any technical problems. The RT-qPCR reaction and the cycling parameters are similar to the one provided in the primer efficiency test given above (3.2.9.3). The data obtained by the Eco Real Time Version 5.0 were analysed by Eco study software Version 5.0 and imported into Microsoft Excel, and quantified using two standard curves. Gene expression data obtained as mass (ng) were converted to copy number using the relation as given below:

Copy number = (mass (g) × Avogadro's number) / (molecular mass of base × template length).

Gene expression was analysed by relative quantification, which was the gene expression in each sample relative to the reference gene. Standard curves were constructed for each GOI and the reference gene. All experimental sample expression rates for each GOI and the reference gene were

quantified from the corresponding standard curve. Data was normalised against the expression of the reference gene β–actin (expressed as a ratio) to compensate for any differences in loading or reverse transcriptase efficiency. The gene copy number was expressed as the target gene copy number normalized over 10^3 β-actin copies.

3.2.9.6 Amplicon quality check by AGE analysis and sequencing

Technical replicates from one biological sample of *T. bernacchii* SCD1a and 1b amplicons were pooled separately following qPCR analysis. The pooled samples were subjected to PCR clean-up and 8 µl of sample was run on a 3.5 % AGE and sequenced on an ABI Prism 3130 sequencer. Sequences obtained were BLASTn searched against GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to confirm their identity.

3.2.9.7 Statistical analysis

Levene's test (Levene, 1960) was used to validate the reference gene for homogeneity in variation across treatment and time. This test was done for the mRNA copy number of β–actin obtained for both species across all time points and temperatures (control as well as acclimated temperatures). The P values for β–actin for both the species were >0.05 indicating that the variation between the treatments was non-significant. Gene expression results obtained as copy number and normalized to 10^3 β–actin copies were also log-transformed to achieve normality and the data was checked for normality. Normalized data were analysed using two way ANOVA to analyse the difference between the temperature (0 and 4 °C) and the interaction between temperature and days of acclimation (1, 2, 3, 7 and 14 or 28 days for *T. bernacchii* and *P.borchgrevinki* respectively. Holm-Sidak was used as a post hoc test (Holm, 1979) to determine which treatments differed from one another. Unpaired t test was used to analyse the significance of SCD1b expression at 6 °C. Statistical analysis was performed using Minitab 17 v17.2.1 (Minitab Pty Ltd, Sydney) software. .

3.3 Results

3.3.1 Tissue expression of SCD1a and SCD1b in *T. bernacchii* and *P. borchgrevinki* by end point PCR.

The tissue expression of SCD isoforms in *T. bernacchii* and *P. borchgrevinki* determined by end point PCR is presented in Figure 14. In *T. bernacchii*, the expression of both SCD1a and SCD1b is observed in all of the 10 tissues analysed (Figure 14A). This was confirmed in a second biological replicate (data not shown). In *P. borchgrevinki*, there was ubiquitous tissue expression for the SCD1b isoform, but

for SCD1a only a very small amount of expression was detected in brain, spleen, white muscle and heart. The other tissues had no detectable expression of SCD1a (Figure 14B). The same expression profiles for SCD1a and SCD1b were observed in the second independent biological replicate of *P. borchgrevinki.*

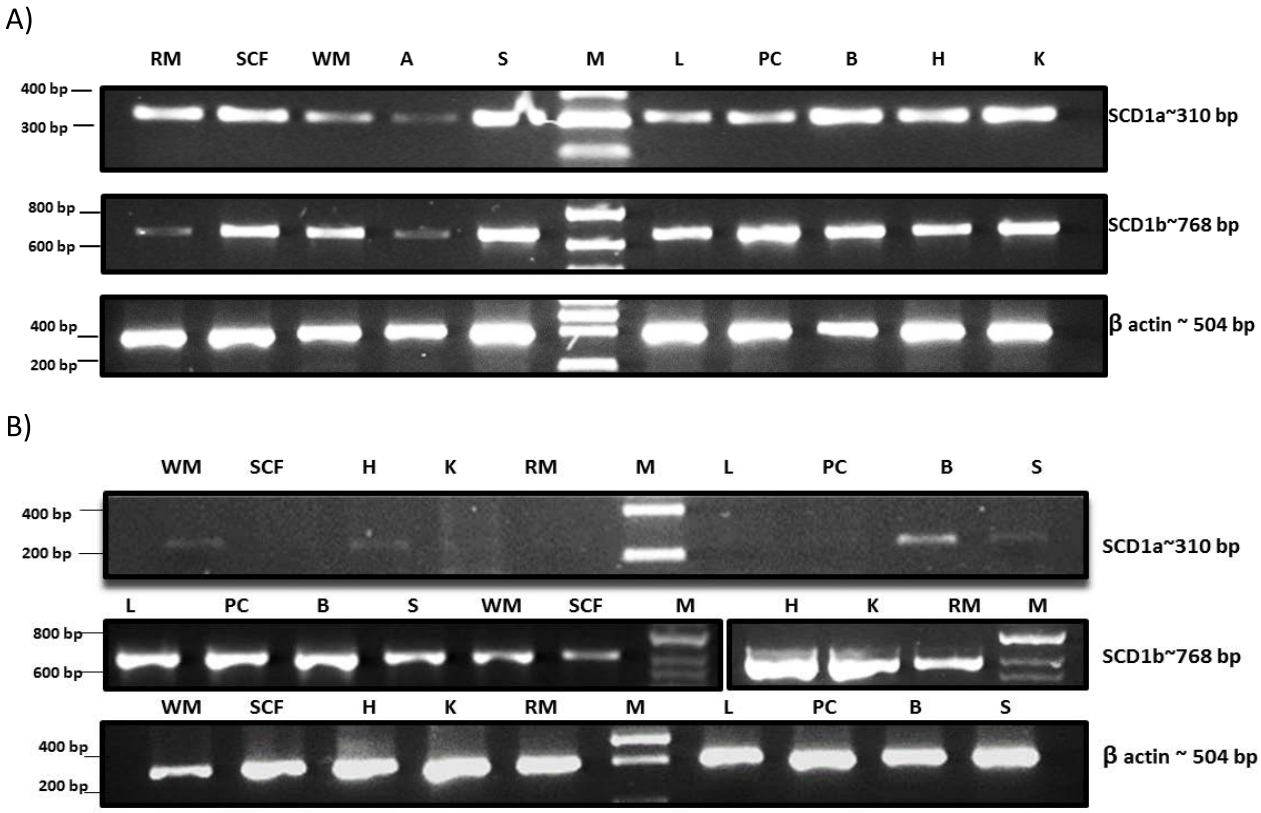


Figure 14 Analysis of SCD isoform expression along with β actin determined by end-point PCR in tissues of Antarctic fish using 1.5% agarose gel electrophoresis. A: *Trematomus bernacchii, B: Pagothenia borchgrevinki.* Five µL of RT-PCR sample was loaded for analysis. (L: Liver, PC: Pyloric caeca, B: Brain, H: Heart, K, Kidney, RM: Red muscle, WM: White muscle, S: Spleen, SCF: Subcutaneous fat, A: Adipose) M: Hyper Ladder I (Bioline), Hyper Ladder II only for SCD1b (Figure 14B)

3.3.1.1.1 Primer efficiency

Melt curve analysis for the target genes (SCD1a, SCD1b and Cu/Zn SOD) and the reference gene all produced a single peak and any sample that showed a deviation from this was not used for analysis. The efficiency of the amplifications is given in Table 22. The efficiency of the PCR ranged from 88-102% and the R^2 coefficient of the assay was >0.99 for all the assays.

Table 22 Details of the efficiency (%) of the PCR, equation for each assay and the R^2 coefficient of the assay. Species TB: *Trematomus bernacchii* and PB: *Pagothenia borchgrevinki* are presented in the parentheses.

Assay Name	Efficiency (%)	Equation	R^2
SCD1b (TB)	92.33	y=-3.52x+6.25	0.998969
SCD1b (PB)	89.97	y=-3.59x+8.51	0.996092
SCD1a (TB)	88.90	y=-3.62x+6.04	0.995212
SOD (PB)	98.46	y=-3.36x+6.91	0.998214
SOD (TB)	98.14	y=-3.37x+6.84	0.998503
β-actin (TB)	102.65	y=-3.26x+6.04	0.999258
β-actin (PB)	102.36	y=-3.27x+5.44	0.999628

3.3.1.1.2 Quality check of amplicons

The molecular weights of *T. bernacchii* SCD1a and 1b amplified by qPCR are presented in Figure 15. SCD1a and 1b PCR products were 178 bp and 195 bp respectively (Figure 15). Blastn search results are presented in Tables 23 and 24. SCD1a was identical to the database *T. bernacchii* sequence with 100% identity, while SCD1b was highly similar to the Antarctic species *Notothenia coriiceps* (97%).

Table 23 Results of the BLASTn search with SCD1a (*T. bernacchii)* as the query sequence of (147bp)

Description	Max score	Total score	Query cover	E value	Identity	Accession
Trematomus bernacchii	267	267	97%	6.00E-68	100%	FJ177513.1
Notothenia coriiceps	261	261	97%	3.00E-66	99%	XM_010780865.1

Table 24 Results of the BLASTn search with SCD1b (*T. bernacchii*) as the query sequence of (160 bp)

Description	Max score	Total score	Query cover	E value	Identity	Accession
Notothenia coriiceps	244	244	90%	3.00E-61	97%	XM_010769852.1
Dicentrarchus labrax	159	159	81%	1.00E-35	89%	FN868643.1

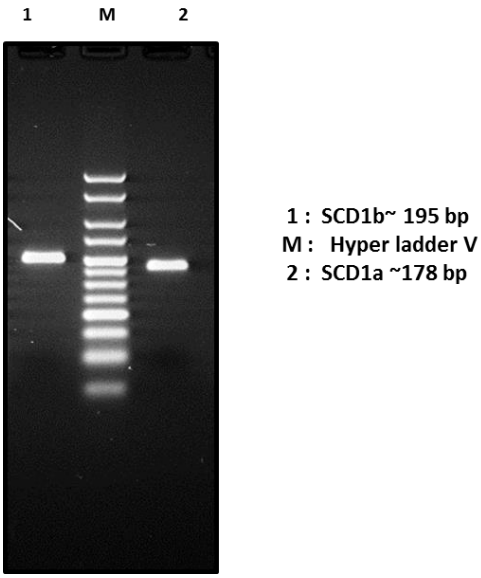


Figure 15 Analysis of SCD1b and SCD1a amplified by qPCR from an experimental sample of *T. bernacchii* liver. 8 µl of the sample was run on a 3.5% agarose gel.

3.3.2 Quantification of SCD1b gene expression in thermally acclimated Antarctic fish

The effect of thermal acclimation on the relative SCD1b gene copy number in liver of *T. bernacchii* at 1, 2, 3, 7 and 14 days is presented in Figure 16. Temperature had a significant effect on the overall SCD1b gene copy number over the 14 day period, with thermal acclimation at 4 °C resulting in a significantly higher gene copy number compared to the control treatment of 0 °C ($P<0.05$; Table 25). The effect of time as well as interaction of time and temperature were statistically non-significant ($P>0.05$; Table 25), although a numerical trend ($P=0.583$) for an increased SCD1b gene copy number in the acclimation treatment on day 1 followed by a decrease in the gene copy number for both treatments over the 14 day acclimation period was observed.

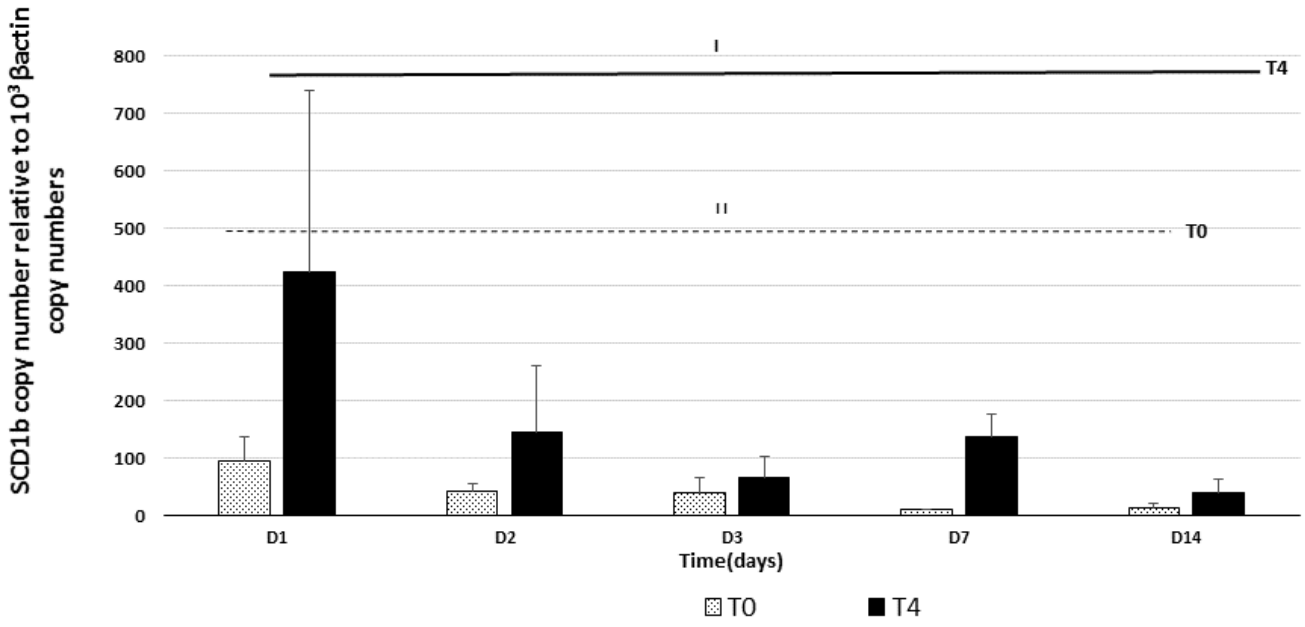


Figure 16 Effect of thermal acclimation on SCD1b gene expression relative to β actin in the liver of *Trematomus bernacchii* at the control temperature (0 °C, T0) and acclimated temperature (4 °C, T4). Control (n=3) and acclimated (n=3) fish were sampled at 1, 2, 3, 7 and 14 days after thermal acclimation. Error bars indicate the standard error of mean. Different Roman numerals indicate a significant temperature effect as determined by Holm-Sidak post hoc test after ANOVA (P<0.05).

Table 25 Analysis of Variance for the effect of thermal acclimation on SCD1b gene expression relative to β actin in the liver of *Trematomus bernacchii,* Adj SS: Adjusted sum of squares, Adj MS: Adjusted mean squares

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature (°C)	1	5.125	5.1255	7.21	0.014
Time (day)	4	5.071	1.2677	1.78	0.172
Temperature (°C)*Time (day)	4	2.070	0.5175	0.73	0.583
Error	20	14.209	0.7105		
Total	29	26.476			

The effect of thermal acclimation on the relative SCD1b gene copy number in the liver of *P. borchgrevinki* is presented in Figure 17. The effect of temperature, time and the interaction between temperature and days of acclimation was significant, although the interaction was significant (P=0.019; Table 26) but the post hoc test after ANOVA was non-significant. A trend of numerical increase in SCD1b gene copy number with temperature acclimation at 4 °C at day 1 and day 2 followed by a decline in SCD1b expression from day 3 onwards was observed. At the end of 28days of thermal

acclimation, SCD1b expression for both the treatment and control had dropped to minimal expression levels compared to day 2 (Figure 17).

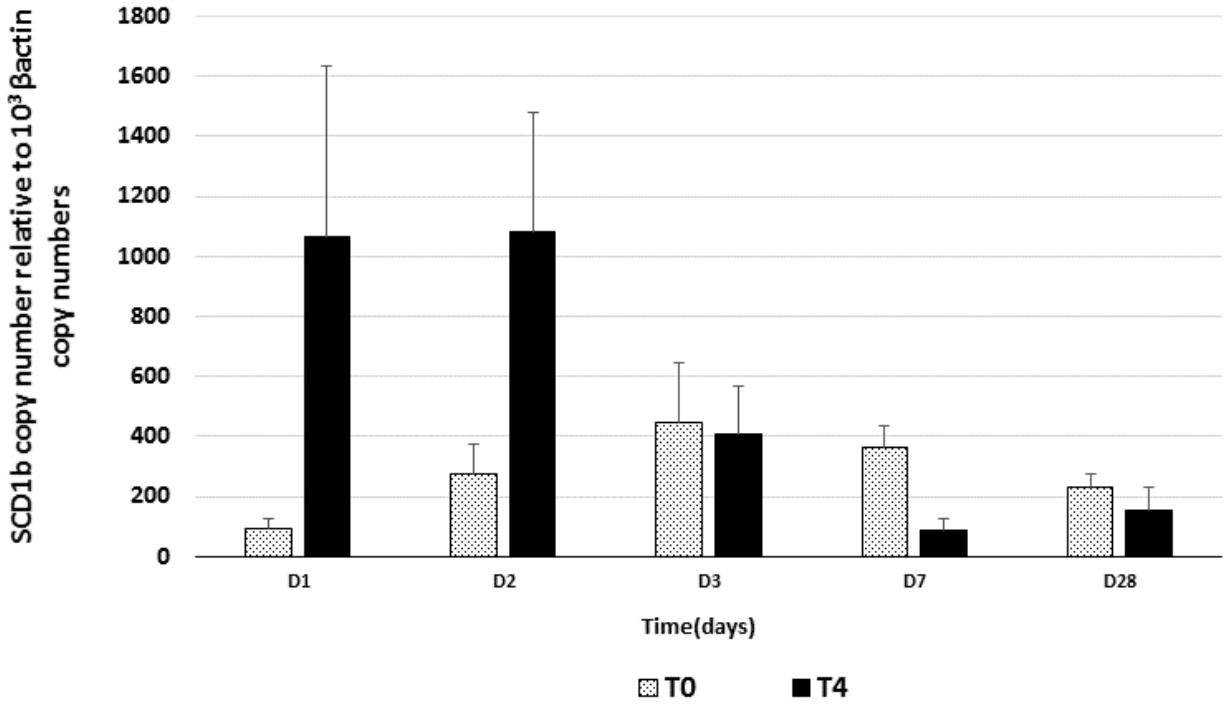


Figure 17 Effect of thermal acclimation on SCD1b gene expression relative to β actin in the liver of *Pagothenia borchgrevinki* at the control temperature (0 °C, T0) and acclimated temperature (4 °C, T4). Control (n=3) and acclimated (n=3) fish were sampled after 1, 2, 3, 7 and 28 days of thermal acclimation. Error bars indicate the standard error of mean.

Table 26 Analysis of Variance of effect of thermal acclimation on SCD1b gene expression relative to β actin in the liver of *Pagothenia borchgrevinki,* Adj SS: Adjusted sum of squares, Adj MS: Adjusted mean squares

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature (°C)	1	0.3939	0.3939	0.63	0.438
Time (day)	4	3.4210	0.8552	1.36	0.283
Temperature (°C)*Time (day)	4	9.5028	2.3757	3.78	0.019
Error	20	12.5696	0.6285		
Total	29	25.8874			

The effect of thermal acclimation at 6 °C**,** on the relative SCD1b gene copy number in the liver of *P. borchgrevinki* and *T. bernacchii* after 7 days of acclimation is presented in Figure 18. Samples for this experiment were only collected at day 7. The effect of temperature was not statistically significant in both the species (P>0.05).

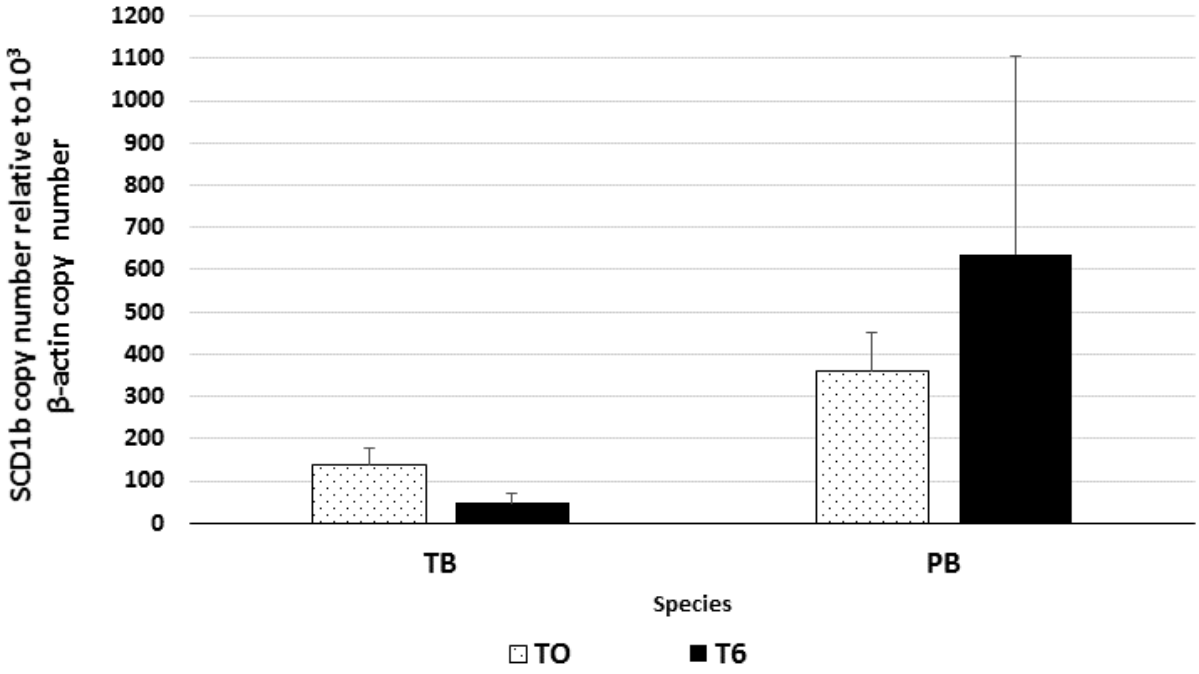


Figure 18 Effect of thermal acclimation on SCD1b gene expression relative to β actin in the liver of *Trematomus bernacchii* and *Pagothenia borchgrevinki* at the control temperature (0 °C, T0) and acclimated temperature (6 °C, T6). Control (n=3) and acclimated (n=3) fish were sampled after 7 days of thermal acclimation. Error bars indicate the standard error of mean. Significance at (P<0.05) determined by an unpaired t test.

3.3.3 Quantification of SCD1a gene expression in thermally acclimated Antarctic fish

The effect of thermal acclimation on the relative SCD1a gene copy number in the liver of *T. bernacchii* is presented in Figure 19. Thermal acclimation at 4 °C resulted in a significant increase in the overall expression of SCD1a, which was significant over the control treatments (P=0.015; Table 27). The effect of days of acclimation was significant with higher gene expression on day 1 and lower gene expression on day 2 and 7 respectively (P=0.003; Table 27) (Figure 19). Interaction between temperature and days of acclimation were not significant (P=0.784; Table 28).

Expression levels of SCD1a isoform were low and as they were not within the limit of detection by qPCR in the cryopelagic species *P. borchgrevinki* and hence was not taken for analysis, this species was not examined within this particular analysis.

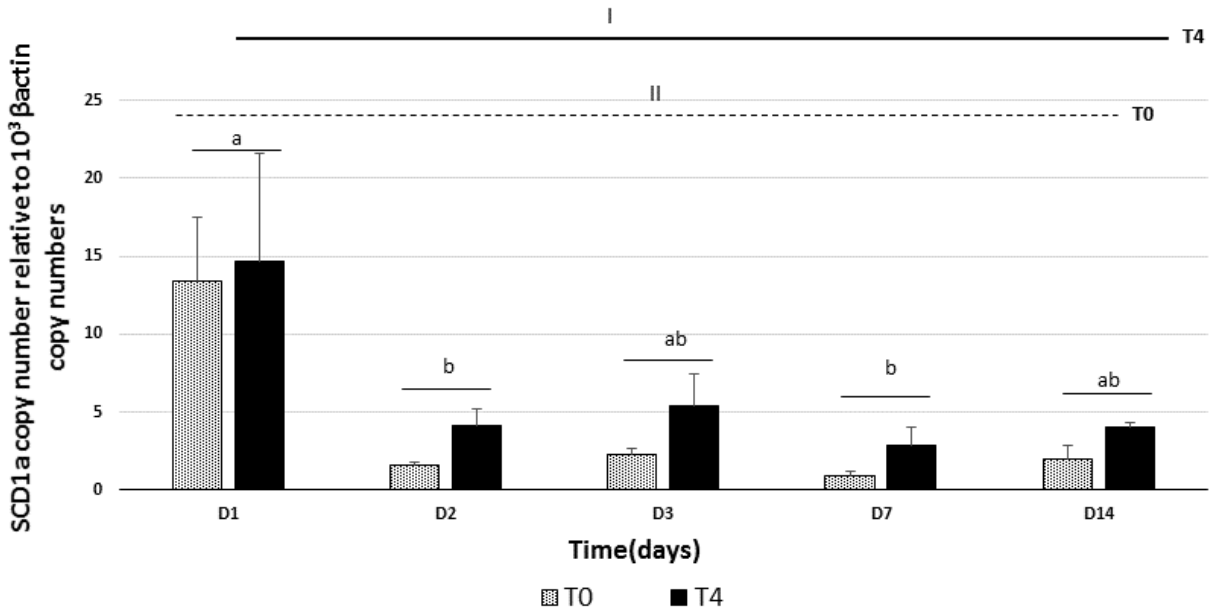


Figure 19 Effect of thermal acclimation on SCD1a gene expression relative to β actin in the liver of *Trematomus bernacchii* at the control temperature (0 °C, T0) and acclimated temperature (4 °C, T4). Control (n=3) and acclimated (n=3) fish were sampled after 1, 2, 3, 7 and 14 days of thermal acclimation. Error bars indicate the standard error of mean. Dissimilar Roman numerals indicate a temperature effect and different letters indicate a time (days) effect as determined Holm-Sidak post hoc test after ANOVA (P<0.05).

Table 27 Analysis of Variance of effect of thermal acclimation on SCD1a gene expression relative to β actin in the liver of *Trematomus bernacchii.* Adj SS: Adjusted sum of squares, Adj MS: Adjusted mean squares

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature (°C)	1	3.8762	3.8762	7.07	0.015
Time (day)	4	12.2346	3.0587	5.58	0.003
Temperature (°C)*Time (day)	4	0.9477	0.236	0.43	0.784
Error	20	10.9591	0.5480		
Total	29	28.0175			

3.3.4 Quantification of SOD gene expression in thermally acclimated Antarctic fish

The effect of temperature on the relative expression of SOD in the liver of *T. bernacchii* is presented in Figure 20. Temperature, time and effect of temperature and time did not result in a significant

change in expression of SOD (P>0.05; Table 28). A trend (P=0.058) of numerical decline in gene expression over the days of acclimation is observed, with higher levels of SOD expression at day 2 and lower levels at day 14.

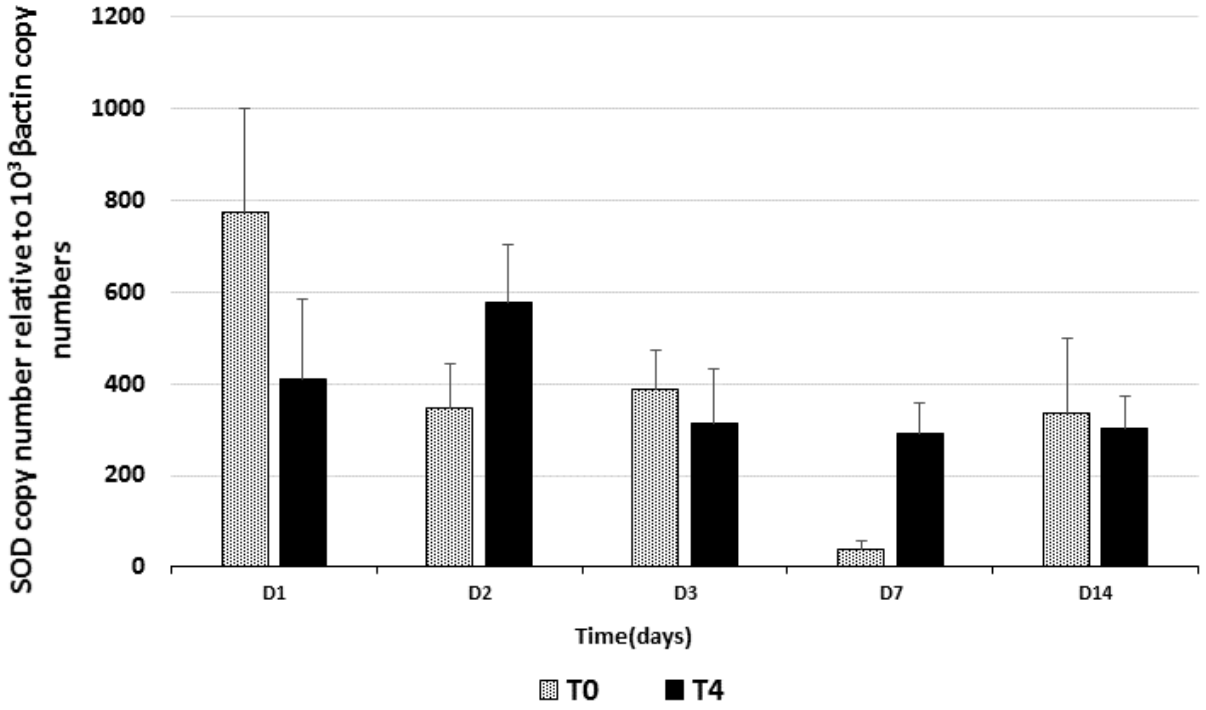


Figure 20 Effect of thermal acclimation on SOD gene expression relative to β actin in the liver of *Trematomus bernacchii* at the control temperature (0 °C, T0) and acclimated temperature (4 °C, T4). Control (n=3) and acclimated (n=3) fish were sampled after 1, 2, 3, 7 and 14 days of thermal acclimation. Error bars indicate the standard error of mean.

Table 28 Analysis of Variance of effect of thermal acclimation on SOD gene expression relative to β actin in the liver of *Trematomus bernacchii.* Adj SS: Adjusted sum of squares, Adj MS: Adjusted mean squares

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature (°C)	1	0.1856	0.1856	0.25	0.624
Time (day)	4	8.1588	2.0397	2.73	0.058
Temperature (°C)*Time (day)	4	5.5052	1.3763	1.84	0.161
Error	20	14.9692	0.7485		
Total	29	28.8188			

The effect of thermal acclimation on SOD expression in the liver of *P. borchgrevinki* is presented in Figure 21, temperature, time and, interaction of time and temperature were statistically non-significant (P>0.05; Table 29). A trend of numerical increase in gene expression in acclimation treatment was observed, with higher gene expression on day 3 and lower gene expression on day 28, while the control treatment changed little over the first four time points, with a numerical increase on day 28.

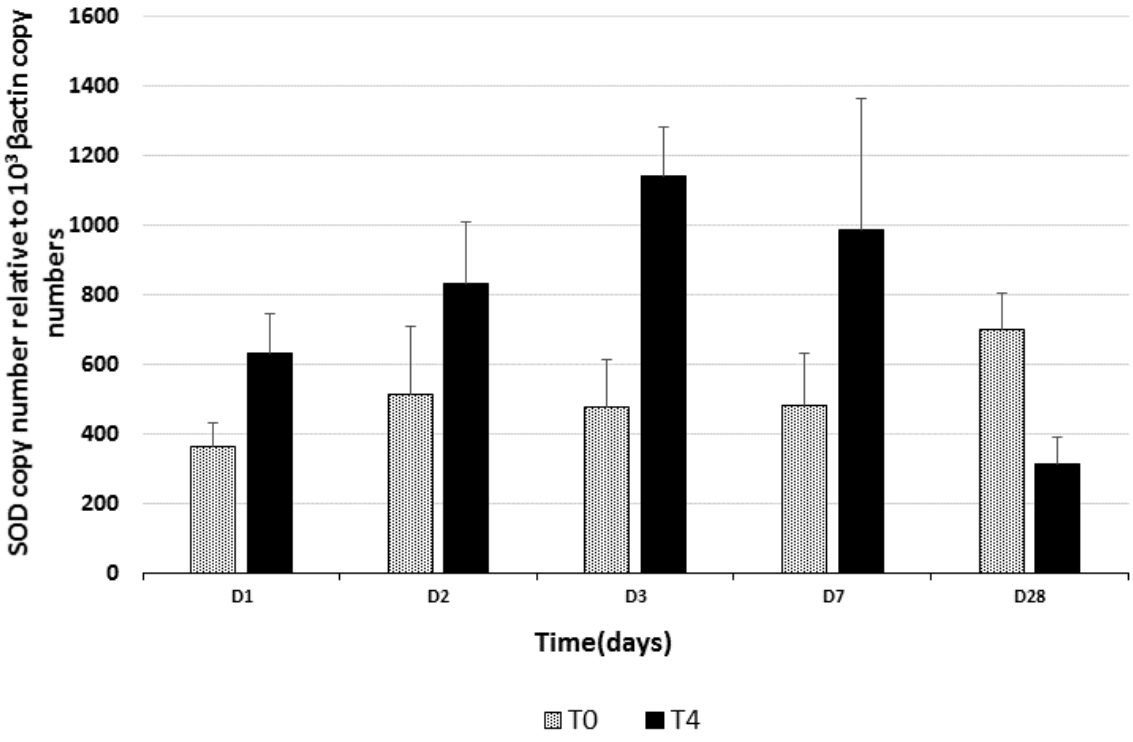


Figure 21. Effect of thermal acclimation on SOD gene expression relative to β actin in the liver of *Pagothenia borchgrevinki* at the control temperature (0 °C, T0) and acclimated temperature (4 °C, T4). Control (n=3) and acclimated (n=3) fish were sampled after 1, 2, 3, 7 and 28 days of thermal acclimation. Error bars indicate the standard error of mean.

Table 29. Analysis of Variance of effect of thermal acclimation on SOD gene expression relative to β actin in the liver of *Pagothenia borchgrevinki.* Adj SS: Adjusted sum of squares, Adj MS: Adjusted mean squares

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature (°C)	1	2.535	2.5349	3.34	0.083
Time (day)	4	1.347	0.3368	0.44	0.776
Temperature (°C)*Time (day)	4	6.867	1.7167	2.26	0.099
Error	20	15.180	0.7590		
Total	29	25.929			

3.4 Discussion

The aims of this study were to (1) analyse the tissue expression of the SCD isoforms in Antarctic fish species by end point RT-PCR and (2) investigate the transcriptional response of SCD isoforms and the antioxidant gene Cu/Zn SOD when subjected to thermal acclimation at 4 °C over a period of 14 days and 28 days, and 6 °C at 7 days in the liver tissue of these species. This is the first study to examine the transcriptional responses of SCD isoforms in Antarctic fish subjected to thermal stress.

Key results

1. The SCD1b isoform had ubiquitous expression in the tissues analysed in both species. SCD1a had ubiquitous expression in the benthic species *T. bernacchii,* while the pelagic, *P. borchgrevinki* species showed minimal expression of SCD1a in only a few tissues (brain, spleen, white muscle and heart) and a complete absence of expression in the other tissues.

2. Temperature acclimation at 4 °C had a significant overall effect on the expression of SCD1a and 1b in the benthic species *T. bernacchii*, but this effect was not observed at individual time points over the acclimation period. Expression of SCD1a was not observed in the pelagic species *P. borchgrevinki* as the expression levels were not in the detectable range. Temperature did not have any effect on SCD1b expression at 6 °C temperature acclimation in either species. The antioxidant gene Cu/Zn SOD lacks a response to thermal acclimation.

The SCD1b isoform had a ubiquitous expression in the tissues analysed in both the species

The research presented in this chapter has expanded the pattern of ubiquitous expression of SCD1b with previous studies in *Gasterosteus aculeatus* (Castro et al., 2011) and in *Sparus aurata* (Benedito-Palos et al., 2014) to the expression shown in our present study in notothenioid Antarctic benthic species *T. bernacchii* and the cryopelagic species *P. borchgrevinki* (Figure 14 A and B). The tissue panel in this study comprised liver, heart, kidney, spleen, adipose tissue, brain, and white muscle as well as red muscle, pyloric caeca and subcutaneous fat, with these latter tissues not previously examined in other species. Phylogenetic analysis of the SCD gene showed that SCD1 in teleosts duplicated into SCD1a and 1b (Castro et al., 2011) due to the third round of genome duplication (3R) which occurred specifically in the teleost lineage, after their divergence from mammals (Jaillon et al., 2004). To determine whether the gene duplicates have a transversal expression pattern within species, an investigation into SCD1 paralog expression in the marine fish *Gasterosteus aculeatus* showed that

SCD1a and 1b are ubiquitously expressed in the ten tissues studied (eye, brain, heart, spleen, liver, ovary, skin, gill, stomach and hind gut), as determined by end-point PCR (Castro et al., 2011). Another study on the fish species *Sparus aurata* used quantitative real time PCR to examine expression of lipid-relevant genes, including SCD1a and 1b, found ubiquitous expression of SCD1b in white muscle, liver, adipose tissue and brain (Benedito-Palos et al., 2014). Other than these studies, tissue expression of the SCD isoforms has not been investigated in any other teleost fish species. In contrast to the data available for other teleost species, ubiquitous expression was not observed in zebrafish (Evans et al., 2008)**.** The phylogenetic study (Figure 4) reported in Chapter 2 suggest that there could have been a loss of SCD1b and duplication of SCD1a in zebrafish which may then explain this discrepancy, as SCD in this species cannot be meaningfully compared with that of other fish due to this difference in the history of duplications and losses.

Following the 3R duplication event, it has been proposed that sub-functionalization of the duplicate paralogs occurred, with SCD1b retaining the ancestral functions of the SCD1 orthologue, which then correlates with its ubiquitous expression. This hypothesis is supported by the SCD1b expression I observed in *T. bernacchii* and *P. borchgrevinki*, suggesting that after gene duplication of SCD1 into SCD1a and 1b, the SCD1b isoform, with its ubiquitous expression, has retained the ancestral functions of SCD1 and SCD1 which is expressed in birds and mammals and is ubiquitously expressed in all of the species examined, with higher expression in the brain and liver (Castro et al., 2011; Lengi & Corl, 2008). Thus, there appears to be retention of ancestral SCD1 functions within Antarctic notothenioid fish SCD1b.

Asymmetric expression of the SCD isoforms: SCD1a has ubiquitous expression in the benthic species *T. bernacchii*, while the pattern differs in the cryopelagic *P. borchgrevinki* species

A pattern of expression referred to as asymmetric expression was previously studied in four teleost species, where 49 paralogous pairs of genes in fish were found to show this pattern of expression as observed in SCD isoforms (Steinke et al., 2006), as was also observed in the present study in the SCD isoforms. According to that previous study (Steinke et al., 2006) following gene duplication, there is sub-functionalization of the paralogs, one of which rapidly evolves resulting in reduced expression and eventually leading to a new function (neo-functionalization) while the other paralog retains the ancestral function and has a high expression. In chapter 2, SCD1a had a higher rate of molecular evolution than SCD1b, as shown by the GA branch analysis in three Antarctic species (Figure 13,

Chapter 2). In another study (Thompson et al., 2014), gene duplicates of the sodium channel gene paralogs Scn4aa/Scn4ab showed a pattern of asymmetric expression, with down-regulation of neo-functionalization of Scn4aa following gene duplication and a parallel loss of Scn4aa expression in muscle tissue of electric fish (Thompson et al., 2014). Thus, low, or absent, expression of SCD1a in some tissues may perhaps correlate with a new function for this isoform. Although, *T. bernacchii* and *P. borchgrevinki* are phylogenetically closely related, they occupy different ecological niches as one is benthic and the other is pelagic, and this may perhaps be the reason for the low requirement of the SCD1a isoform in *P. borchgrevinki*. This correlation could be explored by analysis of SCD1a expression in other Antarctic fish species with differing ecological niches to determine if such losses, or reduction of SCD1a gene expression are the result of neofunctionalization following gene duplication.

Temperature has a significant effect on the expression of SCD1a and SCD1b in the benthic species *T. bernacchii*, but this effect was not observed at different time points over the acclimation period

Currently only one study has shown the expression of two SCD isoforms (*Cds1* and *Cds2*) in a fish (*Cyprinus carprio*) and these are different from the SCD1a and SCD1b isoform genes as they are lineage-specific duplicates of SCD1a (Polley et al., 2003). The regulation of the two isoforms was found to be independent, with one isoform regulated by diet and the other by temperature (Polley et al., 2003). These isoforms are localized on the same chromosome and may result from alternate transcript processing, unlike SCD1a and SCD1b, which are localized on different chromosomes and are genome duplicates as shown by the syntenic analysis (Castro et al., 2011). One of the key questions of this research was to determine any temperature-specific SCD isoform as found in *Cyprinus carprio,* but temperature-specific independent regulatory expression in SCD1a and SCD1b was not observed in the research presented here. Contrary to this, in the benthic species *T. bernacchii*, temperature had a significant effect on the overall increase in expression of SCD1a and 1b over the acclimation period (Figure 16 and 19), although the effect was not significant at the individual time points of the acclimation period, due in part to high variability among the biological replicates. In this study the Antarctic fish samples were obtained from a natural population and perhaps this could be the reason for this variation. A larger sample size would have likely improved the statistical significance of the data. The effects of temperature and time were non-significant at all individual time points for SCD1a as well as SCD1b in the benthic species.

In the present study SCD1a expression in the pelagic species *P. borchgrevinki* was not within the detectable range due to low level of expression in both the control and the acclimated treatment, while expression was detectable and non-significant in *T. bernacchii* (Figure 18). Lower expression levels of SCD1a compared to SCD1b have also been observed in the skeletal muscle of *Sparus aurata* in fish fed to 100% satiation level, and in general, the expression levels are severely affected at lower satiation levels (70%) (Benedito-Palos et al., 2012). Another study by the same research group extended this work to include more tissues and studied lipid-relevant genes in a nutritionally challenged condition. They showed that control samples (samples not subjected to nutritional challenge) had low levels of SCD1a expression in the brain, liver, white muscle and no expression in the adipose tissue (Benedito-Palos et al., 2014).

In the present study duration of acclimation had a significant effect on gene expression for SCD1a in *T. bernacchii*, with significantly higher expression at day 1 for both the control and the treatment than for the later days of acclimation. This suggested that perhaps the pattern of SCD1a gene expression follows a trend irrespective of temperature. Lack of parallel studies of this isoform in response to temperature prevents confirmation of this pattern of expression at this time and a larger number of Antarctic and non-Antarctic fish species should be examined to confirm this pattern of expression for SCD1a.

Numerical trend of acute and transient expression of SCD1b

Studies investigating SCD expression have mostly been done in warm-adapted fish and no study has been conducted in Antarctic fish species. This is the first study to investigate the response of both SCD isoforms in response to thermal acclimation. A non-significant trend of an acute and transient increase in SCD1b expression in response to thermal stress at day 1 in both the species *T. bernacchii* and *P. borchgrevinki* followed by a decline in SCD1b response over the acclimation period, was observed in the present study. This trend is in alignment with transcriptomic profiles in gill tissues of *T. bernacchii* subjected to multiple stresses, viz. high temperature and pCO_2 which showed an upregulation of genes related to lipid metabolism, and specifically genes involved in membrane biosynthesis and maintenance, during the seven days of acclimation followed by a decline over the remainder of the acclimation period, with minimal detectable response at the end of 56 days of thermal acclimation (Huth & Place, 2016b). A similar trend was observed in RNA-seq analysis of gill tissues of *P. borchgrevinki* subjected to thermal and acid stress over 56 days of acclimation showed

an increase in expression of genes related to lipid metabolism for the first 7 days of thermal acclimation and less response over a thermal acclimation period that ended after 56 days (Huth & Place, 2016a). In the present study, an acute transient expression of SCD1b may be induced to meet the lipogenic demands of phospholipid biosynthesis and membrane maintenance in both the Antarctic species following a high degree of oxidative cellular stress. An initial spike, followed by reduction in SCD1b expression in both species of the present study, is also in alignment with the above studies suggestive of a decline in stress response over the acclimation period and a compensatory mechanism to cope with thermal stress in these species (Huth & Place, 2016b).

Temperature did not have any effect on SCD1b expression at 6 °C after seven days of thermal acclimation for either species

The pattern during warm acclimation at temperatures above 4 °C for cold-adapted Antarctic fish is unknown and the research reported here is the first attempt to investigate this. No effect of thermal acclimation was detected at 6 °C but only a single seven day time point was able to be taken due to limitations on Antarctic aquaria space and duration of the field season. SCD transcription could have been activated prior to day seven. High mRNA turnover could also have meant that a transient spike in expression was missed and change in SCD expression could not be detected (Gonzalez et al., 2013). Studies have shown that desaturase activation takes place within 24 hours of cold exposure (Cossins et al., 2002). Initially there is activation of pre-existing inactive desaturase, followed by the activation of desaturase transcription (Cossins et al., 2002).

Antioxidant gene Cu/Zn SOD lacks response to thermal acclimation

Cold temperatures predispose Antarctic fish to high levels of oxidative stress due to higher mitochondrial densities, and oxidation of fatty acids as energy sources make them prone to ROS attack. Antarctic fish have high levels of antioxidant defence systems in place to offset the high amount of ROS (Lesser, 2006). In this study expression of Cu/Zn SOD was unaffected by thermal acclimation in both the benthic species *T. bernachii* and the pelagic species *P. borchgrevinki.* Similar findings of unchanged SOD transcript levels in oxidative tissues (heart muscle) have been observed in red-blooded notothenioids viz, *Gobionotothen gibberifrons* and in *Notothenia coriiceps* when exposed to their respective maximum thermal limit, although there was decrease in SOD activity and transcript levels in the white-blooded icefish, accompanied by increased oxidative damage in the

heart muscle (Mueller et al., 2012). In another study, warm acclimation at 8 °C for 6 days in *Notothenia coriiceps* and *Notothenia rossii* resulted in no change in SOD enzyme activity, but the transcript levels of SOD were not measured (Machado et al., 2014). It is possible that Antarctic fish have pre-existing and sufficient amounts of SOD antioxidant enzyme in place to offset the additional oxidative damage induced by thermal stress. This was suggested by another thermal acclimation study over 56 days, which showed reduced SOD activity in response to temperature acclimation, although there was high oxidative damage during short-term acclimation but a decrease in antioxidant activity over the longer-term acclimation period. Further acclimation reduced cellular damage and this had returned to basal levels by the end of the acclimation period (Enzor & Place, 2014). A similar observation was made in another study, which showed a transient tissue-specific increase in SOD activity during the cold acclimation in three spine stickleback (*Gasterosteus aculeatus*) with SOD activity declining during warm acclimation. This study provides further evidence of a decline in SOD activity in warm acclimation (Grim et al., 2010).The effects of the chemical pollutants polybrominated diphenyl ethers (PBDEs) on SOD activity and expression in *T. bernacchii* show a similar lack of response of SOD expression, although oxidative stress was evident in the liver (Ghosh et al., 2013). In this case the lack of SOD response was attributed to the pre-existing sufficient amount of SOD enzyme that would meet the requirement and that additional synthesis was not required (Ghosh et al., 2013). The findings presented here could also be attributed to sufficient amounts of pre-existing SOD, although SOD activity itself was not measured along with expression analysis. Other antioxidant markers should also be considered in future work.

In summary, the research presented in this chapter has elucidated the tissue expression profile of the two SCD isoforms in two Antarctic notothenioid fish species. With limited previous studies on tissue expression of SCD isoforms in teleost fish, and no studies in Antarctic fish, this information has contributed new knowledge concerning the ubiquitous tissue expression profile of both SCD1a and SCD1b, as observed in the benthic species, and the ubiquitous expression of SCD1b but not SCD1a in the cryopelagic species, demonstrating a pattern of asymmetric tissue expression. Parallel studies on other genes in teleost species strongly supported these observations of an asymmetric expression pattern. SCD gene is one of the major markers for cold tolerance and major concerns about membrane remodelling in response to global warming, specifically in the Antarctic fish, still remain unanswered. This study is the first to provide the expression profile of the SCD isoforms in a thermally challenged Antarctic fish. It should help to establish correlation with the biochemical analysis of

membrane fatty acid profiles examined in the next chapter. An initial spike in SCD1b response during the initial acclimation followed by diminished response over longer-term thermal acclimation is consistent with trends seen in published reports of transcriptomic data from thermally challenged Antarctic fish. Temperature had a significant effect on the overall expression of SCD1a, similar to that seen for SCD1b. Although SCD was the gene used to investigate membrane remodelling capacity in the thermally challenged Antarctic fish in this study, other genes that are involved in the membrane synthesis pathway should also be used for future transcriptional studies of the cellular responses to thermal challenge. Other tissues should also be sampled, along with liver, for the assessment of membrane remodelling in thermally stressed Antarctic fish. The apparent lack of antioxidant response confirms previous studies and there is a need for the transcript levels of other antioxidant markers to also be investigated to determine their response when subjected to thermal stress.

4 Biochemical response of membrane lipid saturation to temperature in Antarctic notothenioid fish

4.1 Introduction

The membranes of stenothermal species which have evolved in a stable, cold environment, such as Antarctic notothenioid fish, have adapted by increasing the proportion of unsaturated fatty acids. This altered membrane composition, or homeoviscous adaptation (HVA), to lower temperatures is observed across all poikilotherms (Hazel & Williams, 1990).Temperate fish have a broad thermal adaptable range and when their membranes are subjected to thermal change, alterations in membrane fluidity take place to bring it to an optimum condition for that temperature. These acclimatory adaptive alterations occur over short periods relative to individual's life time. Acclimation of fish to lower/higher temperatures increases/decreases the proportion of unsaturated fatty acids in the membranes, allowing optimal membrane fluidity to be maintained. This suggests a protective role of the homeoviscous response in short-term acclimation (Skalli et al., 2006; Snyder et al., 2012).

In contrast to acclimatory adaptive changes occurring in temperate fish, the HVA response in Antarctic fish may be a long-term evolutionary adaptive change acquired over the last 30 million years (Hazel, 1995). Homeoviscous adaptation to the constant cold temperatures of the Southern Ocean is one of the key evolutionary adaptive changes in Antarctic notothenioid fish, but it is not known whether they have the capacity to change their membrane saturation states in response to warmer temperatures. Also it is not known, if evolutionary adaptive changes have occurred in the composition of membrane lipids of icefish (Channichthyidae family of notothenioid fish). Icefish, which diversified relatively recently over the last 7.8-4.8 million years (Near et al., 2012) have a suite of physiological adaptations to compensate for the loss of haemoglobin (Kock, 2005). The erythrocyte membranes of icefish have fluidity consistent with those of *T. bernacchii*, but differences were observed between erythrocyte membrane lipids in icefish compared to *T. bernacchii* (Palmerini et al., 2009). It is not known if such changes occur in other icefish tissues.

With anthropogenic global warming (AGW) and the threat this poses to polar species, comes a need to determine their cellular acclimation response of membrane remodelling to warmer temperatures. There is evidence that Antarctic fish may not exhibit an acclimatory HVA response to transient temperature changes. Antarctic fish were acclimated to a higher temperature (4 °C) for five weeks

did not change their membrane lipid saturation states (Gonzalez-Cabrera et al., 1995). However, these fish did show positive compensation by increasing the Na^+-/K^+- ATPase activity and a decline in serum osmolality. This implies that despite a lack of change in unsaturation state, membrane functions were not compromised.

Temperature is also a major determinant of membrane cholesterol content with higher cholesterol in the membranes of warm-acclimated marine copepods (Hassett & Crockett, 2009). Increased cholesterol concentrations will reduce membrane fluidity (Crockett, 1998). It is not known whether Antarctic fish share this adaptive membrane cholesterol concentration change in response to temperature.

This study aimed to establish the normal lipid saturation profile of liver tissue from three Antarctic fish species in comparison with a non-Antarctic New Zealand fish. I bring together existing evidence and new experimental data, to understand the evolutionary adaptive response associated with cold tolerance. To do this, I compared liver membrane lipid profiles of two closely related Antarctic notothenioid fish species *Pagothenia borchgrevinki* and *Trematomus bernacchii* and a more distantly related icefish species (*Chionodraco hamatus)*, as well as with the non-Antarctic Perciformes species *Notolabrus celidotus*. Furthermore, I compared the Antarctic icefish species *C. hamatus* membrane lipid profile to the two closely related Antarctic fish species *P. borchgrevinki* and *T. bernacchii* and examined whether short term thermal stress up to 6 °C resulted in membrane restructuring in two Antarctic fish species *P. borchgrevinki* and *T. bernacchii*. I hypothesise that changes in membrane saturation, the major thermal adaptive mechanism, will occur at reduced capacities in Antarctic notothenioid fish in response to elevated temperatures making them vulnerable to the effects of AGW.

4.2 Methods

4.2.1 Fish and experimental design

The sampled fish species are described in Table 30 and details of the fish harvest and husbandry in the Appendix (A.1.1). Membrane lipid profiles of liver from Antarctic notothenioid fish in normal habitat (pre-acclimation controls for PB and TB of thermal acclimation experiment described in (A.1.1) as well as *C.hamatus* species (Table 1), were compared with those of the non-Antarctic fish species *Notolabrus celidotus,* a common native Perciformes fish located throughout New Zealand (Ayling &

Cox, 1982). This species was chosen because it is easily available and is a perciforme species, the order that notothenioid belong to. Furthermore, they are non-migratory and have a broad range of thermal adaptivity to the variations in temperature from seasonal temperature change (Jones, 1984). *N. celidotus* and *C. hamatus* liver tissues were collected by Dr Victoria Metcalf.

Table 30. Fish location and local temperature range.

Fish species	Family	Location	Adaptation Temperature(˚C)
Trematomus bernacchii	Nototheniidae	McMurdoSound, Antarctica	-1- 1.9
Pagothenia borchgrevinki	Nototheniidae	McMurdoSound, Antarctica	-1- 1.9
Chionodraco hamatus	Channicthyidae	Terra Nova Bay, Antarctica	-1.9
Notolabrus celidotus	Labridae	Kaikoura, New Zealand	9-13

Samples of *Trematomus bernacchii* and *Pagothenia borchgrevinki,* harvested during the thermal acclimation experiments were used for the analysis of lipid profile changes in response to thermal stress on membrane restructuring (Appendices A.1.1 and A.1.2). Fish handling procedures were approved by the Animal Ethics Committee of the University of Canterbury for the thermal acclimation experiment. Standard procedures are given in (A.1.3) for liver sampling.

4.2.2 Treatment details and tissue sampling

Tissues harvested from the temperature acclimation experiment (Table 31) (A.1.2) were freeze-dried in liquid nitrogen and stored at -80°C until further analysis.

Table 31. Samples taken for phospholipid fatty acid analysis at different acclimation temperatures. TB: *Trematomus bernacchii*, PB*: Pagothenia borchgrevinki,* D0, D1, D7, D14 and D28: days after acclimation, T: acclimation temperature, N= number of fish samples taken.

0 ˚C	4 ˚C	6 ˚C
TBD0 (N=4)	TBD1 (N=4)	
TBD7 (N=4)		TBD7 (N=3)
TBD14 (N=4)	TBD14 (N=4)	
PBD0 (N=4)	PBD1 (N=4)	
PBD7 (N=4)		PBD7 (N=4)
PBD28 (N=4)	PBD28 (N=4)	

4.2.3 Phospholipid fatty acid analysis

4.2.3.1 Reagents and standards

The fatty acid standard GLC 463 was obtained from NuChek, Elysian, Minnesota, USA. This standard consists of 54 fatty acid methyl esters (FAMEs). The chromatograms had good resolution for the compound of interest. Major FAMEs were identified based on the retention time and known fatty acids are reported as percentage of fatty acid mixtures. Fatty acids less than 1% were not reported.

4.2.3.2 Equipment cleaning

All plastic-ware and glassware used for the phospholipid extraction was soaked overnight in hot water containing 5% Decon, before rinsing with hot water. This was followed by rinsing with deionised water, three washes with HPLC grade methanol and air dried before use.

4.2.3.3 Lipid Extraction

The lipid extraction method followed that of Folch et al. (Folch et al., 1957) modified as follows. Frozen liver tissue samples weighing approximately 0.2 g were homogenised into a fine powder with liquid nitrogen (Burim et al., 2003). Homogenisation was performed in a sterilized and chilled, pestle and mortar. Following homogenisation, 6 ml of dichloromethane (DCM)/methanol (2:1) with 0.01% butylated hydroxytoluene (BHT) added and the mixture transferred to Oak Ridge tubes (Nalgene™) and sonicated continuously, for 5 min at 4 °C**.** The settings for the sonicator (W-225 from Watson Victor) were, % Duty cycle = 55, Output control = 5 and % Power output = 30. After sonication, 2 ml of 0.88% potassium chloride was added and vortexed for two min followed by centrifugation at 2000 rpm for 5 min at 21 °C. The bottom organic layer was stored and the top layer extracted again with 2 ml of DCM/methanol (2:1) with 0.01% BHT and the phases separated by centrifugation again at 2000 rpm for 5 min at 21°C. The bottom organic layer combined with the first organic layer was transferred to a glass tube and the solvent evaporated off with nitrogen at 30 °C (3L/min). Dried samples were then stored at 4 °C until fractionation. The extraction process included blank samples (negative controls) which underwent all of the extraction procedures but without the addition of a tissue sample. For every eight lipid extractions there was a blank extraction.

4.2.3.4 Lipid fractionation

Fractionation of the lipid samples into different lipid classes was achieved using SPE columns, Isolate SI 500 mg 6 ml SPE (Biotage). Samples were brought to room temperature before fractionation. The column was rinsed with 0.3ml of chloroform followed by 1 ml of chloroform to check the flow rate. It was discarded if elution took longer than 1 min. The evaporated sample was dissolved in 0.1 ml chloroform, vortexed and loaded onto the column. Another 0.1 ml of chloroform was added, vortexed, and loaded onto the column. The sample was allowed to stand for 2 min in the column and then sequentially eluted with 5ml of chloroform to elute the neutral lipids, 5ml of acetone to elute the glycolipids and finally 5ml of methanol to elute the phospholipids. The solvent was evaporated from the phospholipid fraction under nitrogen (Industrial grade) at 30 °C at the rate of (3L/min) then stored at 4 °C before proceeding with methylation.

4.2.3.5 Quality check for the presence of phospholipids

Three fractions *viz.,* neutral lipids, glycolipids and phospholipids, obtained in the fractionation step using silica columns were analysed by thin layer chromatography (TLC) for the qualitative analysis of phospholipids. Lecithin was taken as a standard. Silica plates 10 X 20 cm, silica gel (200mm; Merck #1.057209001) were activated at 110 °C for one hour. Following activation, 0.1 ml of analytes were loaded onto the plates 1.5 cm from the bottom in 10 aliquots of 0.01 ml with drying between each application. The silica plates were developed in chloroform: methanol: water (65:25:4) in a covered chamber saturated with the developing solvent. The plates were developed until the solvent front had migrated to 3 cm from the end of the plate (about 2 hours). Following development, the plates were air-dried for 20 min and immersed in 10% cupric sulphate in 8% aqueous phosphoric acid for 1 min (Churchward et al., 2008) then air-dried at room temperature for 10 min and placed into an oven at 145 °C for 10 min to develop colour.

4.2.3.6 Methylation

The fatty acids in the phospholipids were converted into fatty acid methyl esters and quantified by gas chromatography. Evaporated samples (air was excluded by addition of Nitrogen before storage) were brought to room temperature and 1 ml of tetrahydrofuran: methanol (1:1v/v) added, vortexed for 30 seconds, 1ml of 0.2M potassium hydroxide added, followed by incubation at 37 °C for 15 min. After incubation, 2 ml of hexane: chloroform (4:1) plus 0.3 ml of 1M acetic acid and 2 ml of deionised water were added and mixed followed by centrifugation at 2500 rpm for 5 min. The top organic layer was transferred to a holding tube, the lower layer re-extracted with 2 ml of hexane: chloroform (4:1)

and the organic fractions combined. The organic layer was evaporated under Nitrogen in a water bath at 37 °C, hexane (50 µl) was added to the evaporated organic layer and the solution transferred to a 50µl insert with a poly spring (Global science, Auckland) held in a 2.0 ml amber vial (Restek, Shimadzu, Auckland, NZ) for GC analysis.

4.2.3.7 Gas chromatographic separation

The sequence of samples comprised blank, FAME standard, and eight samples. Chromatography was carried out on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu, Tokyo, Japan) fitted with a Shimadzu AOC-20s auto sampler. The column was 100 m length with a film thickness of 0.25µm (ID 0.25mm Varian CP7420, Serial # 6005241 and helium flow 0.96ml/min). The split ratio was 15:1 and the injector temperature was 250 °C. Detection was by Flame Ionisation Detector (FID) at 310 °C.

4.2.3.8 Column temperature ramping

The initial column temperature of 45 °C for 4 min was followed by a temperature ramp of 13 °C/min to 175 °C. This temperature was held for 27 min followed by another ramp of 4 °C/min to 215 °C, which was held for 35 min before a final ramp 25 °C/min to 245 °C. This temperature was held for 5 min to remove any residues from the column before returning to the initial temperature. Each cycle took 92.2 min. All these GLC conditions were based on adapting the initial conditions indicated by Lee and Tweed (Lee & Tweed, 2008).

4.2.3.9 Analysis of GC data

The fatty acids in the samples were identified by comparison with retention times for the FAME standard using 'GC solution' software (version 2.21.00SUI, © Shimadzu). The list of FAMEs identified in each sample was transferred to Excel spreadsheet. The retention time of each FAME peak in the sample was compared with those of the external standards and if these were the same as those in the FAME standard, the sample peak was identified as the FAME constituent.

4.2.4 Membrane cholestrol analysis

4.2.4.1 Membrane cholesterol extraction

Membrane cholesterol was determined as non-esterfied (free) cholesterol. A Cholesterol extraction method was followed as given in the study of Gonzalez et al. (Gonzalez et al., 2013). About 50 mg of liver tissue was taken and homogenised to a fine powder using liquid nitrogen in a pestle and mortar and 5ml of (2:1) DCM: methanol with 0.01% BHT added, then transferred to an Oak ridge tube and sonicated 2 min at 4 °C. The settings of the sonicator were % duty cycle = 55, Output control = 5 %

power output = 30. After sonication, 2.5 ml of 2M potassium chloride and 5mM EDTA were added, the sample mixed and centrifuged for 10 min at 2000 rpm and the bottom organic phase was separated, dried and re-suspended in 1 ml of 2-methoxyethanol, then stored at -80 °C.

4.2.4.2 Quality check for the presence of cholesterol

TLC Silica gel 60 Aluminium sheet 5x10 cm (Merck KGaA 64271 Darmstadt, Germany, product number1.16835.0001) was used for this analysis. Samples and cholesterol standard (Jt Baker, F676-05 100G-Purity=90-100%, United States) (0.0.1 ml) aliquots were spotted separately 1 cm from the bottom of the plates, then dried. The plates were developed with the mobile phase solvent [chloroform: methanol: water (65:25:4)]. Development was done in a covered glass beaker pre-equilibrated in mobile phase solvent, until the solvent front had migrated to 3 cm from the top of the plate. Plates were air-dried immersed in 10% cupric sulphate in 8% aqueous phosphoric acid for 1 min, air-dried for 10 min and heated at 110 °C for 10 min. Cholesterol was detected by its colour and co-migration with the standard.

4.2.4.3 Cholesterol fluorometric assay

Cholesterol was measured by a fluorimeter (Fluorostar Omega Microplate Reader from BMG Labtech) using a cholesterol fluorometric assay kit from Cayman Chemical (Ann Arbour, MI, USA, and Cat. #. 10007640) and this kit could determine the presence of both esterified as well as free cholesterol. Membrane cholesterol contains free cholesterol and in the present study esterified cholesterol was not determined hence for free cholesterol determination cholesterol esterase which is provided in the kit was not added for this assay. All of the reagents required for this assay were prepared as per the directions provided in the kit (https://www.caymanchem.com/pdfs/10007640). Diluted standards of 0, 2, 4, 6, 8, 12, 16 and 20 µM were prepared using the assay buffer as diluent and each 50µl of standards (Tubes A to H) were added to the designated wells of the flourometric plate (Whatman, GE Healthcare, UK). Samples of 50 µl from the cholesterol extract re-suspended in 1 ml of 2-methoxyethanol) were added in triplicate. The assay cocktail was prepared, which consisted of assay buffer (4.75 ml), cholesterol detector (150 µl), horse radish peroxidase (HRP: 50 µl), and cholesterol oxidase (50 µl), 50ul of freshly prepared assay cocktail added to all the wells. The plate was covered and incubated for 30 min at 37 °C in the dark. The plate cover was removed and the fluorescence was read using excitation wavelengths between 530 - 540 nm and emission wavelengths between 585 – 595 nm. The average fluorescence was calculated for each standard and sample, and

standard curve was plotted. The concentration of cholesterol in µmol/L obtained using the standard curve was converted to µmol/g of tissue using the equation:

$$\text{µmol cholesterol per g tissue} = \frac{[(\text{µmol/L of cholesterol}) \times (\text{µL in entire extract})]}{[(1{,}000{,}000\text{µL/L}) \times \text{g tissue used}]}$$

4.2.5 Plasma osmolality determination

4.2.5.1 Collection and storage of plasma samples

Blood samples from the experimental fish described in the Appendix (A.1.3) were collected at Scott Base Wet Laboratory where the temperature was constantly below 5 °C. Fish were anaesthetised for 5 min, by administration of 0.1g L^{-1} solution of MS222 (ethyl m-amino benzoate methanesulphonate) dissolved in sea water. Blood samples were obtained by cardiac puncture with a 25 gauge needle, volume of 0.5 to 1.0 ml was collected into tubes containing anticoagulant (EDTA) which were centrifuged at 3000g for 2 min to separate plasma which was snap frozen in liquid nitrogen and transported to New Zealand in an insulated container containing dry ice and then stored at -80 °C.

4.2.5.2 Samples taken for the osmolality analysis

Plasma was collected from both the species *Trematomus bernacchii* and *Pagothenia borchgreviniki* acclimated at 4 °C, 6 °C and the control temperature of 0 °C at all the time-points (Table 32).

Table 32. Plasma samples taken for the osmolality analysis from different acclimation temperatures. TB: *Trematomus bernacchii*, PB*: Pagothenia borchgreviniki,* D0, D1, D3, D7, D14 and D28: days after acclimation, T: acclimation temperature

Acclimation temperature	0 °C	4 °C	6 °C
	TBD1 (N=4)	TBD1 (N=4)	
	TBD2 (N=4)	TBD2 (N=4)	TBD7 (N=4)
	TBD3 (N=4)	TBD3 (N=4)	
	TBD7 (N=4)	TBD7 (N=4)	
	TBD14 (N=4)	TBD14 (N=4)	
	PBD1 (N=4)	PBD1 (N=4)	
	PBD2 (N=4)	PBD2 (N=4)	
	PBD3 (N=4)	PBD3 (N=4)	
	PBD7 (N=4)	PBD7 (N=4)	PBD7 (N=4)
	PBD28 (N=4)	PBD28 (N=4)	

4.2.5.3 Plasma osmolality determination

Plasma samples were thawed to room temperature and 10 µl plasma aliquots used for osmolality determinations using a Wescor 5520C vapour pressure osmometer, calibrated with standard solutions (concentration of 100,290 and 1000 mmol/Kg) before the measurements.

4.2.6 Calculations and statistics

Analysis for the comparison of lipid profiles of different species was done using a principal component analysis with a correlation matrix. The PCA used percent phospholipid fatty acids for each sample of the species and the data was not transformed prior to analysis. In addition, a one way ANOVA followed by a Holm-Sidak post hoc test (Holm, 1979) was performed to determine the significance among the species. Desaturation indices (DSI) were calculated as C16:1c9/C16:0 and C18:1c9/C18:0. These two particular unsaturated fatty acids (C16:1c9 and C18:1c9) were used for DSI, as the ratio of (C16:1c9/C16:0) and (C18:1c9/C18:0) have been shown to be correlated with Stearoyl-CoA desaturase activity, degree of desaturation and membrane fluidity in the study (Hsieh & Kuo, 2005). Data were analysed by two way ANOVA followed by a Holm-Sidak post hoc test (Holm, 1979) to determine the significance of the means of the treatments. Data included temperature (control 0 °C and acclimated 4 °C), time (days) and interaction of temperature and time. Data analysis at 6 °C thermal acclimation was done using an unpaired Student t test. All statistical analyses were done using Minitab v17.2.1 (Minitab Pty Ltd, Sydney) software.

4.3 Results

4.3.1 Quality check for the phospholipid extraction

It was necessary to separate the phospholipids from the lipid extract and results showing the separation of phospholipids after fractionation (Fraction 1, 2 and 3) using the silica column are presented in Figure 22. Successful separation of lipid classes from *T. bernacchii* liver was confirmed by TLC in comparison with the standard (soya lecithin), sample 1. The standard (Lane 1) separated into the different phospholipid classes. Lane 2, fraction 1 comprised the neutral lipids and lane 3, fraction 2, the glycolipids. The phospholipid fraction (Lane 4, Fraction 3) was devoid of neutral lipids or glycolipids and aligned with the different phospholipids of the standard soya lecithin (Lane 1). Lane 5, the lipid extract without fractionation, contained all of the lipid classes.

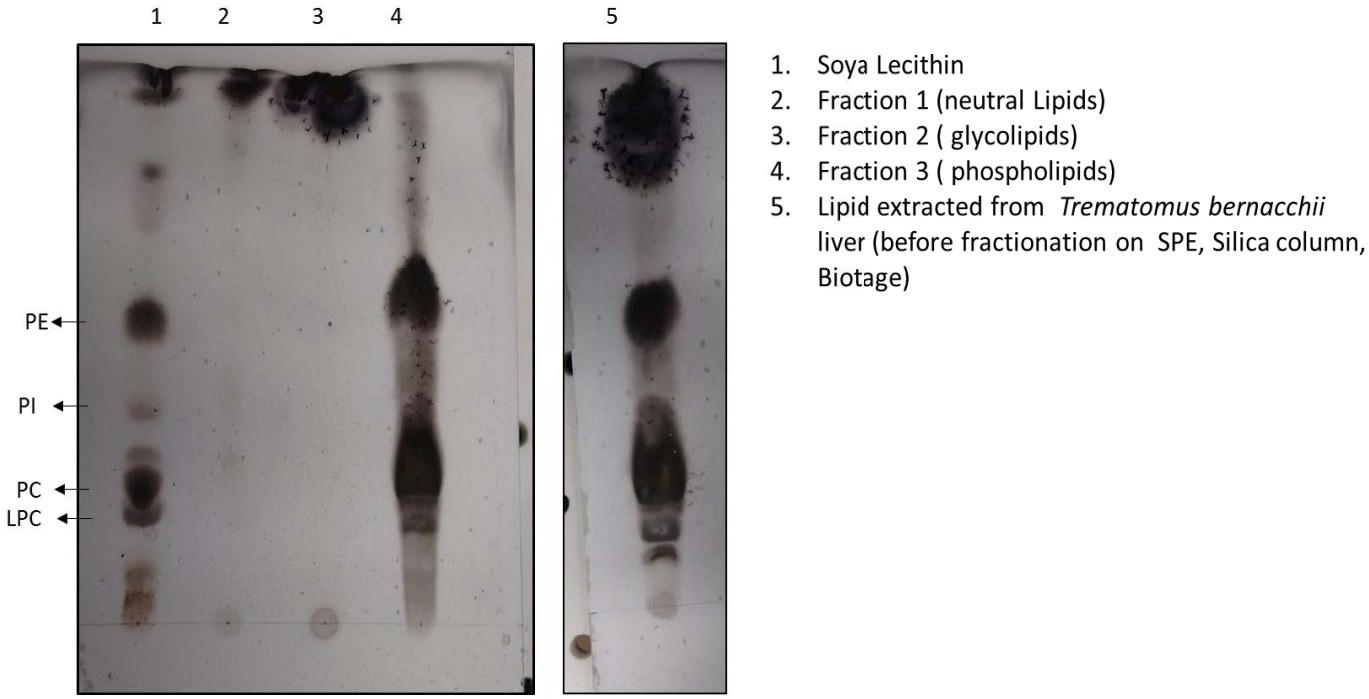


Figure 22. Analysis of lipid fractions from *Trematomus bernacchii* liver by TLC along with standard (soya lecithin).
Fractions 1, 2 and 3 were obtained after lipid was extracted from *Trematomus bernacchii* liver, fractionated with the silica column and eluted with different organic solvents. Abbreviations of the phospholipid classes shown on the left are PE: Phosphotidylethanolamine, PI: Phosphatidylinositol, PC: Phosphotidylcholine, LPC:

4.3.2 Profile of the major phospholipid fatty acid in *Trematomus bernacchii* liver and the reference standard GLC- 463

The major phospholipid fatty acids detected in the Antarctic species and non-Antarctic species along with their retention time are presented in Table 33, representative chromatogram of the reference standard GLC-463 is presented in the Appendix (D) and the phospholipid fatty acid profile of *T. bernacchii* liver is given in Figure 23. Fatty acids that were present in less than 1% of the sample are not reported. An unknown fatty acid, which did not co-elute with standard GLC-463, and had a retention time of 56.352 min, was present only in Antarctic fish and eluted after C20:1c11 (Figure 23). The PUFA docosapentaenoic acid (DPA), C22:5 were detected only in the non-Antarctic species and is not shown in the Table 33.

Table 33. List of major fatty acids and retention time detected in the phospholipid profile of the Antarctic and the non-Antarctic fish. Fatty acids less than 1% are not shown.

Systematic name	Trivial Name	Notation from COOH end of fatty acid	Retention time(min)
Hexadecanoic acid	Palmitic acid	C16:0	36.510
c-9-Hexadecenoic acid	Palmitoleic acid	C16:1c9	38.826
Octadecanoic acid	Stearic acid	C18:0	46.898
c-9-Octadecenoic acid	Oleic acid	C18:1c9	48.691
c-11-Octadecenoic acid	Vaccenic acid	C18:1c11	49.021
Eicosenoic acid	Eicosenoic acid	C20:1c11	55.997
c-11,14-Eicosadienoic acid	Eicosadienoic acid	C20:2c11,14	58.641
c-5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	C20:4c5,8,11,14	61.899
c-5,8,11,14,17-Eicosapentaenoic acid	Timnodonic acid	C20:5c5,8,11,14,17	65.663
c-4,7,10,13,16,19-Docosahexaenoic acid	Cervonic acid	C22:6c4,7,10,13,16,19	79.466

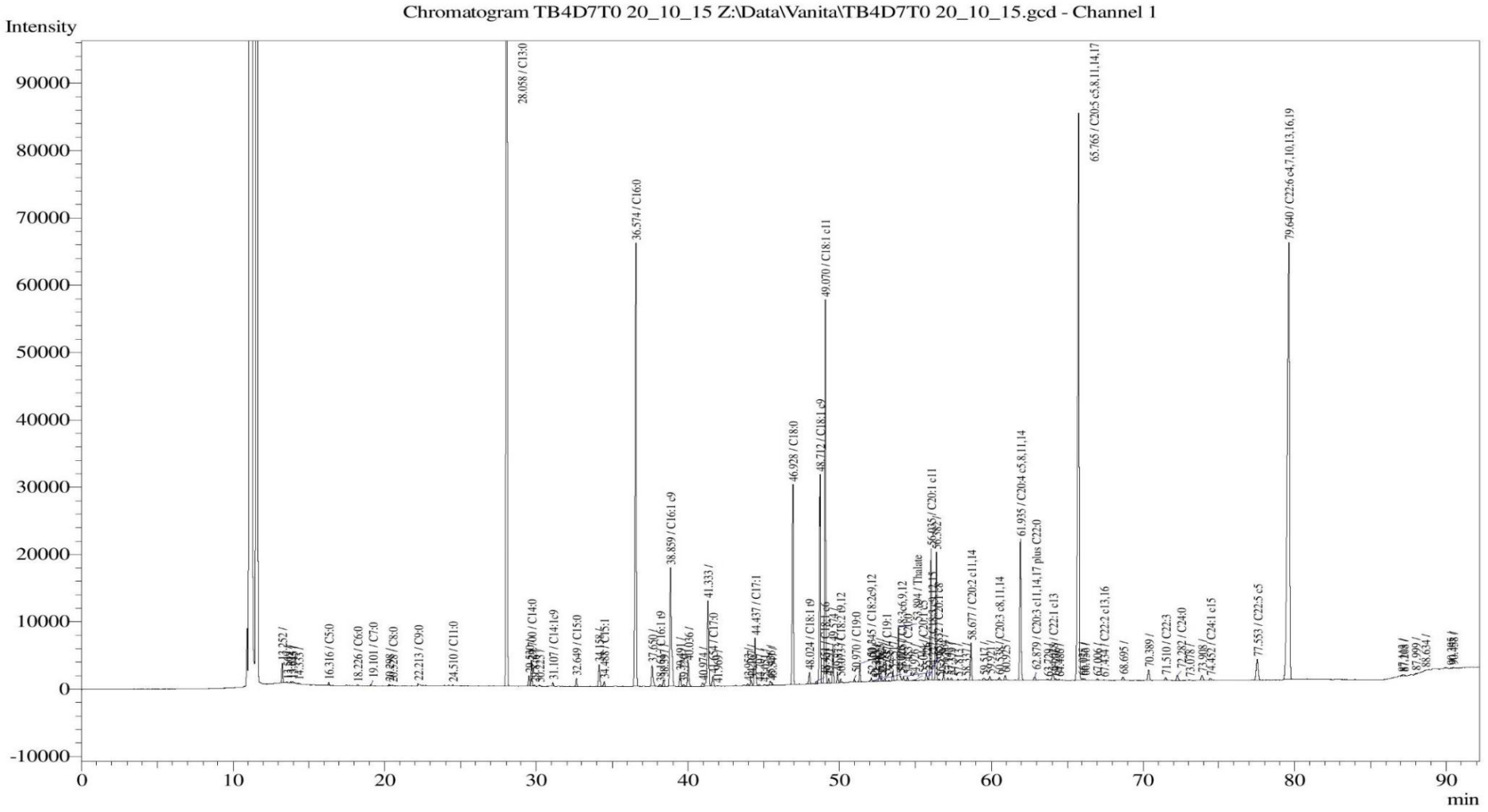


Figure 23. Representative chromatogram of the phospholipid fatty acid profile from *Trematomus bernacchii* liver.

4.3.3 Multivariate analysis of phospholipid fatty acid profiles in Antarctic and non-Antarctic species

Principal component analysis (PCA) of the data on the phospholipid profiles in Antarctic and non-Antarctic fishspecies in the first two principal components (PCs) explained 76.26 % of the variance in the data matrix (Table 34). The contribution of the variables (phospholipids fatty acids in liver tissue) to the two principal components (PCs) for the Antarctic species (*P. borchgrevinki, T. bernacchii* and *C.hamatus*) and non-Antarctic fish (*Notolabrus celidotus*) is provided as a graphical representation in Figure 24. PC1 represented 58.1% of the variables, while PC2 represented 18.2% of the variables. In PC1 the SFA (C18:0) and the PUFA (C20:4) were positively correlated while MUFAs (C16:1c9, C18:1c11, C20:1c11 and an unknown MUFA) and PUFA (C20:5) were negatively correlated. In PC2 the PUFA (C22:6) was positively correlated, while SFA (C16:0) and MUFA (C18:1c9) were negatively correlated. The non-Antarctic fish *N. celidotus* had high proportions of saturated fatty acids (C18:0) and the PUFA (C20:4), while the Antarctic species (all three) were high in MUFAs, such as (C16:1c9, C18:1c11, C20:1c11 and an unknown MUFA), and PUFA (C20:5). Along with this only the Antarctic species *C.hamatus* had high proportions of the SFA (C16:0) and the MUFA (C18:1c9). The PUFA (C22:6) were in high proportions in both the Antarctic (*P. borchgrevinki* as well as *T. bernaccchii*) and non-Antarctic species.

Table 34. Eigenvalues of the correlation matrix of the phospholipid fatty acid profiles in the Antarctic and non-Antarctic fish species.

Principal component	Eigenvalues	Cumulative eigenvalue	% of Total Variance	Cumulative % of Total Variance
1	5.80686	5.80686	58.0686	58.0686
2	1.81947	7.62633	18.1947	76.2633
3	0.838814	8.46515	8.38814	84.6515
4	0.560584	9.02573	5.60584	90.2573
5	0.438527	9.46426	4.38527	94.6426
6	0.283992	9.74825	2.83992	97.4825
7	0.135192	9.88344	1.35192	98.8344
8	0.080882	9.96432	0.80882	99.6432
9	0.024074	9.9884	0.24074	99.884
10	0.011604	10	0.116037	100

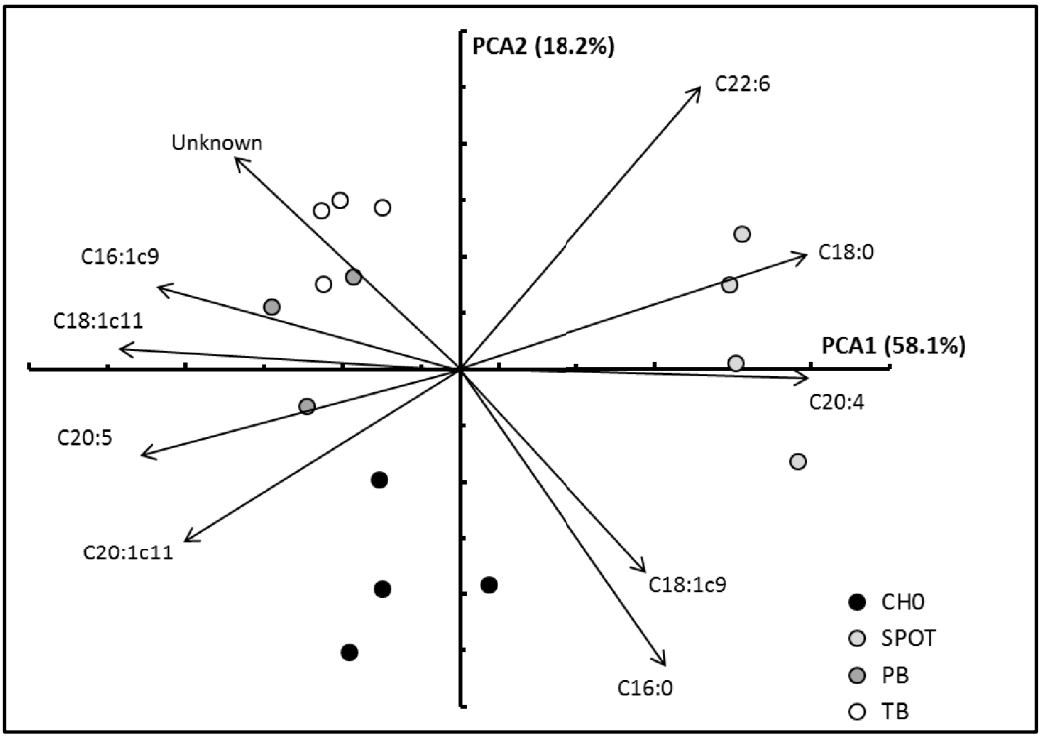


Figure 24. PCA plot of the contribution of the phospholipid fatty acids to the principal components in liver tissue of the Antarctic species (PB: *Pagothenia borchgrevinki*,TB: *Trematomus bernacchii* and CH : *Chionodraco hamatus*) and non-Antarctic species (NC: *Notolabrus celidotus*).

4.3.4 Phospholipid fatty acid profiles of Antarctic and non-Antarctic fish species

The fatty acid profiles of the phospholipids in the liver of the Antarctic and non-Antarctic species are presented in Table 35. In general, the phospholipids from Antarctic species had lower levels of SFAs and higher levels of MUFAs. The SFAs of the non-Antarctic species *N. celidotus* had a significantly higher percentage of stearate (C18:0) when compared to the Antarctic fish species. The SFA palmitic acid (C16:0) and the total SFA contents of Antarctic species *C. hamatus* and non-Antarctic species *N. celidotus* were significantly higher than the other two Antarctic species ($P<0.05$) (Figure 25). The MUFA profiles of the Antarctic species were significantly higher in palmitoleate (C16:1), *cis* vaccenic acid (C18:1c11), eicosenoate (C20:1) and in the percentage of total MUFAs. The PUFAs of non-Antarctic fish species had a significantly higher percentage of arachidonic acid (C20:4) and significantly lower levels of EPA (C20:5) compared to the Antarctic species ($P<0.05$) (Figure 25). The total PUFAs of the non-Antarctic species *N. celidotus* were significantly higher than the Antarctic species *C. hamatus.*

Table 35. Fatty acid composition of phospholipids fatty acids in liver of Antarctic and non-Antarctic fish expressed as % of total membrane fatty acids.

	N. celidotus	***C. hamatus***	***P. borchgreviniki***	***T. bernacchhii***
C16:0	20.05 ± 0.45	20.43 ± 0.60	13.16 ± 0.79	13.08 ± 0.45
C18:0	12.64 ± 1.01	3.05 ± 0.49	4.16 ± 0.46	5.01 ± 0.26
SFA	**32.69 ± 1.26**	**23.47 ± 2.56**	**17.32 ± 1.10**	**18.09 ± 0.59**
C16:1c9	0.48 ± 0.42	3.30 ± 0.63	4.01 ± 0.29	4.97 ± 0.30
C18:1c9	11.07 ± 1.47	10.51 ± 1.73	9.64 ± 0.79	7.42 ± 0.25
18:1c11	3.22 ± 0.43	7.69 ± 0.51	9.84 ± 0.53	9.11 ± 0.43
C20:1c11	nd	5.04 ± 0.85	2.78 ± 0.10	3.62 ± 0.33
Unknown	0.00	0.87 ± 0.43	2.93 ± 1.30	4.24 ± 0.71
MUFA	**14.77 ± 1.21**	**27.40 ± 1.72**	**30.55 ± 0.65**	**31.99 ± 0.63**
C18:2c9,12	0.62 ± 0.54	1.17 ± 0.36	0.00	0.00
C20:4c5,8,11,14	9.08 ± 0.44	4.97 ± 0.31	3.13 ± 0.18	4.79 ± 0.29
C20:5c5,8,11,14,17	14.00 ± 0.66	19.83 ± 0.70	22.52 ± 1.36	19.63 ± 0.94
C22:5c5	1.41 ± 0.41	nd	nd	nd
C22:6c4,7,10,13,16,19	28.06 ± 1.33	20.84 ± 1.25	23.54 ± 0.97	25.50 ± 0.29
PUFA	**52.55 ± 1.83**	**46.05 ± 1.14**	**51.40 ± 0.36**	**49.92 ± 0.63**

Values are mean ± SEM (n=4), except for *Pagothenia borchgrevinki* (n=3), nd=not detected

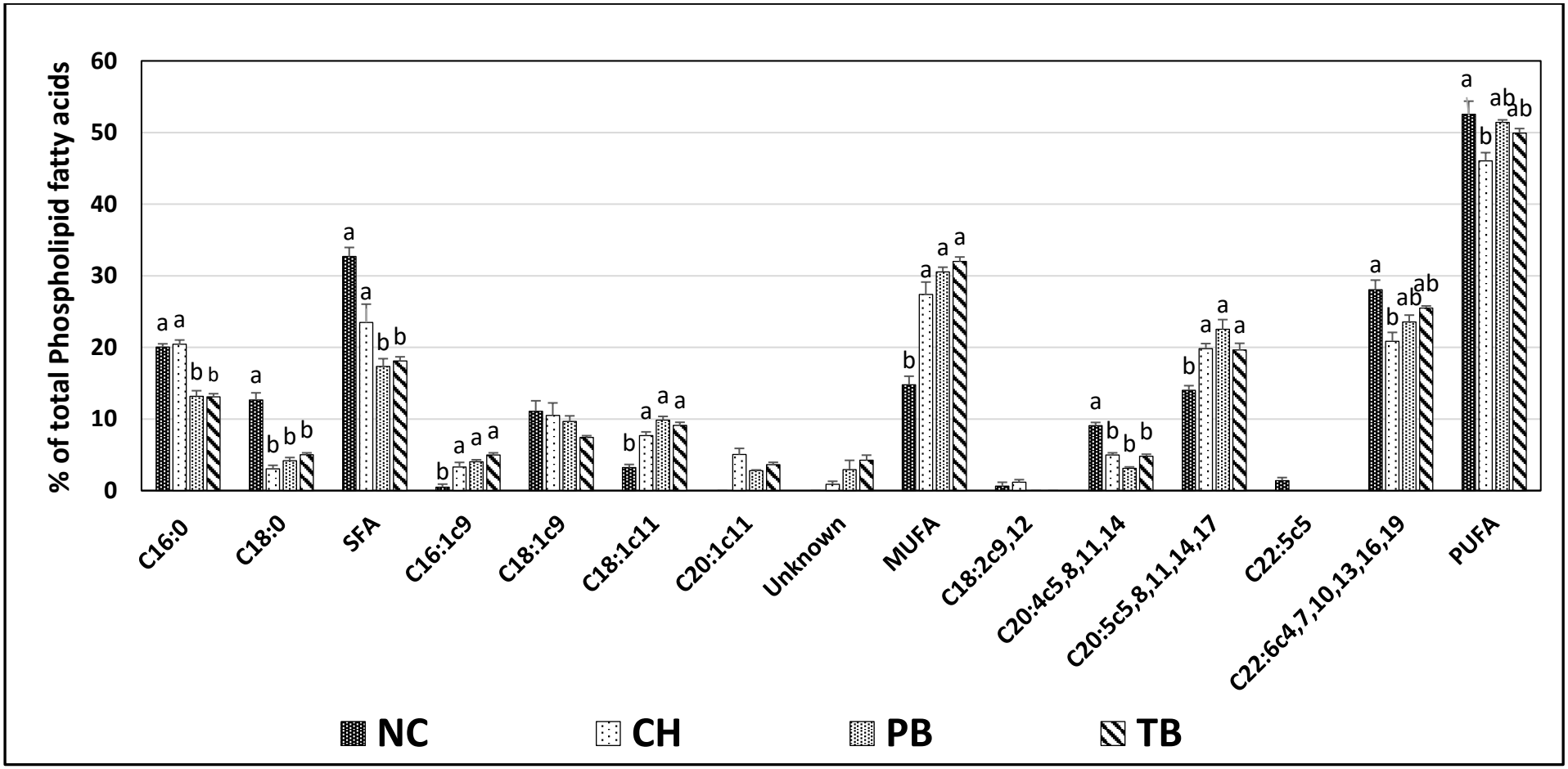


Figure 25. Phospholipid fatty acid profile of Antarctic species *Chionodraco hamatus* (CH), *Pagothenia borchgrevinki* (PB), *Trematomus bernacchii* (TB) and non-Antarctic species *Notolabrus celidotus* (NC) in liver. Significance among the species is indicated by different letters (P<0.05).

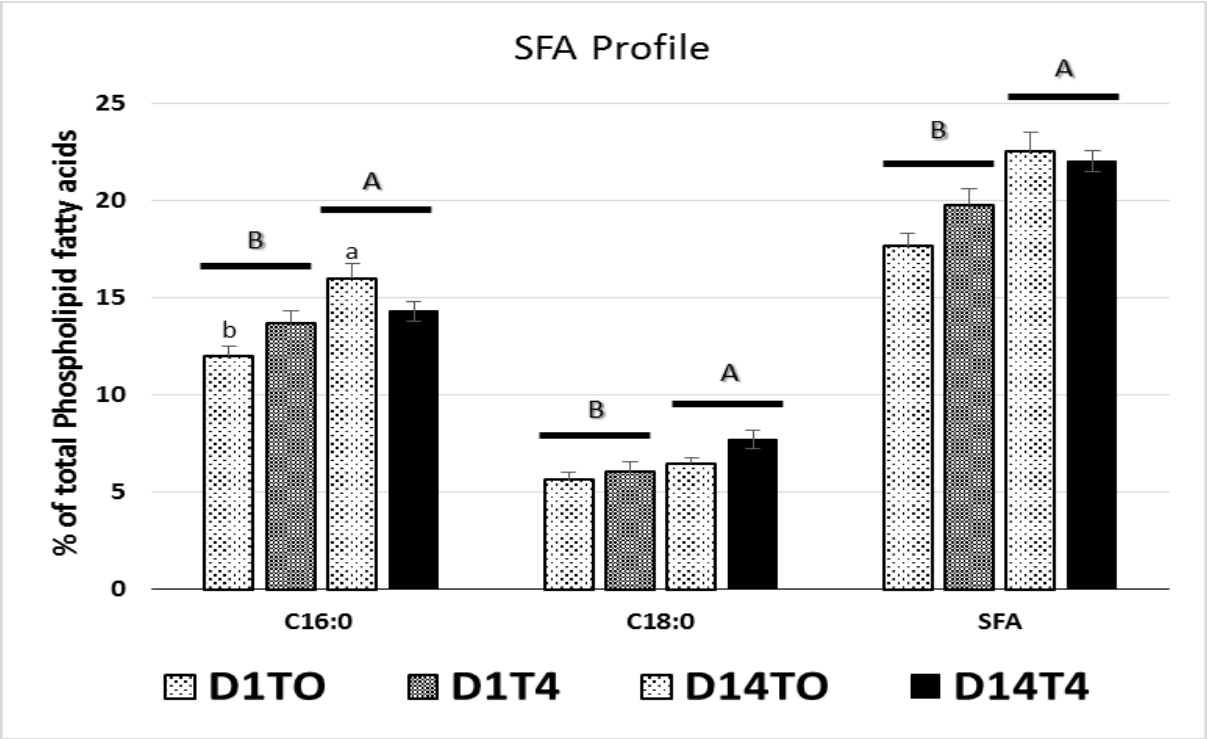
4.3.5 Membrane fatty acid profile in *T. bernacchii* after 1 and 14 days of thermal acclimation

The effect of thermal acclimation in the liver of the benthic Antarctic species *T. bernacchii* on the phospholipid profile is presented in Table 36. There was a significant effect of days of acclimation but there was no significant effect of the interaction of days of acclimation and temperature for both the SFAs and MUFAs. Palmitic acid (C16:0) and stearic acid (18:0) were significantly higher on day 14 than on day 1, indicating an acclimation time, rather than a temperature effect. The total SFAs were also significantly higher on day 14 (Fig.26) ($P<0.05$). Similarly, monounsaturated fatty acid (MUFAs) profile showed a significant decrease in oleic acid (C18:1), eicosanoic acid (C20:1) and an unknown fatty acid on day 14 compared to day 1, and the same was observed with total MUFAs, which were significantly higher ($P<0.05$) on day 1 than on day 14 (Figure 26), while significant difference was not observed between the control treatment and the acclimated treatment over the period of 14 days. The effect of thermal acclimation was seen with the PUFAs, shown by a significant decrease in eicosapentaenoic acid (EPA) (C20:5) and a significant increase in the docosahexaenoic acid (DHA) (C22:6) on day 14 in the thermally acclimated treatment (Figure 27) ($P<0.05$). There was no significant difference at day 1 between the control and the thermally acclimated treatment (Figure 27) ($P>0.05$).

Table 36. Fatty acid composition of phospholipids fatty acids in the liver of *Trematomus bernacchii* acclimated for 14 days at 0 °C and 4 °C, and expressed in % of total membrane phospholipid fatty acids.

	Day 1		Day 14	
	T0	**T4**	**T0**	**T4**
C16:0	12.01 ± 0.48	13.72 ± 0.61	16.01 ± 0.76	14.30 ± 0.53
C18:0	5.63 ± 0.41	6.04 ± 0.56	6.49 ± 0.30	7.71 ± 0.47
SFA	**17.64 ± 0.68**	**19.76 ± 0.84**	**22.50 ± 1.00**	**22.01 ± 0.54**
C16:1c9	3.31 ± 0.28	4.33 ± 0.70	4.42 ± 0.78	3.09 ± 0.47
Unknown	nd	nd	2.44 ± 0.87	2.68 ± 0.11
C18:1c9	7.21 ± 0.42	6.87 ± 0.41	5.68 ± 0.26	5.98 ± 0.87
C18:1c11	9.05 ± 0.29	9.33 ± 0.31	8.55 ± 0.23	9.03 ± 0.83
C20:1c11	4.11 ± 0.11	3.62 ± 0.31	3.07 ± 0.27	3.26 ± 0.31
Unknown	5.39 ± 0.55	3.44 ± 0.15	1.93 ± 1.20	2.83 ± 1.10
MUFA	**31.81 ± 1.10**	**29.88 ± 0.87**	**26.08 ± 1.20**	**26.87 ± 1.00**
C20:4c5,8,11,14	4.75 ± 0.58	4.99 ± 0.27	4.75 ± 0.73	4.68 ± 0.34
C20:5c5,8,11,14,17	20.18 ± 0.71	20.28 ± 1.50	24.24 ± 1.10	19.05 ± 0.72
C22:6c4,7,10,13,16,19	25.62 ± 0.79		22.43 ± 0.64	27.38 ± 0.93
PUFA	**50.55 ± 0.53**	**50.36 ± 0.15**	**51.42 ± 2.00**	**51.11 ± 0.55**
C16:1c9/C16:0	0.33 ± 0.06	0.49 ± 0.13	0.27 ± 0.04	0.21 ± 0.03
C18:1c11/C18:0	1.44 ± 0.24	1.73 ± 0.29	0.88 ± 0.06	0.78 ± 0.11

Values are mean ± SEM (n=4), nd: not detected



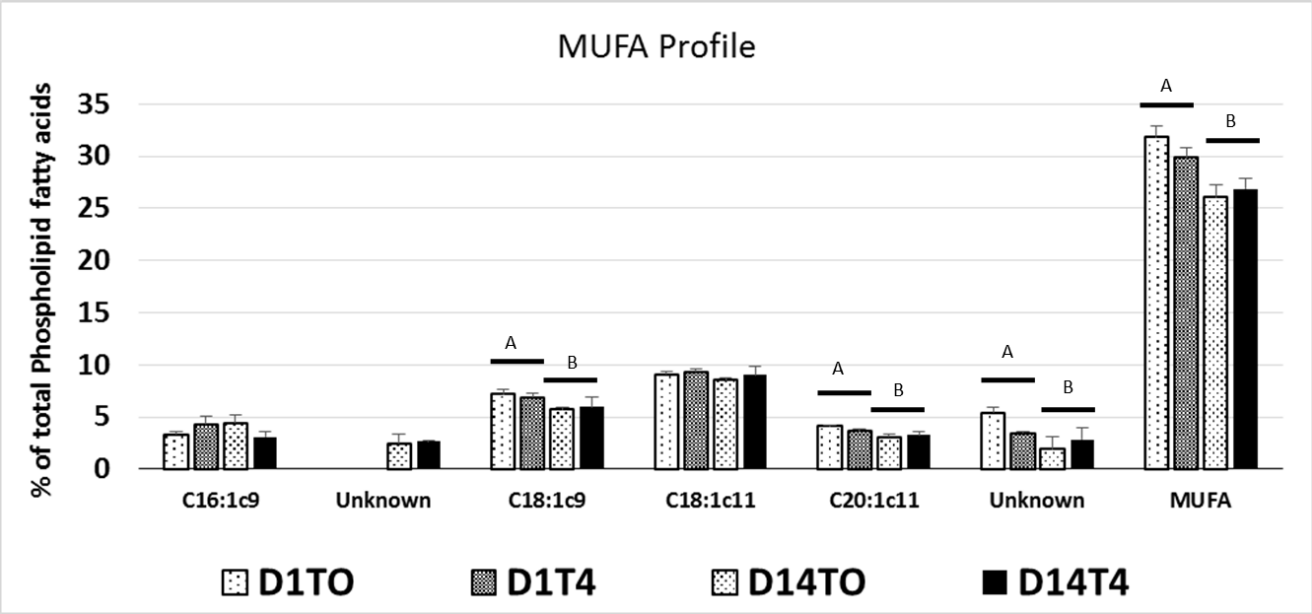


Figure 26. SFA and MUFA profile of *Trematomus bernacchii* (TB) in liver after 1 day and 14 days of thermal acclimation at 4 °C. Values are means ± SEM (N=4) for control temperature (T0: 0 °C) as well as for warm acclimation temperature (T4: 4 °C). TB: *Trematomus bernacchii*, D1 and D14: 1 day and 14 days after thermal acclimation. Significant effects of days of acclimation are indicated by different letters (upper case) (P < 0.05) and significant effects of thermal acclimation and time are indicated by different letters (lower case).

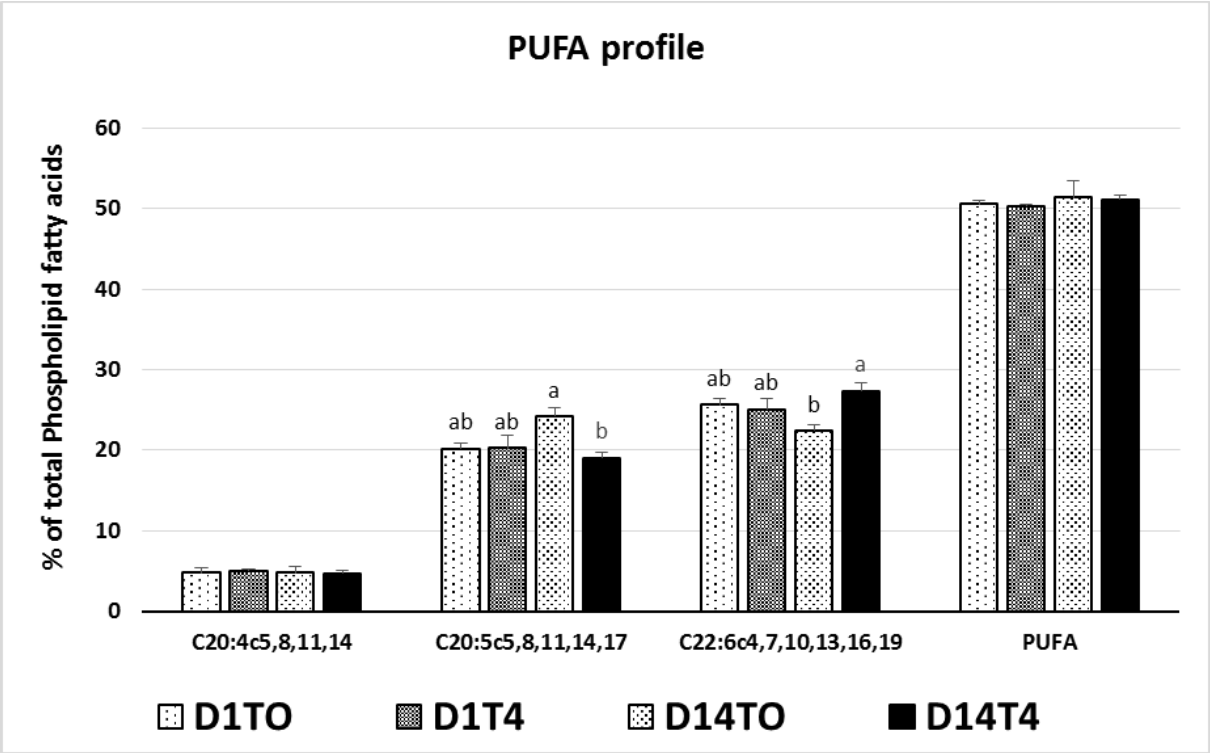


Figure 27. PUFA profile of *Trematomus bernacchii* (TB) in liver phospholipid after 1 and 14 days of thermal acclimation at 4 °C. Values are mean ± SEM (N=4) for control temperature (T0: 0 °C) as well as for warm acclimation temperature (T4: 4 °C). TB: *Trematomus bernacchii*, D1 and D14: 1 and 14 days after thermal acclimation. Significant effect of temperature and days of acclimation are indicated by different letters (P<0.05).

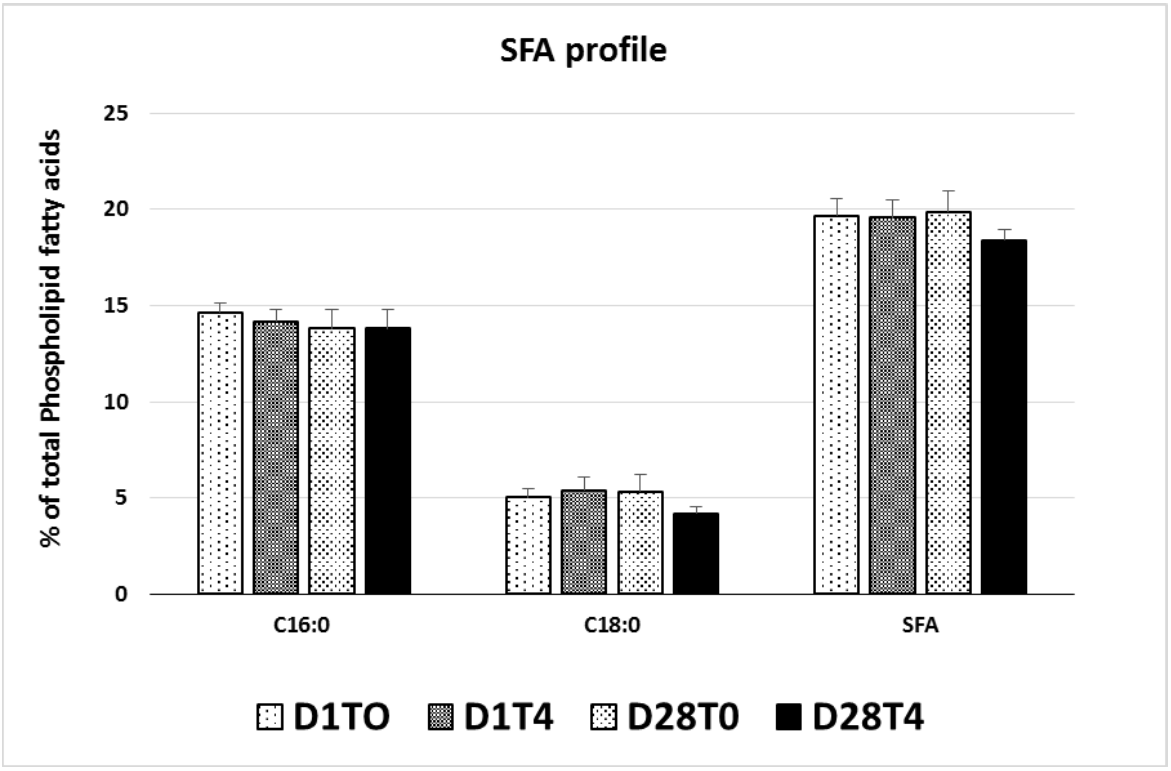
4.3.6 Membrane fatty acid profile after 1 and 28 days of thermal acclimation in the cryopelagic species *Pagothenia borchgrevinki*

The effect of thermal acclimation on the cryopelagic species *Pagothenia borchgrevinki* is presented in Table 37. Thermal acclimation for 28 days did not change the saturation state of the phospholipids there being no significant differences between control and thermal acclimated treatment in either PUFAs or SFAs (Figure 28 and 29, P>0.05) There was no change in the MUFAs with the exception of eicosonoic acid and total MUFAs. Eicosonoic acid significantly decreased on day 28, but there was no significant difference between the control and the acclimation treatment, whereas differences over time (days of acclimation) were significant (Figure29) (P<0.05), while temperature acclimation resulted in a significant increase in total MUFAs at day 1 and day 28 (Figure 28) (P<0.05).

Table 37. Fatty acid composition of phospholipids fatty acids in the liver of *Pagothenia borchgrevinki* acclimated for 28 days at 0 °C and 4 °C, expressed in % of total membrane fatty acids.

	Day 1		Day 28	
	T0	T4	T0	T4
C16:0	14.65 ± 0.46	14.18 ± 0.61	13.79 ± 1.00	13.79 ± 1.00
18:0	5.03 ± 0.44	5.39 ± 0.67	5.33 ± 0.86	4.20 ± 0.37
SFA	**19.68 ± 0.89**	**19.56 ± 0.92**	**19.86 ± 1.10**	**18.38 ± 0.58**
C16:1c9	2.90 ± 0.98	3.60 ± 0.21	3.66 ± 0.38	3.66 ± 0.38
Unknown	nd	nd	0.43 ± 0.43	1.81 ± 0.67
C18:1c9	11.00 ± 0.66	11.42 ± 0.50	10.05 ± 0.68	11.27 ± 0.32
C18:1c11	10.05 ± 0.44	10.52 ± 0.38	9.50 ± 0.50	9.69 ± 0.39
C20:1c11	2.61 ± 0.25	2.47 ± 0.08	2.11 ± 0.20	2.05 ± 0.23
Unknown	0.88 ± 0.54	1.54 ± 0.53	0.72 ± 0.72	1.17 ± 0.70
MUFA	**27.46 ± 1.20**	**29.54 ± 0.70**	**26.47 ± 0.83**	**30.15 ± 1.40**
C20:4c5,8,11,14	3.20 ± 0.47	3.04 ± 0.16	2.56 ± 0.10	3.49 ± 0.36
C20:5c5,8,11,14,17	21.85 ± 1.20	20.40 ± 1.40	18.40 ± 1.30	17.64 ± 2.00
C22:6c4,7,10,13,16,19	27.81 ± 1.10	27.46 ± 1.40	29.51 ± 2.90	30.35 ± 3.20
PUFA	**52.86 ± 0.51**	**50.90 ± 0.38**	**50.47 ± 2.30**	**51.47 ± 1.50**
C16:1c9/C16:0	0.20 ± 0.07	0.25 ± 0.02	0.27 ± 0.02	0.30 ± 0.04
C18:1c11/C18:0	2.24 ± 0.26	2.26 ± 0.36	2.11 ± 0.52	2.76 ± 0.31

Values are mean ± SEM (n=4), nd: not detected



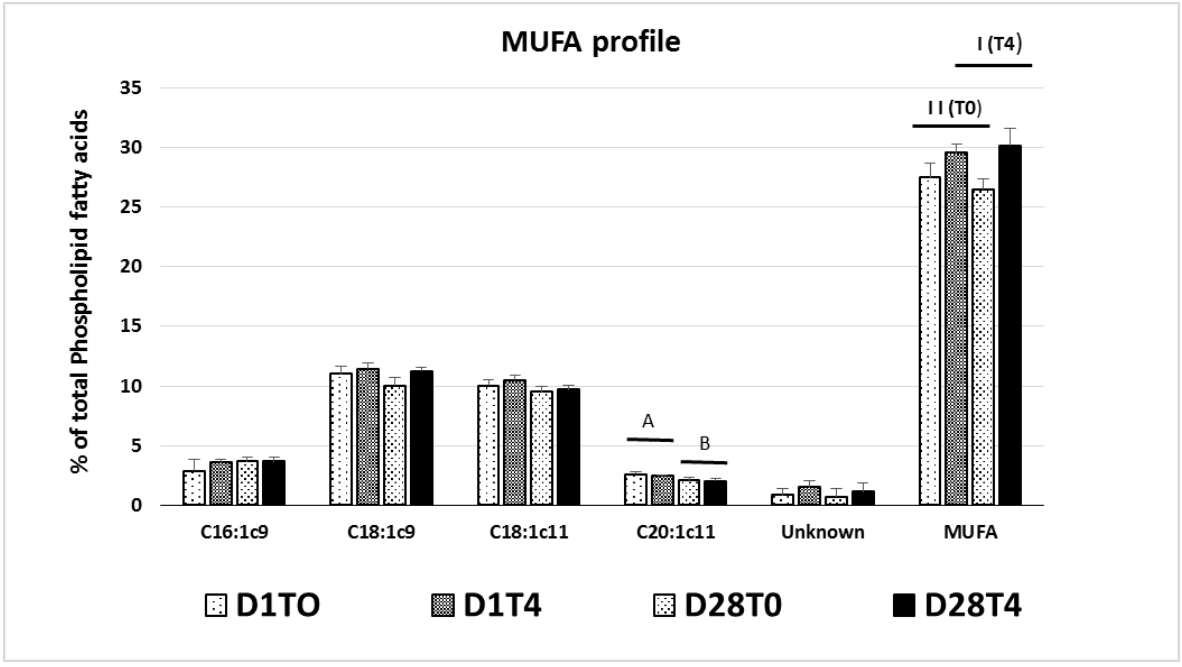


Figure 28. SFA and MUFA profile of *Pagothenia borchgrevinki* (PB) in liver after 1 and 28 days of thermal acclimation at 4 °C. Values are mean ± SEM (N = 4) for control temperature (T0:0 °C) and warm acclimation temperature (T4: 4 °C). PB: *Pagothenia borchgrevinki*, D1 and D28: 1 day and 28 days after thermal acclimation. Significant effects of thermal acclimation are indicated by different Roman numerals (P< 0.05). Significant effects of days of acclimation are indicated by different letters (P< 0.05).

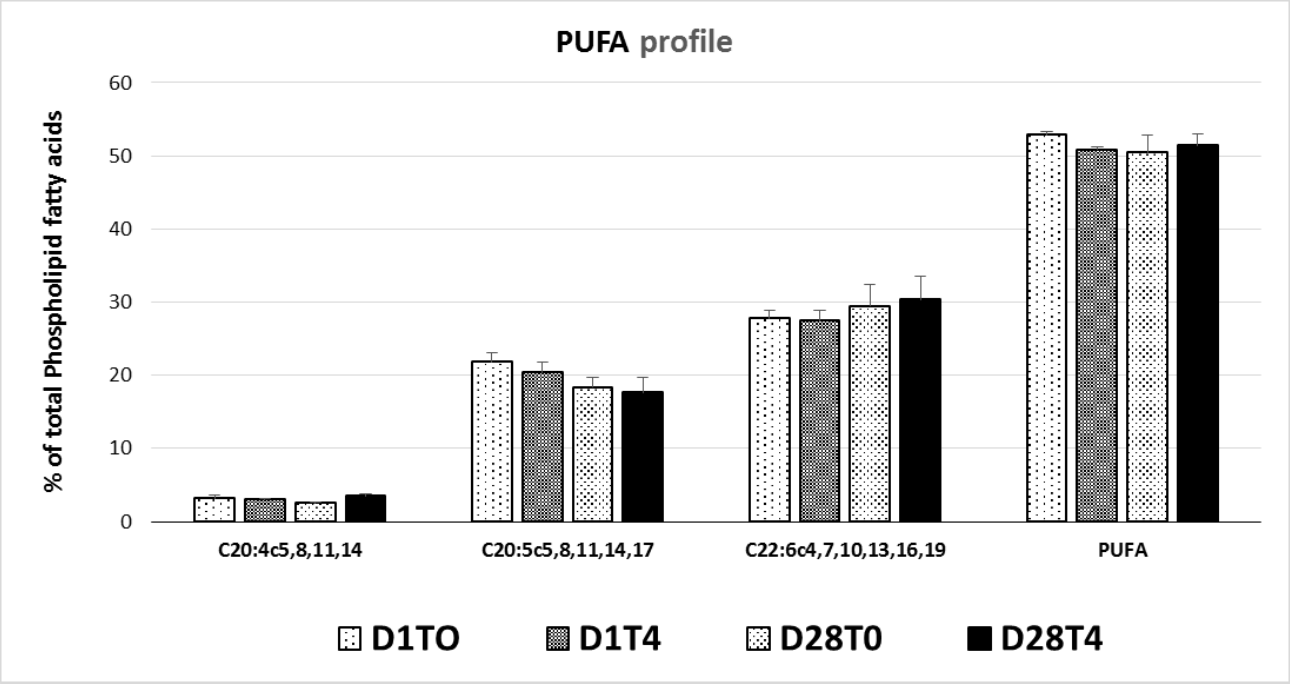


Figure 29. PUFA profile of *Pagothenia borchgrevinki* (PB) in liver after 1 day and 28 days of thermal acclimation at 4 °C. Values are mean ± SEM (N=4) for control temperature (T0: 0 °C) as well as for warm acclimation temperature (T4: 4 °C), PB: *Pagothenia borchgrevinki*, D1 and D14: 1 day and 28 days after thermal acclimation.

4.3.7 Membrane fatty acid profile after 7 days of thermal acclimation at 6 °C in *T. bernacchii* and in *P. borchgreviniki*

The effects of thermal acclimation on the fatty acid compositions after 7 days at 6 °C in *T. bernacchii* are presented in Table 38. Thermal acclimation resulted in a significant increase in stearate (C18:0) ($P<0.01$) and in total SFAs ($P<0.01$). In MUFAs, thermal acclimation resulted in a significant decrease in eicosenoate (C20:1) and in total MUFAs (Figure 30; $P<0.05$). There were significant changes in components of the PUFAs following thermal acclimation, although the total PUFAs remained unchanged by temperature. The changes in the components of PUFAs (Figure 30) included a significant decrease in the EPA (C20:5; $P<0.05$) and a significant increase in the DHA (C22:6; $P<0.05$).

The saturation profile of the cryopelagic species *Pagothenia borchgreviniki* remained unchanged after thermal acclimation at 6 °C for 7 days (Table 39), except for a significant decrease in eicosenoate (C20:1) which is part of the MUFA profile ($P<0.05$)(Figure 30). The other MUFA components, and the total MUFAs, were unchanged, nor were SFA and PUFA profiles ($P>0.05$)(Figure 30).

Table 38. Fatty acid composition of phospholipids fatty acids in the liver of *Trematomus bernacchii* acclimated 0 °C and 6 °C after 7 days of thermal acclimation, expressed in % of total membrane phospholipid fatty acids.

	T0	T6	P- value
C16:0	13.70 ± 0.44	16.49 ± 0.9	0.11
C18:0	5.28 ± 0.49	8.24 ± 0.58	0.018
SFA	**18.99 ± 0.61**	**24.727 ± 0.34**	**0.001**
C16:1c9	5.341 ± 0.24	4.543 ± 0.24	0.072
C18:1c9	6.677 ± 0.36	5.888 ± 0.45	0.245
C18:1c11	9.42 ± 0.57	8.807 ± 0.3	0.396
C20:1c11	3.833 ± 0.18	3.185 ± 0.084	0.032
Unknown	4.63 ± 1.2	2.571± 0.19	0.18
MUFA	**29.9 ± 1.0**	**23.08 ± 1.4**	**0.017**
C20:4c5,8,11,14	4.243 ± 0.38	4.944 ± 0.1	0.17
C20:5c5,8,11,14,17	21.79 ± 0.45	18.99 ± 0.75	0.049
C22:6c4,7,10,13,16,19	25.08 ± 0.76	28.25 ± 0.7	0.037
PUFA	**51.11 ± 0.5**	**52.19 ± 1.1**	**0.456**

Values are mean ± SEM (n=4), TO: 0 °C and T6: 6 °C acclimation temperature respectively. Significant P-values are highlighted in green.

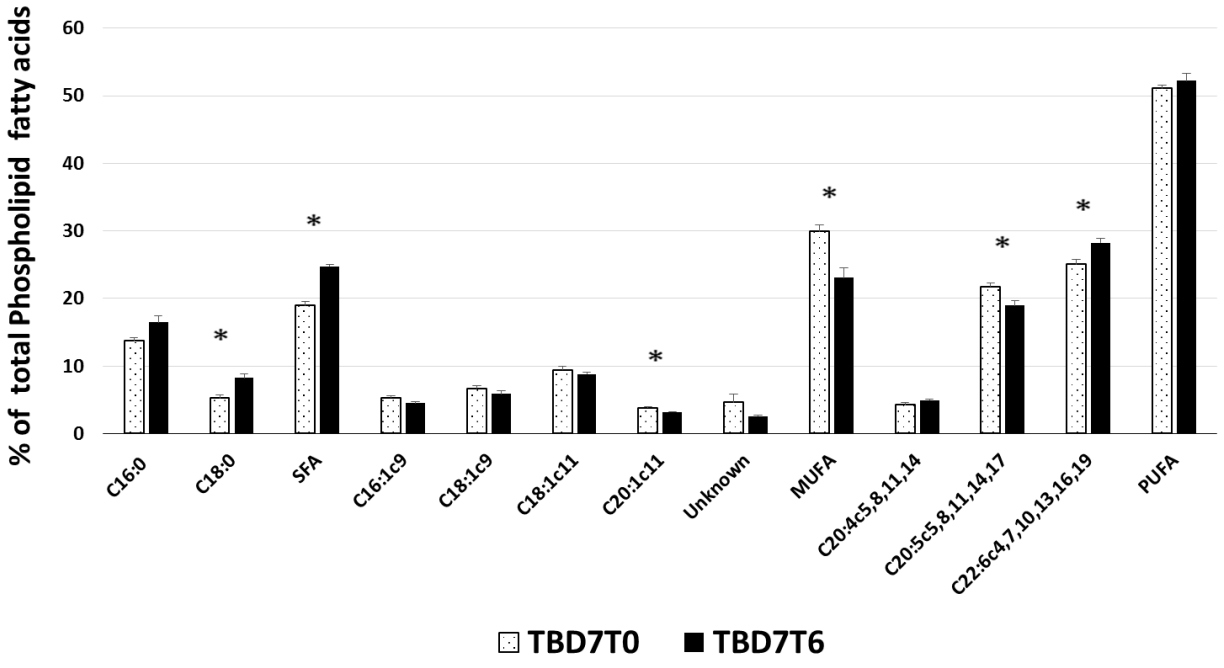


Figure 30. Phospholipid fatty acid profile of *Trematomus bernacchii* (TB) liver after 7 days (D7) of thermal acclimation at 6 °C. Values are mean ± SEM (N=4) for control temperature (T0: 0 °C) and warm acclimation (T6: 6 °C) (N=3). Significant effects of thermal acclimation are indicated by asterisks (P<0.05).

Table 39. Fatty acid composition of phospholipids fatty acids in liver of *Pagothenia borchgrevinki* acclimated at 0 °C and 6 °C after 7 days of thermal acclimation, expressed in % of total membrane fatty acids.

	T0	T6	P- value
C16:0	14.67 ± 0.29	13.71 ± 0.6	0.220
C18:0	4.54 ± 0.24	5.36 ± 0.68	0.339
SFA	**19.22 ± 0.51**	**19.08 ± 0.86**	**0.890**
C16:1c9	3.896 ± 0.34	4.267 ± 0.16	0.381
C18:1c9	11.018 ± 0.23	11.85 ± 0.68	0.332
C18:1c11	9.695 ± 0.14	10.01 ± 0.54	0.617
C20:1c11	3.052 ± 0.3	1.976 ± 0.08	0.041
Unknown	1.385 ± 0.47	1.644 ± 0.11	0.629
MUFA	**29.046 ± 0.27**	**29.74 ± 0.83**	**0.486**
C20:4c5,8,11,14	2.916 ± 0.12	3.09 ± 0.23	0.544
C20:5c5,8,11,14,17	20.11 ± 1.5	17.47 ± 1.80	0.317
C22:6c4,7,10,13,16,19	28.7 ± 2.0	30.62 ± 2.40	0.560
PUFA	**51.73 ± 0.55**	**51.19 ± 1.50**	**0.752**

Values are mean ± SEM (n=4), TO: 0 °C and T6: 6 °C acclimation temperature respectively. Significant P-values are highlighted in green.

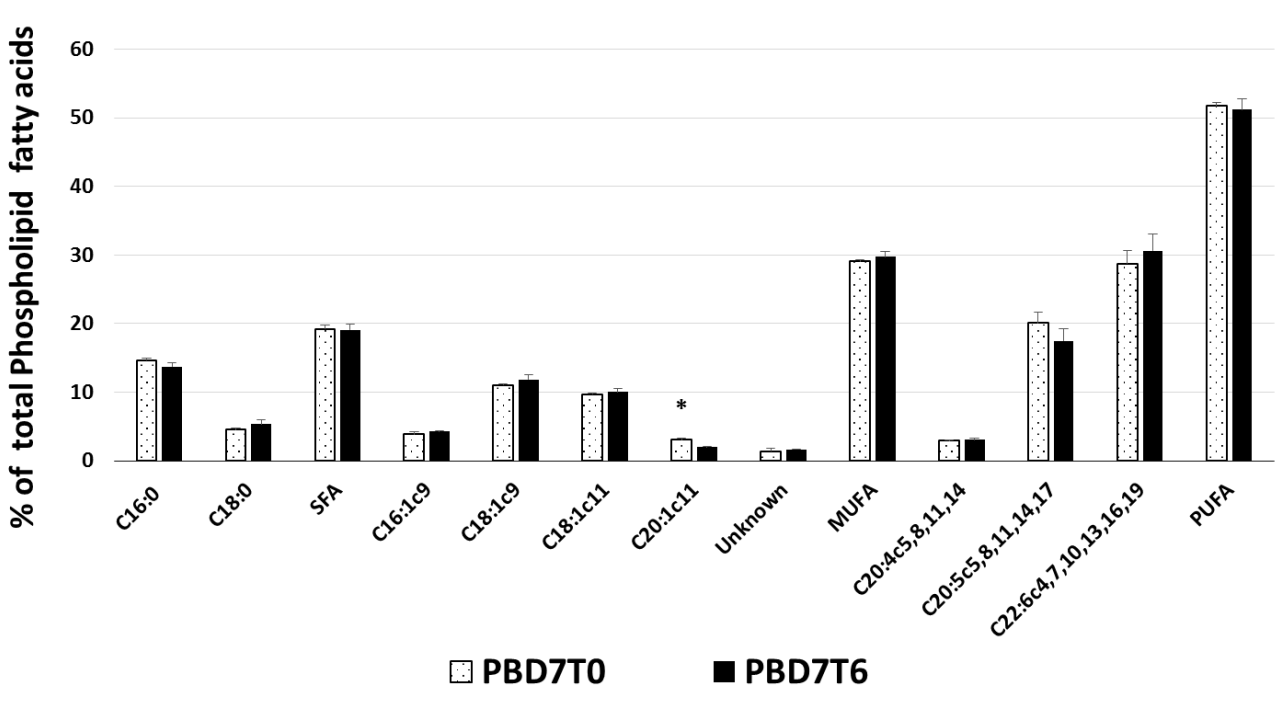


Figure 31. Phospholipid fatty acid profile of *Pagothenia borchgreviniki* (PB) in liver after 7 days (D7) of thermal acclimationat 6 °C. Values are mean ± SEM (N=4) for control temperature (T0: 0 °C) as well as warm acclimation (T6: 6 °C). Significant effects of thermal acclimation are indicated by asterisks (P<0.05).

4.3.8 Desaturation Indices in *T. bernacchii* and *P. borchgrevinki* acclimated to 4 °C

The effect of thermal acclimation for 14 days in *T. bernacchii* and 28 days in *P. borchgrevinki* on the desaturation index (DSI) (C18:1c9/C18:0) (C16:1c11/C16:0) are presented in Figures 32 and 33. For *T. bernacchii,* in general irrespective of temperature, acclimation time (days) for 14 days caused a significant decrease in the desaturation index for (C18:1c9/C18:0) as well as (C16:1c9/C16:0) (Fig.32 and 33). In the case of C16:1c9/C16:0 and C18:1c9/C18:0, the effect of thermal acclimation and the interaction between time (days) and temperature was not significant (P>0.05) (Figures 32 and 33). There was no difference in DSI (both C18:1c9/C18:0 and C16:1c9/C16:0) for the cryopelagic species *P. borchgrevinki* between day 1 and day 28 for either the control or the acclimated temperature (Figures 32 and 33).

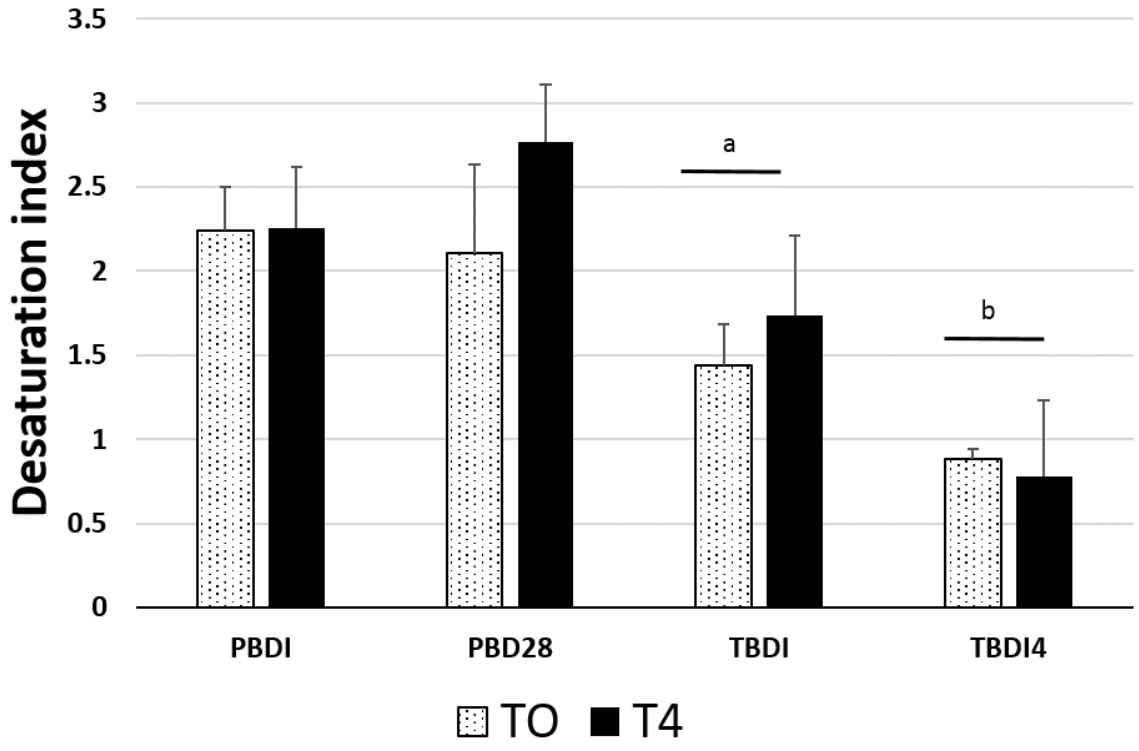


Figure 32. Changes in the desaturation Index (C18:1c9/C18:0) in the livers of *Pagothenia borchgrevinki* and *Trematomus bernacchii* acclimated at 4 °C for 28 days and 14 days, respectively. Values are mean ± SEM (n=4). Significant effects of days of acclimation are indicated by different letters.

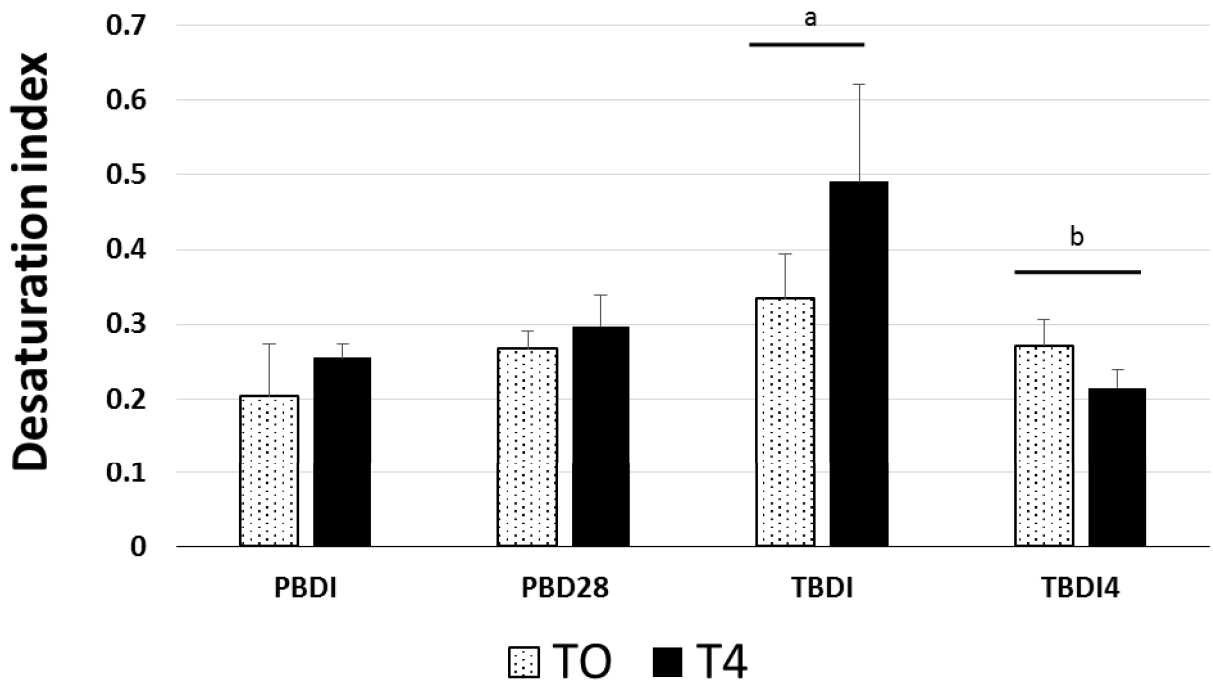


Figure 33. Changes in the desaturation Index (C16:1c9/C16:0) in the livers of *Pagothenia borchgrevinki* and *Trematomus bernacchii* acclimated at 4 °C for 28 days and 14 days, respectively. Values are mean ± SEM (n=4). Significant effects of acclimation time (days) are indicated by different letters.

4.3.9 Desaturation Indices in *T. bernacchii* and *P. borchgrevinki* acclimated at 6 °C for 7 days

The desaturation Index after 7 days of thermal acclimation at 6 °C is presented in Figure 34. In the case of (C16:1c9/C16:0) there was no significant change after 7 days of acclimation for either species (Figure 34, left). However, the DSI (C18:1c9/C18:0) for *T. bernacchii* decreased significantly after 7 days of thermal acclimation at 6 °C (Figure 34, right; $P<0.05$). No change was detected in the cryopelagic species *P. borchgrevinki* as there was no difference between the DSI of the control and the acclimated treatment (Figure 34, right).

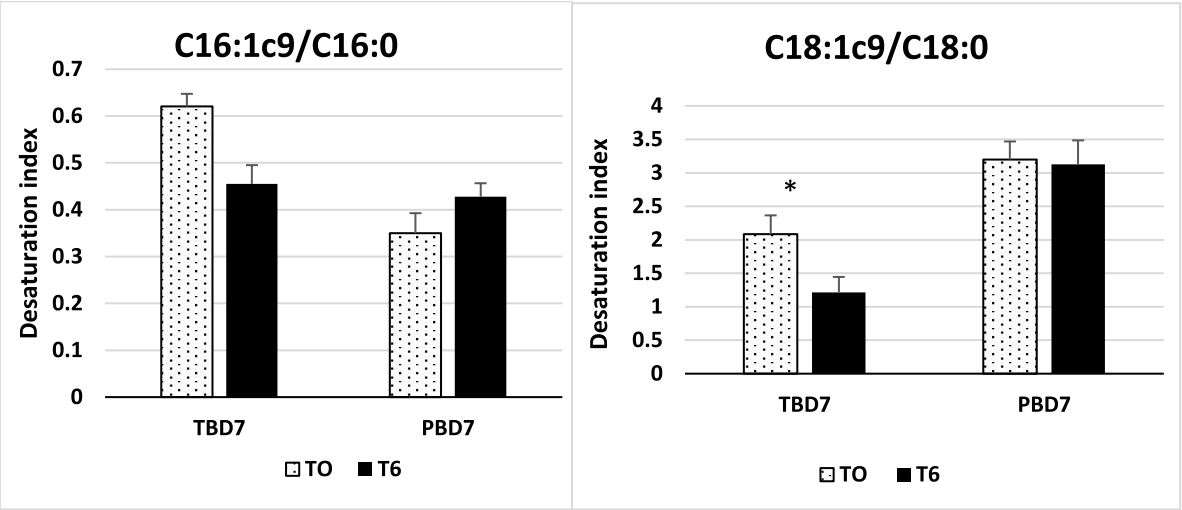


Figure 34. Changes in the desaturation Index (C16:1c9/C16:0, C18:1c9/C18:0) in the livers of *Pagothenia borchgrevinki* and *Trematomus bernacchii* acclimated at 6 °C for 7 days. Values are mean ± SEM (n=4). Significant effects of thermal acclimation are indicated by asterisks (P<0.05).

4.3.10 Desaturation Indices of Antarctic and non-Antarctic species

The Desaturation Indices for the Antarctic species and the non-Antarctic species are presented in Figure 35. The Antarctic species had higher DSIs (C16:1c9/C16:0), compared with the non-Antarctic species *Notolabrus celidotus* (Figure 35, left; P<0.05). Among the Antarctic species, *T. bernacchii* had the highest DSIs and *C. hamatus* had the lowest (Figure 35, left; P<0.05). The Antarctic species also had higher DSIs for (C18:1c9/C18:0). Among them *C. hamatus* had the highest DSI followed by *P. borchgrevinki,* while the other Antarctic and the non-Antarctic species had similar DSIs.

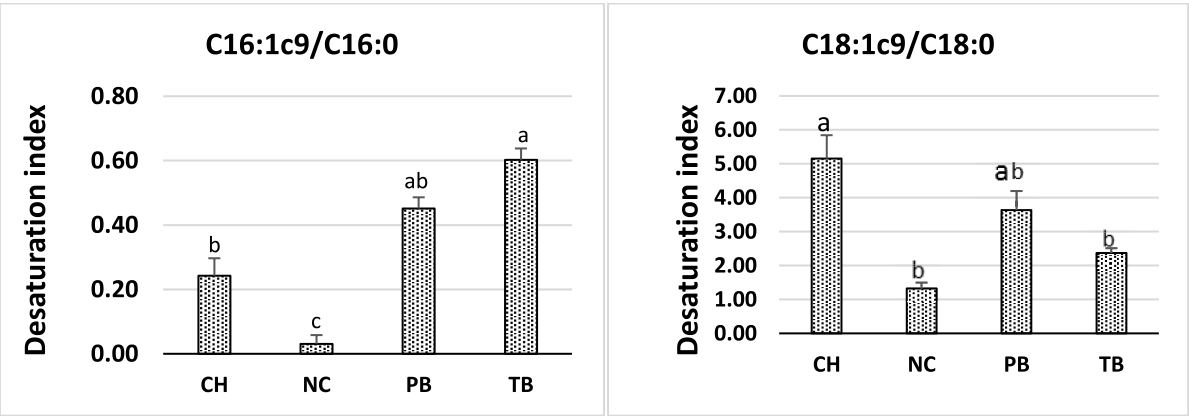


Figure 35. Changes in the desaturation Index (C16:1c9/C16:0, C18:1c9/C18:0) in livers of Antarctic species *Chionodraco hamatus* (CH), *Pagothenia borchgrevinki* (PB), and *T. bernacchii* (TB) and the non-Antarctic species *Notolabrus celidotus* (NC). Values are mean ± SEM (n=4). Significant effects among species are indicated by different letters (P<0.05).

4.3.11 Quality check for the presence of cholesterol

The TLC quality check for the presence of cholesterol in the lipids extracted from *T. bernacchii* liver and *P. borchgrevinki* using TLC is presented in Figure 36. The samples were analysed along with

cholesterol standard which migrates to the same position as cholesterol in the lipid fractions of *T. bernacchii* and *P. borchgrevinki*, which stained blue after development. Free fatty acids migrated above cholesterol and the phospholipids below, as expected.

1. 11 mM cholesterol
2. 1.1 mM cholesterol
3. 10 µl of lipid extract isolated from *Trematomus bernacchii* liver
4. 10 µl of lipid extract isolated from *Pagothenia borchgrevinki* liver

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Figure 36. Analyses of lipid fractions of *Trematomus bernacchii* and *Pagothenia borchgrevinki* along with standard cholesterol at two different concentrations on TLC.

4.3.11.1 Membrane cholesterol in Antarctic and non-Antarctic species

Membrane cholesterol contents of all the Antarctic and the non-Antarctic species are presented in Figure 37. The non-Antarctic species *Notolabrus celidotus* had significantly more membrane cholesterol compared to the Antarctic species *P. borchgrevinki* ($P<0.05$).

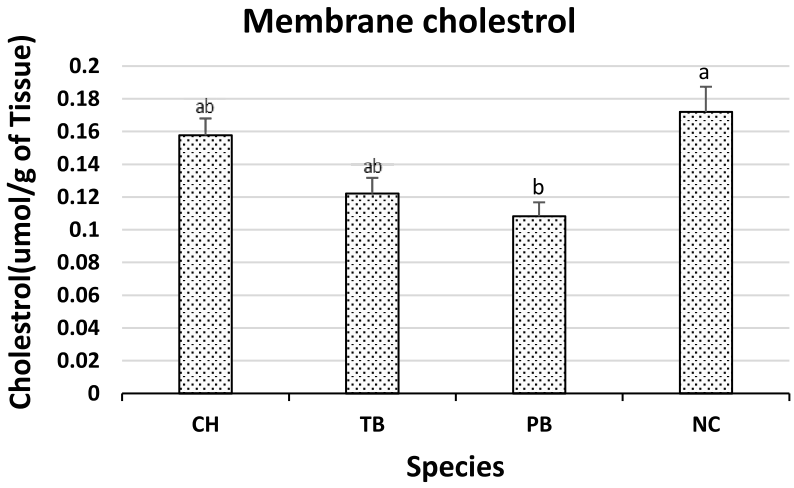


Figure 37. Membrane cholesterol concentration in the livers of Antarctic species *Chionodraco hamatus (*CH), *Pagothenia borchgrevinki* (PB), *Trematomus bernacchii* (TB) and non-Antarctic species *Notolabrus celidotus* (NC). Significance among species are indicated by different letters.

4.3.11.2 Membrane cholesterol in *Trematomus bernacchii* and *Pagothenia borchgrevinki* after thermal acclimation

The effect of 14 and 28 days temperature acclimation on membrane cholesterol in *T. bernacchii* are presented in Figure 38. The interaction of temperature and days of acclimation was non-significant in *T. bernacchii*. Further, irrespective of temperature, membrane cholesterol decreased from day 1 to day 14 (P<0.05; Figure 37, left). Thermal acclimation for 28 days at 4 °C had no significant effect on membrane cholesterol in *P. borchgrevinki*.

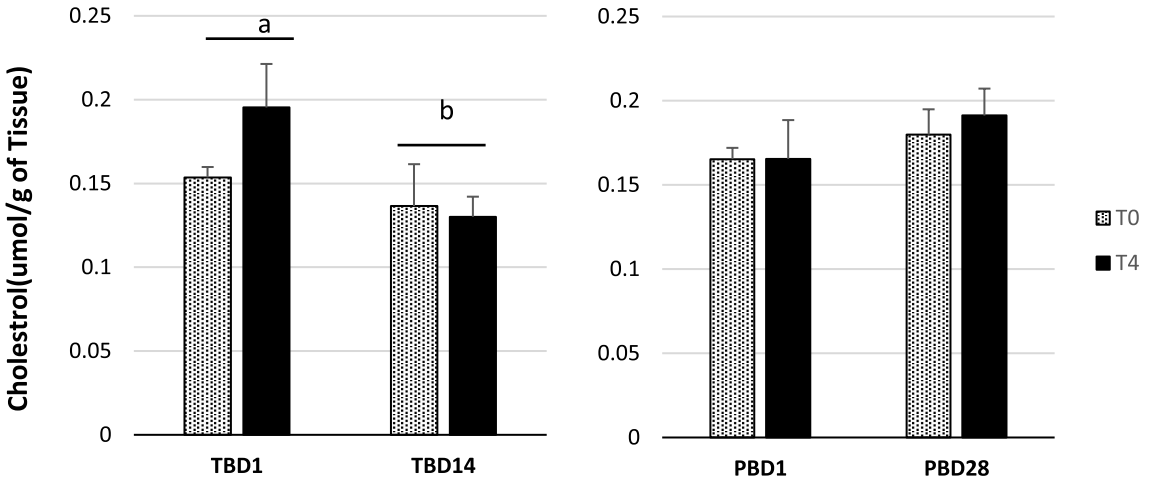


Figure 38. Effect of thermal acclimation on membrane cholesterol concentration in the livers of *Trematomus bernacchii* (TB) and *Pagothenia borchgrevinki* (PB). Membrane cholesterol was determined at various time points (days after thermal acclimation; D1, D14 and D28). Significant effects of acclimation time (days) are indicated by different letters (P<0.05).

4.3.12 Plasma osmolality

The plasma osmolality change in *T. bernacchii* after 14 day acclimation is presented in Figure 39. The effect of temperature was non-significant on the plasma osmolality while effect of temperature and time (days) were significant (P<0.01). Osmolality significantly decreased on day 3 and day 7 for the thermally acclimated treatment when compared to the control at day 14 (P<0.01; Figure 39).

The plasma osmolality change in *P. borchgrevinki* over the 28 days of thermal acclimation is presented in Figure 39. The effect of temperature led to significant reduction in osmolality for thermal acclimation treatment (P<0.01). Also there was a significant effect of temperature and time (days) which resulted in a significant decrease in plasma osmolality after day 2 of thermal acclimation. At day 2 the plasma osmolality was significantly the highest of thermally acclimated *P. borchgrevinki* plasma samples; following which there was a decrease in plasma osmolality at day 7

and day 28 (P<0.01). Plasma osmolality in *P. borchgrevinki* at 0 °C over the 28 days of acclimation was not statistically significant and remained unchanged (P>0.05).

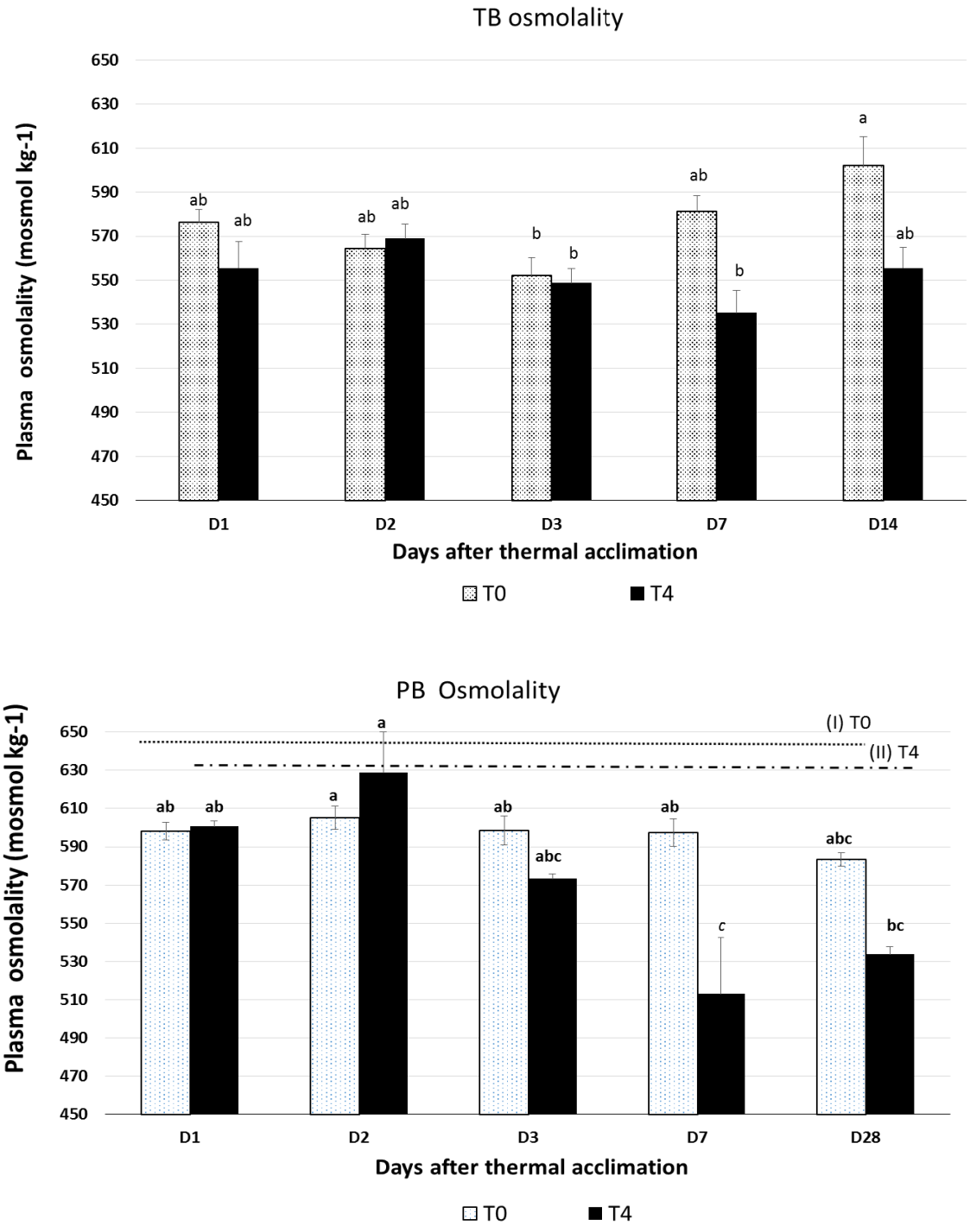


Figure 39. Plasma osmolality determined at various times (days after thermal acclimation; D1, D2, D3, D7 and D28) at 4 °C (T4) and the control temperature of 0 °C (T0) in *Pagothenia borchgrevinki* (PB) and *Trematomus bernacchii* (TB). Significant effect of temperature are indicated by different Roman numerals and effect of temperature and days of acclimation are indicated by different letters (P<0.05).

4.4 Discussion

Changes in the membrane components of liver tissue in three Antarctic fish were compared with those in one non-Antarctic species to look for signatures of thermal adaptation. The thermal acclimatory process of membrane remodelling in the stenothermal Antarctic fish species was also examined. This is the first study to examine the homeoviscous adaptive response (HVA) of Antarctic species acclimated to 4 and 6 °C. The phospholipid fatty acid profile was analysed along with membrane cholesterol, and osmolality was also determined as an indicator of membrane functions.

Key results

1. The membrane phospholipid fatty acid compositions of all three Antarctic fish differed from non-Antarctic fish species.

2. Thermal acclimation tested in two species to 4 °C did not induce the HVA response in two of the Antarctic species. HVA response was induced in one of the Antarctic fish species, *Trematomus bernacchii*, when it was acclimated to a warming temperature of 6 °C.

Distinct phospholipid fatty acid composition of Antarctic species

In the present study phospholipid fatty acid profiles of the Antarctic species differed from the non-Antarctic species, containing significantly more unsaturated fatty acids. This was consistent with earlier reports where other Antarctic species, higher latitude species, and cold water fish species have higher proportions of unsaturated fatty acids (Logue et al., 2000). Such changes are part of the homeoviscous response by which ectotherms remodel their membrane lipids to offset the detrimental effects of temperature changes. In general, phospholipid saturation strongly correlates with environmental temperature, and increased unsaturation has been observed with a drop in temperature. Temperature-induced phospholipid fatty acid unsaturation changes have been well established in thermal acclimation studies (Cossins, 1977; Hsieh & Kuo, 2005; Trueman et al., 2000; Williams & Hazel, 1995). Antarctic fish are stenothermal and do not experience seasonal variation. They have a narrow thermal adaptable range and are in the constant cold, but have an adaptive homeoviscous response that has been acquired over their evolutionary history. A key question of this study was to determine whether the recently diversified icefish (*C. hamatus)* have changes in the membrane lipids when compared to the other closely related Antarctic species. This study shows

that proportions of SFAs including palmitic acid (C16:0), were similar in the Antarctic species *C. hamatus and* non-Antarctic species *N. celidotus.* Both were significantly higher when compared to the other Antarctic species (Figure 25). Also shown in the PCA analysis the icefish group separately from the other two Antarctic species and had high proportions of palmitic acid (C16:0). Higher proportions of the SFA, palmitic acid in the liver membranes of the icefish could be one of the features acquired after diversification from the other Antarctic species. This is the first study to analyse the liver membranes of icefish. However, in another study changes were observed in the erythrocyte membrane lipids of the icefish *C. hamatus.* Contrary to our findings changes involved, destauration of longer chain fatty acids such as C:20–C:22 in icefish, while shorter chain fatty acids such as C:16 and C:18 became unsaturated in *T. bernacchii* and both species had consistent membrane fluidity (Palmerini et al., 2009). The recently diversified icefish species could have evolved with specific adaptations, such as more palmitic acid in liver membranes shown in the present study and unsaturation of longer chain fatty acids in ghost cell membranes (Palmerini et al., 2009). In the present study, concentration of saturated fatty acid stearic acid (C18:0), was consistently lower in all the Antarctic species and overall, palmitic acid (C16:0) formed the major fraction of the total saturated fatty acids in Antarctic fish species and this aligned with a study comparing the fatty acid compositions of phospholipid from muscle tissue in 15 marine species from the Southeast Brazilian coast and two from East Antarctic (Visentainer et al., 2007). Palmitic acid was the major SFA and represented 16 – 30 % of the total FA content for all organs in *Notothenia codiceps* and *Notothenia rossii* (Magalhaes et al., 2010). In the present study higher proportions of palmitic acid and low stearic acid (C18:0) fractions could perhaps be due to increased desaturation of stearic to oleic acid.

A high concentration of *cis*-vaccenic acid was a feature of all the Antarctic fish species in the present study. High *cis*-vaccenic acid contents have also been observed in the Antarctic fish *Pleuragramma antarcticum* in two stages of its life cycle (larvae and juvenile stage) (Mayzaud et al., 2011). Similarly, high- latitude fish of the sub-Arctic have significantly higher *cis*-vaccenic acid contents in muscle tissue (Murzina et al., 2013). Studies examining the fatty acid profile of liver microsomes in Antarctic *Dissostichus mawsoni* also found higher contents of *cis*-vaccenic acid compared to non-Antarctic fish (Romisch et al., 2003). These studies indicate a linkage between temperature and the proportion of *cis*-vaccenic acid in the membrane of the cold-adapted fish. The conformation of unsaturated *cis*-vaccenic acid presents a possible structural advantage and has a potential role in maintaining membrane fluidity, which may be the reason for its selective incorporation in the

membranes of Antarctic fish. Studies have shown that *cis* vaccenic acid can act as a membrane fluidizing agent, which enhances transport of glucose in adipocytes (Pilch et al., 1980). In another study, *cis*–vaccenic acid increased fluidity in porcine pulmonary endothelial cells and thus increased serotonin transport (Block & Edwards, 1987). In this study, other monounsaturated fatty acids, such as eicosaonate were present in higher proportions in Antarctic fish but not detected in non-Antarctic fish. Eicosaonate may also aid in the expanded membrane composition to provide the required fluidity.

In this study composition of PUFAs varied between Antarctic and non-Antarctic species, although total PUFAs were not significantly different. Antarctic fish had significantly less arachidonic acid (ARA) and more of eicosapentaenoic acid (EPA) than non-Antarctic species. However, the amounts of docosahexaenoic acid (DHA) were similar in both Antarctic and non-Antarctic species. These data align with other studies of Antarctic fish species that found more EPA in the membranes and less ARA. This has been observed in the muscle phospholipids of five species of Antarctic fish with different life histories (pelagic, benthopelagic and benthic) from the Weddell and Lazarev Seas (Hagen et al., 2000). More EPA and low ARA has been observed in both the juvenile and larval stages in the Antarctic silverfish, *Pleuragramma antarcticum* (Mayzaud et al., 2011). Similar findings were observed in the liver microsomal lipids of the Antarctic *D. mawsoni,* which had less DHA compared to trout and slightly less than the carp species, whereas the EPA content was higher than in the non-Antarctic species (Romisch et al., 2003) suggestive of a specific role for EPA in the Antarctic fish membranes. In general, cold-adapted species incorporate (n-3) PUFAs and have depleted (n-6) long chain PUFAs in their phospholipids. This was shown in the cold thermal acclimation studies of gill phospholipids of fresh water alewives (*Alosa pseudoharengus*) (Snyder et al., 2012). Cold transfer of the *C. elegans* worms, resulted in an increase in EPA, which was transient and substantial (Murray et al., 2007). The proportions of DHA in this study are not significantly different across the Antarctic and non-Antarctic species, suggesting that EPA could offer additional roles associated with cold tolerance whereas DHA, having an expanded conformation, has a structural advantage over EPA in contributing to the membrane fluidity. Other than membrane fluidity, EPA may help stabilization of the hyperfluid membranes, indicated in the study of *Shewanella violacea*,a psychrophilic piezophile that can grow at high pressures and low temperatures and has high amounts of EPA (Usui et al., 2012). Mutants of this strain that lack the omega-3 PUFA synthase gene have disturbed membrane over a range of pressures compared to the wild type whose membranes are unperturbed. This suggests that maintaining membrane stability at high pressure is more important than fluidity (Usui et al., 2012). Hypercholesteraemic rats whose membrane fluidity is reduced, have been shown to

have increased platelet membrane fluidity when fed DHA, but not when fed EPA (Hashimoto et al., 2006). A similar effect has been shown in aortic endothelial cells (Hashimoto et al., 1999). EPA, but not DHA, has been shown to be a potent anti-inflammatory agent, whereas ARA is highly pro-inflammatory (Sears & Ricordi, 2011; Seki et al ., 2009). EPA is one of the major (n-3) PUFAs present in the membrane of the Antarctic fish and the specific roles of this fatty acid need further investigation in a larger range of fish species.

Lack of homeoviscous response in Antarctic species at 4 °C of thermal acclimation

In the present study, thermal acclimation at 4 °C did not induce the major common cellular response of homeoviscous response in either the benthic Antarctic *T. bernacchii* or the pelagic Antarctic *P. borrchgrevinki*. Changes in the phospholipid fatty acid profile were observed in *T. bernacchii* but not the expected homeoviscous response. The changes were associated with the duration of acclimation rather than the effect of temperature. Total SFAs, and their major components *viz.,* palmitic acid and stearic acid were high on day 14, and the same trend was observed for MUFAs where oleic acid was high on day 14. It is unclear why the acclimation time, rather than the temperature, resulted in such a change. In the case of PUFAs, thermal acclimation resulted in a decrease in EPA and an increase in DHA. As explained above, EPA could be associated with specific functions in the extreme cold, perhaps in stabilizing membranes (Usui et al., 2012) or in protective roles associated with extreme cold by reducing inflammation (Sears & Ricordi, 2011; Seki et al., 2009). Membrane remodelling does not appear to be required and the saturation states are sufficient for the membranes to function at 4 °C. In the pelagic species, *P. borchgrevinki*, increased temperature resulted in a significant increase in the MUFAs at day 1 as well at day 28, but the saturation states of the PUFAs and SFAs were unchanged. These findings align with thermal acclimation experiments in Antarctic species at 4 °C, which resulted in unchanged membrane states showing no signs of a HVA response. This was shown in the benthic *Trematomus bernacchii* and *T. newnesi* in the membranes of gills, kidneys, liver and muscle (Gonzalez-Cabrera et al., 1995). The current findings have extended this observation to a cryopelagic species as well as confirming them in the same benthic species, as the saturation states upon acclimation were unchanged. Another study aligning with these findings is that mitochondrial membrane saturation states were also unchanged upon thermal acclimation in two Antarctic fish species (Strobel et al., 2013).

Thermal acclimation at 6 ˚C results in HVA response in *T. bernacchii*, but not in the pelagic species *P. borchgrevinki*

A homeoviscous response was shown in *T. bernacchii* upon thermal acclimation at 6 °C. This response was dominated by a significant increase in overall SFAs the major increase being stearic acid rather than palmitic acid (C16:0). There was a decline in unsaturated fatty acids, especially total MUFAs and the PUFA component EPA and an increase in DHA, but there was no change in total PUFAs. Saturated fatty acids reduce membrane fluidity and result in highly ordered membranes that are rigidified. This response is induced upon warm acclimation as a protective mechanism to offset the deleterious effects on the membrane lipids, which become more fluid upon warm acclimation (Hazel, 1995). It has been seen in the highly ordered synaptic membranes of warm-adapted fish (Logue et al., 2000).

There are very few reports of membrane composition after warm acclimation in Antarctic species, and no study to date has been done at 6 °C for these Antarctic species. Previous conclusions have been based on studies of non-Antarctic fish. Warm acclimation of *Dicentrarchus labrax* for 84 days at 29 °C compared to a control temperature of 14 °C resulted in responses that were tissue-specific, with changes observed in brain membrane lipids rather than in the liver. There was an increase in saturated fatty acids (C18:0) of the brain polar lipids and a decline in PUFAs *viz.,* EPA, DHA and ARA (Skalli et al., 2006). Liver and muscle tissue have been shown to be more responsive than other tissues to warm acclimation in fresh water alewives (*Alosa pseudoharengus*) where warm acclimation resulted in a significant increase in SFAs and a decline in PUFAs (Snyder et al., 2012). These studies are in alignment with the predicted trend of HVA response upon warm acclimation and the results of this study. Together, the body of research indicates that this response varies with species and among tissues. Also in contrast to *T. bernacchii,* the cryopelagic species *P. borchgrevinki* do not have the HVA response upon thermal acclimation at 6 °C. In another study, warm acclimation of the Antarctic species *Notothenia rossii* to 7 °C and *Lepidonotothen squamifrons* to 9 °C did not result in a HVA response in mitochondrial membranes. However, the proton leak capacities were unaffected, indicating high plasticity to warm acclimation (Strobel et al., 2013). *P. borchgrevinki* has a high degree of thermal plasticity (Franklin et al., 2007) and a higher upper lethal temperature compared to *T. bernacchii* (Somero & DeVries, 1967), which could perhaps result in an HVA response beyond 6 °C.

Desaturation index correlates to the membrane saturation status

In the present study the desaturation indices (C16:1c9/C16:0) and (C18:1c9/C18:0) were shown to correlate with the saturation states of the membrane. The Antarctic species had a high desaturation index (C16:1c9/C16:0) compared to the non-Antarctic species, whereas this trend is not specific for the DSI (C18:1c9/C18:0). High DSI (C16:1c9/C16:0) in the Antarctic fish species could be attributed to an increase in the MUFA palmitoleic acid C16:1c9 reflecting higher desaturation of palmitic acid by stearoyl-CoA desaturase**.** Furthermore, in this study there was significant decline in DSI (C18:1c9/C18:0) upon thermal acclimation at 6 °C in *T. bernacchii*. The HVA response with a significant increase in the SFA stearic acid (C18:0) could perhaps be due to a lowering of desaturase activity upon thermal acclimation. This decline in DSI was not observed at 4 °C in either of the species tested as they did not exhibit the HVA response. A positive correlation does exist between the SCD activity with the desaturation index, as previously established in two fish species, milk fish and the grass carp when subjected to cold acclimation from 25 °C to 15 °C over 21 days (Hsieh & Kuo, 2005). In milk fish and grass carp, the change in SCD activity was parallel to the desaturation index (ratio of C16:1c9/C16:0 and C18:1c9/C18:0). Milk fish had a significant increase of SCD activity and decline one day earlier than the DSI where as in grass carp the SCD activity and the increase in DSI was gradual and maintained at a high level over the acclimation period (Hsieh & Kuo, 2005). Thus, the present findings in this study of correlation with the desaturation index and membrane saturation upon thermal acclimation at 6 °C and in the Antarctic species could also correlate with the SCD activity.

Thermal acclimation has no effect on membrane cholesterol in the Antarctic species

Temperature of acclimation had no effect on membrane cholesterol in the benthic species *T bernacchii*. Irrespective of temperature, there was a significant decline in the membrane cholesterol after 14 days of thermal acclimation. There was an effect of duration of acclimation rather than temperature. The membrane cholesterol content in the pelagic species *P. borchgrevinki* was unaffected either by temperature or duration of acclimation. Very few studies have examined the effect of thermal acclimation on membrane cholesterol. A study that examined the effect of a chemical fluidizer and temperature on goldfish membranes showed that increased temperature resulted in a significant decline of cholesterol in the gill membranes, but it had no effect on the brain and liver cholesterol concentration (Gonzalez et al., 2013). They attributed the decline to an interaction with osmoregulation. In the same study, the membrane fluidizer PCB 153 was found to cause an increase in the cholesterol in liver and brain tissue. The structure of cholesterol mimics

phospholipid structure and intercalates in the phospholipid membrane bilayer resulting in an increase in membrane order and a reduction in membrane fluidity (Crockett, 1998). Cholesterol is known to counter the effects of increased temperature on membrane lipids and an increase in cholesterol is often observed at high temperatures (Crockett, 1998). Cholesterol did not change in my study, suggesting that the fluidity is unchanged at 4 °C under thermal acclimation for both Antarctic species examined.

Analysis of membrane cholesterol across the four species showed no overall differences between Antarctic and non-Antarctic species, although more membrane cholesterol was observed in the non-Antarctic species (*Notolabrus celidotus*) followed by the Antarctic species *C. hamatus* and *T. bernacchii,* and significantly more than *P. borchgrevinki.* In another study, the Cholesterol in erythrocyte ghost membranes was high in *C. hamatus*, followed by the non-Antarctic species *Anguilla anguilla,* while there was less cholesterol in other Antarctic and non-Antarctic species. These findings suggest that cholesterol contents could vary with species rather than temperature (Palmerini et al., 2009).

Thermal acclimation results in a decline in plasma osmolality in both Antarctic species

An inverse relationship exists between serum osmolality and water temperature. Inorganic serum ions tend to accumulate at lower temperatures and result in high osmolality. Antarctic fish have high serum osmolality. In an analysis of 11 teleost species, the serum concentration of Antarctic species was higher than the temperate species (Dobbs & DeVries, 1975). It was also observed that fish inhabiting cold waters, other than Antarctic fish, had high serum inorganic ion concentrations, and these inorganic ions have been shown to have protective roles in freezing avoidance by decreasing the melting point (O'Grady & DeVries, 1982). In my study, thermal acclimation caused a significant decline in serum osmolality in *P. borchgrevinki.* Other studies have also shown reduced osmolality upon thermal acclimation (Gonzalez-Cabrera et al., 1995; Guynn et al., 2002; Hudson, Brauer et al., 2008; Lowe & Davison, 2005). A transient increase in osmolality in *P. borchgrevinki*, but not in *T. bernacchii*, at day 2 was observed here. This increase has been attributed to increased efflux of water and retention of ions, mainly due to the alteration of permeability to ions brought about by the release of stress hormones, cortisol and catecholamine (Lowe & Davison, 2005). It is unclear why this transient increase in osmolality is seen in *P. borchgrevinki* but not *T. bernacchii*. The osmolality fell in both species after day 3 of thermal acclimation and the reduction was significant at day 7. This decline in the osmolality is mainly attributed to increased Na^+/K^+- ATPase

activity as a result of an increase in the turnover of the enzyme, as previously found in a study of Na^+-/K^+- ATPase pumps in gill tissue of the Antarctic notothenioid *T. bernacchii* and the non-Antarctic notothenioid *Notothenia angustata* (Guynn et al., 2002). This study, and the other studies mentioned above, further establishes the effective functioning of membranes and membrane fluidity at 4 °C and can be attributed to the functioning of the Na^+-/K^+- ATPase enzyme, which has resulted in hyper-osmoregulation and decrease of serum osmolality.

In summary, this study has established distinct thermal adaptive signatures reflected in the varying membrane lipid composition across Antarctic species and non-Antarctic species. Along with the presence of unsaturated fatty acids, there is selective increase of *cis*-vaccenic acid and EPA in the membranes of Antarctic fish. An HVA response was not seen in either of the Antarctic species at 4 °C of thermal acclimation. There was evidence that the membrane-associated functions were not affected as plasma osmolality was reduced upon thermal acclimation. An HVA response was exhibited only at 6 °C in the benthic species *T. bernacchii* suggesting that this species has acclimatory response to warming temperatures. HVA was not observed in the cryopelagic species *P. borchgrevinki* at 6 °C. Membrane cholesterol was unchanged upon thermal acclimation, suggestive of unchanged membrane fluidity upon thermal acclimation at 4 °C. Membrane cholesterol concentration were not different across Antarctic and non-Antarctic species, but membrane cholesterol varied across species.

5 Summary of the key findings and future lines of enquiry

Brief overview

The research presented in this thesis has investigated both (1) the adaptive responses to reduced temperatures acquired by Antarctic fish over their evolutionary history and (2) the short-term acclimatory response of homeoviscous adaptations when Antarctic fish are exposed to acute thermal stress. From a broader perspective, this research uncovers the process of homeoviscous adaptation (HVA) at three basic levels of the central dogma of molecular biology that exist in biological systems. First, at the DNA level, this study has unravelled the evolutionary history of the SCD gene in teleost fish, and in Antarctic fish in particular. Second, at the transcriptome level, the response of the SCD gene to warming acclimation has provided evidence of the response to thermal stress. Third, at the protein level, the effect of SCD on membrane saturation change in response to thermal stress has shown evidence of acclimatory HVA response in Antarctic fish

Summary of key contributions of this thesis

This study has extended the data set of SCD isoform sequences with the isolation of partial SCD paralog sequences from notothenioid fish species from Antarctic and non-Antarctic habitats. Castro also presented evidence of SCD gene duplication from a limited data set of teleost species (Castro et al., 2011). The extended data set of this study, along with other published sequences, has reconstructed a comprehensive SCD gene phylogeny in teleosts, with strong support for ancestral SCD gene duplication, more recent lineage-specific duplicates restricted to certain species and the loss of duplicates in specific orders of fish. Cold adapted Notothenioid SCD of Antarctic and non-Antarctic fish species (with an Antarctic ancestry but subsequent dispersal to non-Antarctic waters) were evolutionarily distinct and form separate clades within the SCD1a and 1b phylogenies.

The present study has demonstrated SCD evolution as a model for increased understanding of thermal adaptation to extreme environments. Evolutionary analysis of the SCD gene using phylogenetic, amino acid composition, and codon usage analyses in combination with tests of positive selection have shown how temperature can shape the SCD gene, allowing it to acquire adaptive responses, such as the homeoviscous response. Partial primary amino acid sequences show different amino acid compositions within the isoforms and between Antarctic and, non-Antarctic fish, which could increase the physical flexibility of the SCD in polar conditions. This study

has further identified thermal adaptive signatures in the SCD isoforms, such as specific amino acid replacements and localization of replacements in the loop regions of the protein. These patterns are comparable to other cold-adapted proteins and present a possible evolutionary advantage in the structure of SCD for conferring flexibility at reduced temperatures.

This study has identified more evolutionary change in the SCD1a isoform than the SCD 1b isoform. Sites under positive selection were detected in the SCD1a isoform specific for Antarctic fish, but much less so in the SCD1b isoform. Furthermore, the unique histidine pair found across Antarctic SCD1a isoforms, and changes in specific residues in the SCD1a that determine the length of fatty acyl chain to be desaturated, have provided evidence of the diversity of the SCD isoforms in teleost fish, and Antarctic fish in particular. The tissue expression study suggested that SCD1a and SCD1b fit a pattern of asymmetric evolution for the species *P. borchgrevinkii.* This pattern results in reduced expression of SCD1a, shown by absence of expression in all tissues by endpoint PCR and reduced expression in liver by qPCR. SCD1b, however, was ubiquitously expressed at high levels in most tissues in both the species examined, suggesting that the ancestral function had been retained by SCD1b isoform. This is the first study to demonstrate asymmetric species-specific tissue expression in SCD. The study has contributed new information on tissue expression patterns of SCD isoforms. In general, after gene duplication, one of the duplicates often retains the ancestral functions with increase in expression while the other duplicate can rapidly evolve a new function or a loss of function often accompanied by a decrease in expression. This is asymmetric evolution.

This is the first study in teleost fish, and Antarctic fish in particular, to investigate the transcriptional response of both SCD isoforms in fish subjected to thermal stress. This study has determined the pattern of expression of SCD isoforms over time with a thermal exposure of 4 °C. SCD1a and SCD1b showed an overall increase in expression in response to thermal treatment in the benthic species, whereas SCD1a levels were low to be detected in the cryopelagic species. Pattern of expression over the acclimation period was non-significant. Perhaps a larger sample size could have compensated for the high variation associated with obtaining samples from a wild population. SCD1b did not show any response after seven days of acclimation at 6 °C in either of the species. However, a significant change in the membrane saturation state was observed in the benthic species, suggesting a homeoviscous response that does not correlate with SCD expression.

This study provides substantial evidence of altered membrane compositions, dominated by an increase in unsaturated fatty acids, in Antarctic species when compared to non-Antarctic fish (New Zealand species), presumably due to an evolutionary adaptive response. Antarctic icefish species

have high proportions of saturated fatty acids in comparison to other closely related Antarctic species signalling evolutionary diversification of these species. Membrane remodelling was not observed in either species examined at 4 °C thermal acclimation, but membrane remodelling did occur in the benthic species at 6 °C thermal acclimation, although this response is not seen in the cryopelagic species, *P. borchgrevinki*. In the context of anthropogenic global warming, the stenothermal nature of Antarctic fish is a cause for major concerns with respect to thermal adaptability. However, this investigation has established a short-term acclimatory homeoviscous response is possible in an Antarctic benthic fish species, suggesting that thermal plasticity could play a role in adapting Antarctic benthic species to warming temperatures.

In summary, the key findings of this study are listed below:

1. Temperature leaves signatures of thermal adaptation and asymmetric evolution on the SCD genes of Antarctic fish that help them to function in a constant, cold environment.

2. Evolutionary analysis of the SCD gene in Antarctic teleosts has provided a framework for future functional analyses and has contributed to the understanding of thermal adaptation in extreme environments.

3. The effects of temperature on the membrane remodelling capacity in two Antarctic fish species, the benthic *T. bernacchii* and the cryopelagic *P. borchgrevinki,* have been characterised. Homeoviscous response was observed in *T. bernacchii* at 6 °C but not at 4 °C, while *P. borchgrevinki* lacked this response at 6 °C.

4. Membrane remodelling is dominated by changes in phospholipid desaturation, but not by changes in SCD gene expression, suggesting that other genes may also be involved in the homeoviscous response.

Temperature leaves thermal adaptive signatures in biological macromolecules, such as the DNA and the primary protein structure of the enzyme SCD.

SCD isoforms of cold adapted Notothenioid display signatures of thermal adaptation and these features were unique to the Notothenioid species. Whether these features are displayed in the SCD isoforms of Arctic fish, where they also experience narrow and cold temperature ranges, would be worth investigating to examine how common thermal adaptive signatures are across all cold adapted fish species. However, the general patterns of thermal adaptation seen in other studies,

such as specific amino acid changes (Windisch et al., 2012) and localization of changes in loops of protein structure (Romisch et al., 2003), align with our findings. Also in the present study clustering of SCD1a isoform with the cold adapted notothenioid species as well as the non-notothenioid cold adapted species in the PCA analysis implies general thermal adaptive signatures in the SCD1a isoform. However, the amino acid replacements at specific sites in the cold adapted notothenioids were different from that observed in *Pachycara brachycephalum* (non-Notothenioid Antarctic fish) for the SCD1b isoform shown in our study. While the PCA analysis of this study showed clustering of SCD1b isoform of only cold adapted notothenioid and was separate from the non-Notothenioid cold adapted species implying specific thermal adaptive signatures. Similarly AFGP, although apparently similar for the Arctic and Antarctic fish display differences indicating independent evolution of AFGP in Arctic and Antarctic (Chen et al., 1997a). Thus the present study upheld the hypothesis of thermal adaptive signatures specific for the SCD of cold adapted notothenioids.

Membrane saturation, a major thermal adaptive mechanism, will occur at reduced capacities in Antarctic notothenioid fish in response to elevated temperatures and make them vulnerable to the effects of AGW.

Thermal acclimation at 4 °C did not result in membrane remodelling, which was supported by the biochemical analysis. Membrane functions were not affected as shown by the decline of serum osmolality for *P. borchgrevinki.* Membrane remodelling may not be required for membranes to function at 4 ℃. Previous studies have been over a broad thermal range and the difference between the normal and acclimation temperature was 6 ℃ (Skalli et al., 2006) to 19 ℃ (Snyder et al., 2012). A lack of studies on membrane saturation states of fish at 4 °C makes it difficult to predict the saturation states required for 4 °C. At 6 °C thermal acclimation, we observe a HVA response in the benthic species *T. bernacchii,* while this was not observed in the pelagic species *P. borchgrevinkii*. The present study has provided preliminary evidence to disprove the hypothesis for one species.

Future avenues of work

This study has determined the key changes that have occurred in the primary amino acid sequence of the SCD isoforms, as well as the sites under positive selection, and these have been mapped onto the structure of the mouse SCD. However, to establish the functional consequence of these amino acid changes these sites could be potential targets for mutational studies to test whether these sites are associated with cold tolerance or any other functions associated with extreme cold environments. Such findings could contribute to the development of cold tolerance in commercial

warm-adapted fish species that currently face huge mortalities during colder than typical winters. One of the unique finding is the presence of a histidine pair in the SCD 1a isoform. Histidine motifs form the active sites of the enzyme and are highly conserved in the desaturases present. A functional study to decipher whether histidine offer a catalytic advantage or a structural advantage to function in the extreme cold environment would be useful.

Asymmetric rates of molecular evolution need to be investigated in a larger panel of Antarctic species. Along with quantitative real-time tissue expression analysis, this could decipher the functional consequences of this type of evolution in SCD1.

Cold-adapted fish have high proportions of unsaturated fatty acids in their membranes. There is also a selective incorporation of *cis*-vaccenic acid and EPA in the membranes of cold-tolerant species that needs further investigation to determine the roles of these lipids in cold tolerance. Studies could be undertaken to investigate whether incorporation of these components in the diets of fish could improve their cold tolerance.

The biochemical analysis of membrane saturation did not correlate with SCD1b expression at 6 °C which showed a significant change in saturation states in *T. bernacchhii* after 7 days of thermal acclimation. These findings suggest that perhaps the sampling time at day 7 could have missed the initial period of SCD expression (Cossins et al., 2002) and also that other gene targets involved in the pathway for membrane syntheses need assessment these involve gene targets, e.g. LPCAT 1, 2 and 3 and PEMT (Benedito-Palos et al., 2012) should be examined for a better correlation between gene expression and biochemical response.

Gene expression to study the process of membrane remodelling in the thermally stressed Antarctic fish need to be done in liver and muscle tissue by RNA seq analysis and further validation of the transcript data need to be supported by digital quantification of mRNA such as NanoString nCounter system. Transcriptomic data would generate information of all the key genes involved in the membrane biogenesis, repair as well as the SCD isoforms.

Cost of Physiological plasticity in Antarctic fish needs assessment

Preliminary evidence of membrane remodelling upon thermal acclimation in this study has shown Antarctic fish are likely to be able to cope with a temperature rise. Antarctic fish display cellular physiological plasticity upon thermal acclimation which primarily involve a shift from aerobic lipid metabolism in Antarctic fish to anaerobic carbohydrate metabolism upon thermal acclimation (Windisch et al., 2011; Huth & Place, 2016) . Antarctic fish can alter their cardiac functions to support

the physical activity at higher temperature acclimation (Franklin et al., 2007) and also have an antioxidant capacity in place to mitigate long term thermal stress (Enzor & Place, 2014). Antarctic fish show a reduction of serum osmolality upon thermal acclimation, a good indicator for membrane functions at higher temperature (Gonzalez-Cabrera et al., 1995 ; Lowe & Davison, 2005). Finally, transcriptomic profiles show diminished cellular stress responses in Antarctic fish after of long term thermal acclimation (Huth & Place, 2016a ; Huth & Place, 2016b). In addition to this my research adds new information of membrane protection in response to temperature rise in Antarctic fish demonstrate plasticity at a cellular level to cope with the warming temperature but the effect of thermal stress on the whole organism performance, such as growth and reproduction, needs assessment. Whether challenges associated with maintenance of cellular homeostasis upon warming could result in energetic trade-offs is currently unknown (Enzor & Place, 2014). Future studies need to be directed to answer these questions to predict the vulnerability of Antarctic fish to prolonged warming.

Appendix A

Thermal acclimation Experimental Methods

A.1 Method details:

The fieldwork components, comprising the thermal acclimation experiment and harvesting of fish tissues in Antarctica, were conducted by Dr Victoria Mecalf in 2007. This was covered by Animal Ethics (University of Canterbury). My PhD study used fish samples obtained during this Antarctic fieldwork as well as fish samples obtained during other seasons and in other locations, including by other scientists (details in subsequent chapters). Details of the thermal acclimation experiment and harvesting of fish tissues are presented below.

A.1.1 Collection of fish samples for the thermal acclimation experiment

Fish were primarily caught within McMurdo Sound, Antarctica, or the wider Ross Sea area. Fish collection was carried out during the austral summer of 2007 (October-November). Fish were caught using small fishing rods baited with either small fish pieces or soft bait on barbless hooks. Typically at the fishing sites, 10 cm diameter holes were drilled through the sea ice (which ranged from 2-8 metres thick) using a motorised jiffy drill and fish were accessed through these holes. Alternatively, a hole-melter was used to create a 1 metre wide hole in the sea ice through which fish were accessed, sometimes with a heated wannagin over top. Holes were kept clear of sea ice by regular use of small dip-nets. Individuals of the benthic species, *T. bernacchii*, were caught in inshore waters at a water depth of 15-40 m. Individuals of the pelagic and schooling species, *P. borchgrevinki,* were collected from deeper water locations (up to 300m deep) near the ice runway in McMurdo Sound with sea ice cover less than 4m thick (typically 2 m) and away from seal activity. Following capture, and quick and careful hook removal, fish were immediately placed in an insulated container of seawater and transported to laboratory facilities at Scott Base, where they were held in flow–through aquaria with sea water temperature maintained at -1.0 ± 0.3 °C. Fish were held post-catching for a minimum period of three days to allow them to recover from handling stress. During the recovery period, fish were not fed and were maintained in a 24 hour light regime to replicate the austral summer conditions of Antarctica. Following the recovery period, and when

sufficient fish had been caught for the experiment, fish were pre-acclimated to a temperature of -1 °C for a period of 15 days and were fed *ad libitum* twice weekly.

A.1.2 Thermal acclimation experimental design and sampling

Following the pre-acclimation period of 15 days, five randomly chosen individuals of each fish species were euthanised before the thermal acclimation experiment started and their tissues were harvested as an initial control prior to temperature treatment. The remaining fish of each species were placed in either static or flow-through tanks (limitations of the aquaria facilities meant some treatments were in static tanks) and kept in groups of no more than 10 fish per tank. For all acclimation temperature treatments, the water temperature was then gradually increased from -1 °C to 4°C, or 6 °C. The experimental temperature regime consisted of stepwise increases in temperature to the target temperature over a 24h period (except in the case of the 6 °C treatment, in which case slower acclimation over three days was employed). The tanks were mantained at the treatment temperature ± 0.3 °C using two heat exchangers connected to a feeder tank that contained thermostatically-controlled water heater. Where possible, flow-through tanks were used, but by necessity some of the heat treatments required the use of static tanks with oxygen bubblers. For static tanks, daily replacement of 25% of the tank capacity was done to avoid both (1) the accumulation of waste products and (2) decreases in oxygen concentration. A cohort of fish held at as close to environmental temperature as possible was used as a control treatment; in which case fish remained at -1 °C for the duration of the experiment. Further, they were fed *ad libitum* twice weekly during the acclimation period.

A.1.3 Collection and transport of tissue and plasma samples

Fish (n=5 of each treatment, including controls) were euthanised, blood samples collected and tissues harvested at 1, 2, 3, 7, 14 and 21 days post-acclimation. All routine procedures were followed as per the Animal Ethics approval and sampling procedures were performed at the Scott Base Wet Laboratory, with air temperature below 5°C. Routine anaesthetic exposure via transfer to seawater containing MS-222 (ethyl m-amino benzoate methane-sulfonate) was performed. Fish were anaesthetised for five min in a 0.1 g/L solution of MS222 dissolved in sea water. Details of the individual fish were then recorded. Blood was collected from the caudal vein with a needled syringe and the fish was euthanised by severing the spinal cord. Fish were dissected using standard dissection procedures under sterile conditions and tissue samples were transferred into labelled vials and immediately frozen in liquid nitrogen. Tissues (liver, brain, heart, kidney, white muscle, red

muscle, subcutaneous fat and adipose tissue) were rapidly removed, snap frozen in liquid nitrogen and stored at -80˚C for later biochemical and genetic analyses.

Appendix B

Details of sequences and alignments used for the bioinformatics analysis of Chapter 2

B.1 Details of species along with their nomenclature included in the phylogenetic and other bioinformatics analysis

S.No	Species	Common name	Accession number (Protein sequence)	Gene name	Accession number(DNA sequence)	Family	Order	class
1	*Danio rerio*	Zebrafish	ENSDART00000044009	SCDb001	ENSDART00000044009	Cyprinidae	Cypriniformes	Actinopterygii
2	*Danio rerio*	Zebrafish	ENSDART00000050006	SCD201	ENSDART00000050006	Cyprinidae	Cypriniformes	Actinopterygii
3	*Gadus morhua*	Cod	ENSGMOT00000013805	SCD-201	ENSGMOT00000013805	Gadidae	Gadiformes	Actinopterygii
4	*Gadus morhua*	Cod	ENSGMOT00000000411	SCDb-201	ENSGMOT00000000411	Gadidae	Gadiformes	Actinopterygii
5	*Astyanax mexicanus*	Cavefish	ENSAMXT00000004466	SCD201	ENSAMXT00000004466	Characidae	Characiformes	Actinopterygii
6	*Astyanax mexicanus*	Cavefish	ENSAMXT00000012724	SCDb-201	ENSAMXT00000012724	Characidae	Characiformes	Actinopterygii
7	*Latimeria chalumnae*	Coelacanth	ENSLACT00000021043	SCD201	ENSLACT00000021043	*Coelacanthidae*	Coelacanthiformes	Sarcopterygii
8	*Cyprinus carpio*	Common carp	Q9PU86	CDS2	6018387:229-1203	Cyprinidae	Cypriniformes	Actinopterygii
9	*Oryzias latipes*	Medaka	ENSORLG00000006839	SCDb-201	ENSORLT00000008593	Adrianichthyidae	Beloniformes	Actinopterygii
10	*Oryzias latipes*	Medaka	ENSORLT00000011566	SCD-201	ENSORLT00000011566	Adrianichthyidae	Beloniformes	Actinopterygii
11	*Sparus aurata*	Gilt-head bream	AFP97551.1	SCD1a	JQ277703.1	Sparidae	Perciformes	Actinopterygii
12	*Sparus aurata*	Gilt-head bream	AFP97552.1	SCD1b	JQ277704.1	Sparidae	Perciformes	Actinopterygii
13	*Oreochromis niloticus*	Tilapia	ENSONIT00000023312	SCD -201	ENSONIT00000023312	Cichlidae	Perciformes	Actinopterygii
14	*Oreochromis niloticus*	Tilapia	ENSONIT00000023313	SCD -202	ENSONIT00000023313	Cichlidae	Perciformes	Actinopterygii
15	*Oreochromis niloticus*	Tilapia	ENSONIT00000020272	SCDb-201	ENSONIT00000020272	Cichlidae	Perciformes	Actinopterygii
16	*Trematomus_bernacchii*	Emerald rockcod	ACI16378.1	SCD	FJ177513.1	Nototheniidae	Perciformes	Actinopterygii
17	*Gasterosteus aculeatus aculeatus*	Stickleback	ENSGACT00000003160	SCD-202	ENSGACT00000003160	Gasterosteidae	Gasterosteiformes	Actinopterygii
18	*Gasterosteus aculeatus aculeatus*	Stickleback	ENSGACT00000003154	SCD-201	ENSGACT00000003154	Gasterosteidae	Gasterosteiformes	Actinopterygii
19	*Gasterosteus aculeatus aculeatus*	Stickleback	ENSGACT00000011284	SCDb_201	ENSGACT00000011284	Gasterosteidae	Gasterosteiformes	Actinopterygii
20	*Salmo salar*	Atlantic salmon	ACN11041.1	Acyl -CoA desaturase	BT059328.1	Salmonidae	Salmoniformes	Actinopterygii
21	*Salmo salar*	Atlantic salmon	NP_001133452.1	Acyl -CoA desaturase	NM_001139980	Salmonidae	Salmoniformes	Actinopterygii
22	*Takifugu rubripes*	Fugu	ENSTRUT00000031408	SCD-201	ENSTRUT00000031408	Tetraodontidae	Tetraodontiformes	Actinopterygii
23	*Takifugu rubripes*	Fugu	AAU89872.1	SCD2	AY741384.1	Tetraodontidae	Tetraodontiformes	Actinopterygii
24	*Homo sapiens*	Human	ENST00000370355	SCD001	ENST00000370355	Hominidae	Primates	Mammalia
25	*Polypterus_senegalus_*	Gray bichir	AEG25345.1	SCD 1	JF729410.1	Polypteridae	Polypteriformes	Actinopterygii
26	*Mus_musculus*	Mouse	CAJ18540.1	SCD 1	CT010332.1	Muridae	Rodentia	Mammalia
27	*Rattus_norvegicus*	Rat	NP_631931.2	Acyl -CoA desaturase 1	148747463	Muridae	Rodentia	Mammalia
28	*Ctenopharyngodon idella*	Grass carp	CAB53008.1	SCD	AJ243835.1	Cyprinidae	Cypriniformes	Actinopterygii
29	*Scyliorhinus_canicula*	lesser spotted dogfish	AEG25343.1	SCD1	JF729408.1	Scyliorhinidae	Carcharhiniformes	Chondrichthyes
30	*Cyprinus caprio*	Common carp	AER39747.1	SCD1a	JF836162.1	Cyprinidae	Cypriniformes	Actinopterygii
31	*Xenopus_laevis*	African clawed frog	NP_001087809.1	SCD	NM_001094340.1	Pipidae	Anura	Amphibia
32	*Sebasticus marmoratus*	Marble trout	AFK08797.1	SCD	JQ663624.1	Salmonidae	Salmoniformes	Actinopterygii
33	*Chanos chanos*	Milk Fish	AAL99291.1	SCD1b	AY082003.1	Chanidae	Gonorynchiformes	Actinopterygii
34	*Dicentrarchus labrax*	European sea bass	CBM40644.1	SCD1b	FN868643.1	Moronidae	Perciformes	Actinopterygii
35	*Dicentrarchus labrax*	European sea bass	CBN81527.1	SCD1a	374428415	Moronidae	Perciformes	Actinopterygii
36	*Oreochromis mossambicus*	Mozambique tilapia	Q8AWI1	SCD	AY150696.1	Cichlidae	Perciformes	Actinopterygii
37	*Chionodraco hamatus*	Crocodile icefish	Q9PU84	SCD	AJ249579.1	Channichthyidae	Perciformes	Actinopterygii
38	*Takifugu rubripes*	Puffer fish	Q5XQ38	SCD1	NM_001078577.1	Tetraodontidae	Tetraodontiformes	Actinopterygii
39	*Xiphophoru maculatus*	Platyfish	ENSXMAT00000003100	SCDb201	ENSXMAT00000003100	Poeciliidae	Cyprinodontiformes	Actinopterygii
40	*Xiphophoru maculatus*	Platyfish	ENSXMAT00000014994	SCD201	ENSXMAT00000014994	Poeciliidae	Cyprinodontiformes	Actinopterygii
41	*Gallus gallus*	Chicken	Q9YGM2	SCD	45382442	Phasianidae	Galliformes	Aves
42	*Mus musculus*	Mouse	P13011	SCD2	200943	Muridae	Rodentia	Mammalia

B.2 Summary of SCD gene organization in teleost species obtained from ENSEMBL

S.No	Species(SCD1b)	E	I	E	I	E	I	E	I	E	I	E	I	E	I	E	I	E	I	E	Name	Transcript ID	Length (bp)	Length (aa)	Chromosome location	Exons	Coding exons	Gene size(kb)
1	*Sparus aurata SCD1b*					238		131		206		233		200							SCD1b							
2	*Gadus moruhua SCDb 201*	53	2	4	10	166	919	131	679	206	1163	233	1763	200							SCDb-201	ENSGMOT00000013805	993	330	1579: 564,952-570,480	7	7	5.53
3	*Gasterosteus aculeatus SCDb 201*			295	928	239	795	131	481	206	851	233	1105	200							scdb-201	ENSGACT00000011284	1304	330	GroupVI: 9,801,170-9,806,633	6	5	5.46
4	*Orechromis niloticus SCDb 201*					556	1540	131	192	206	691	233	1170	197							SCDb-201	ENSONIT00000023313	999	333	GL831242.1: 1,635,536-1,640,314	6	6	4.78
5	*Orechromis niloticus SCDb 202*			35	187	197	1540	131	192	206	691	233	1170	197							SCDb-202	ENSONIT00000023312	1323	324	GL831242.1: 1,635,536-1,640,451	5	5	4.92
6	*Oryzias latipes SCDb201*					347	3219	131	283	206	479	233	2261	200							SCDb-201	ENSORLT00000008593	1117	347	15: 20,935,680-20,943,038	5	5	7.36
7	*Takifugu rubripes SCD2*					232	483	131	31	260	481	233	833	200							SCD 2							
8	*Tetraodon nigroviridis SCDb 201*					372	602	131	233	206	357	145	1	88	219	218					SCDb-201	ENSTNIT00000003617	1160	334	17: 6,034,049-6,036,620	6	6	2.57
9	*Xiphophoru maculatusSCD201*					250	2219	131	1514	206	513	233	1579	200							SCD-201	ENSXMAT00000003100	1020	339	JH556725.1: 1,662,350-1,669,194	5	5	6.84
S.No	Species(SCD1a)	E	I	E	I	E	I	E	I	E	I	E	I	E	I	E	I	E	I	E								
1	*Astyanax mexicanus SCDb 201*			660	213	276	1923	131	330	206	1561	233	624	3831							SCDb-201	ENSAMXT00000012724.1	5337		KB882125.1: 1,940,942-1,950,929	6	5	9.99
2	*Astyanax mexicanus SCDb 201*			247	1400	205	553	131	262	206	1071	233	144	218	133	1135					SCDb-201	ENSAMXT00000004466.1	2375	350	KB882310.1: 790,503-796,440	7	5	5.4
3	*Chionadraco hamatus*					237	1171	160	130	205	405	236	416	195							SCD1a						5	3.15
4	*Danio rerio SCD 001*			217	4748	224	2786	131	665	206	2269	233	1953	526							SCD-201	ENSDART00000050006	2830	326	to be updated	5	5	10.5
5	*Danio rerio SCDb 201*			245	189	206	2955	131	2510	206	2574	233	7840	1025							SCDb-201	ENSDART00000044009	2046	316	13: 29,879,167-29,897,280	6	5	18.1
6	*Gadus moruhua SCD 201*			7	1	154	126	131	148	206	323	233	315	194	2009	3	3	4	1	8	SCD-201	ENSGMOT00000000411	984	328	1971: 909,033-912,944	10	10	3.91
7	*Gasterosteus aculeatus SCD 201*			70	254	249	894	131	118	206	372	233	568	737							SCD-201	ENSGACT00000003160	1124	349	GroupV: 842,137-847,047	8	7	4.91
8	*Gasterosteus aculeatus SCD 202*			33	254	249	894	131	118	206	372	233	568	196	497	19	1084	57			SCD-202	ENSGACT00000003154	1626	330	GroupV: 842,100-845,931	6	5	3.83
9	*Orechromis niloticus SCD201*					259	904	131	153	206	382	233	2863	200							SCD-201	ENSONIT00000020272	1029	335	GL831148.1: 2,480,949-2,486,279	5	5	5.33
10	*Oryzias latipes SCD201*			92	221	260	406	131	338	206	357	233	1842	536							SCD-201	ENSORLT00000011566	1458	333	19: 10,021,042-10,025,663	6	5	4.62
11	*Sparus aurata 1a*					238		131		206		237		280														
12	*Takifugu rubripes SCD2*					232	483	131	31	260	481	233	833	200							SCD-2	ENSTRUT00000031408	1056	351	Scaffold_59: 918,747-921,630	5	5	2.88
13	*Tetraodon nigroviridis SCD 201*					241	604	131	90	206	331	233	793	200							SCD-201	ENSTNIT00000022839	1011	336	2_random: 1,160,737-1,163,565	5	5	2.83
14	*Trematomus bernacchii*					237		131		206		236		192														
15	*Xiphophoru maculalatus*			305	234	258	1433	131	83	206	674	233	2246	2780							SCD-201	ENSXMAT00000014994	3913	334	JH556661.1: 741,858-750,440	6	5	8.58

I: Intron, E: Exon

B.3 List of species used for amino acid composition analysis

Non-Antarctic sequences (SCD1a)

1	*Astyanax mexicanus*_SCDb-201 ENSAMXT00000012724
2	*Astyanax mexicanus*_SCD-201_ENSAMXT00000004466
3	*Chanos chanos*_ SCD_AAL99291
4	*Ctenopharyngodon idella* SCD1_CAB53008.1|
5	*Danio rerio SCDb001*_ENSDART00000044009
6	*Danio rerio SCDb201*_ENSDART00000050006
7	*Dicentrarchus labrax* SCD1a_CBN81527.1|
8	*Gadus Morhua* SCDb-201_ ENSGMOT00000000411
9	*Gasterosteus aculeatus_ SCDb-202* ENSGACT00000003160
10	*Gasterosteus aculeatus_SCDb-201* ENSGACT00000003154
11	*Orechromus niloticus SCDb-201_* ENSONIT00000020272
12	*Oreochromis mossambicus SCD__*Q8AWI1
13	*Oryzias latipes_ SCDb-201_* ENSORLT00000011566
14	*Sparus aurata SCD1a* _AFP97551.1|
15	*Takifugu rubripes _SCDb-201_* ENSTRUT00000031408
16	*Takifugu rubripes SCD1_AAU89872.1*
17	*Xiphophorus maculatus SCD201_* ENSXMAT00000014994

Non-Antarctic sequences (SCD1b)

1	*Bovichtus variegatus* SCD1b
2	*Gadus Morhua* SCD_ENSGMOT00000013805
3	*Cottoperca gobio*_SCD1b
4	*Dicentrarchus labrax* SCD1b_CBM40644.1
5	*Eliginops maclovinus* SCD1b
6	*Gasterosteus aculeatus* SCD-201_ENSGACT00000011284
7	*Orechromus niloticus SCD201*_ENSONIT00000023312
8	*Orechromus niloticus*_ SCD202_ ENSONIT00000023313
9	*Oryzias latipes SCD-201* ENSORLG00000006839
10	*Sparus aurata SCD1b*_ FP97552.1|
11	*Takifugu rubripes*_SCD2_NM001078577.1
12	*Xiphophorus maculatus*_SCDb201_ ENSXMAT00000003100.

Antarctic sequences (SCD1a)

1	*Trematomus bernacchii* SCD1a_ ACI16378.
2	*Chionodraco hamatus* SCD1a_Q9PU84
3	*Notothenia angustata* SCD1a*
4	*Champsocephalus esox* SCD1a*
5	*Chaenocephalus aceratus*_ SCD1a
6	*Champsocephalus gunnari_ SCD1a*
7	*Pagothenia borchgrevinki SCD1a_*
8	*Notothenia_rossii_SCD1a*
9	*Notothenia_coriiceps_736218284_151-1158_SCD1a*

* Non-Antarctic sequences but having an Antarctic ancestry

Antarctic sequences (SCD1b)

1	*Chaenocephalus.aceratus* SCD1b
2	*Champsocephalus.esox* SCD1b *
3	*Champsocephalus.gunnari* SCD1b VIRT13522
4	*Chionodraco_hamatus_*SCD1b
5	*Dissostichus_eleginoides_*SCD1b *
6	*Gobionothen_gibberifrons_*SCD1b
7	*Gobionothen_marionensis_*SCD1b
8	*Gobionotothen acuticeps* SCD1b
9	*Gymnadraco_acuticeps_*SCD1b
10	*Lepidonotothen squamifrons* SCD1b
11	*Notothenia angustata* SCD1b *
12	*Notothenia_coriiceps_736179142_219-1220_SCD1b*
13	*Notothenia_rossii_*SCD1b
14	*Pagothenia borchgrevinki* SCD1b
15	*Pseudochoenicthys_georgius_*SCD1b
16	*Trematomous nicolai* SCD1b
17	*Trematomus_hansoni_*SCD1b

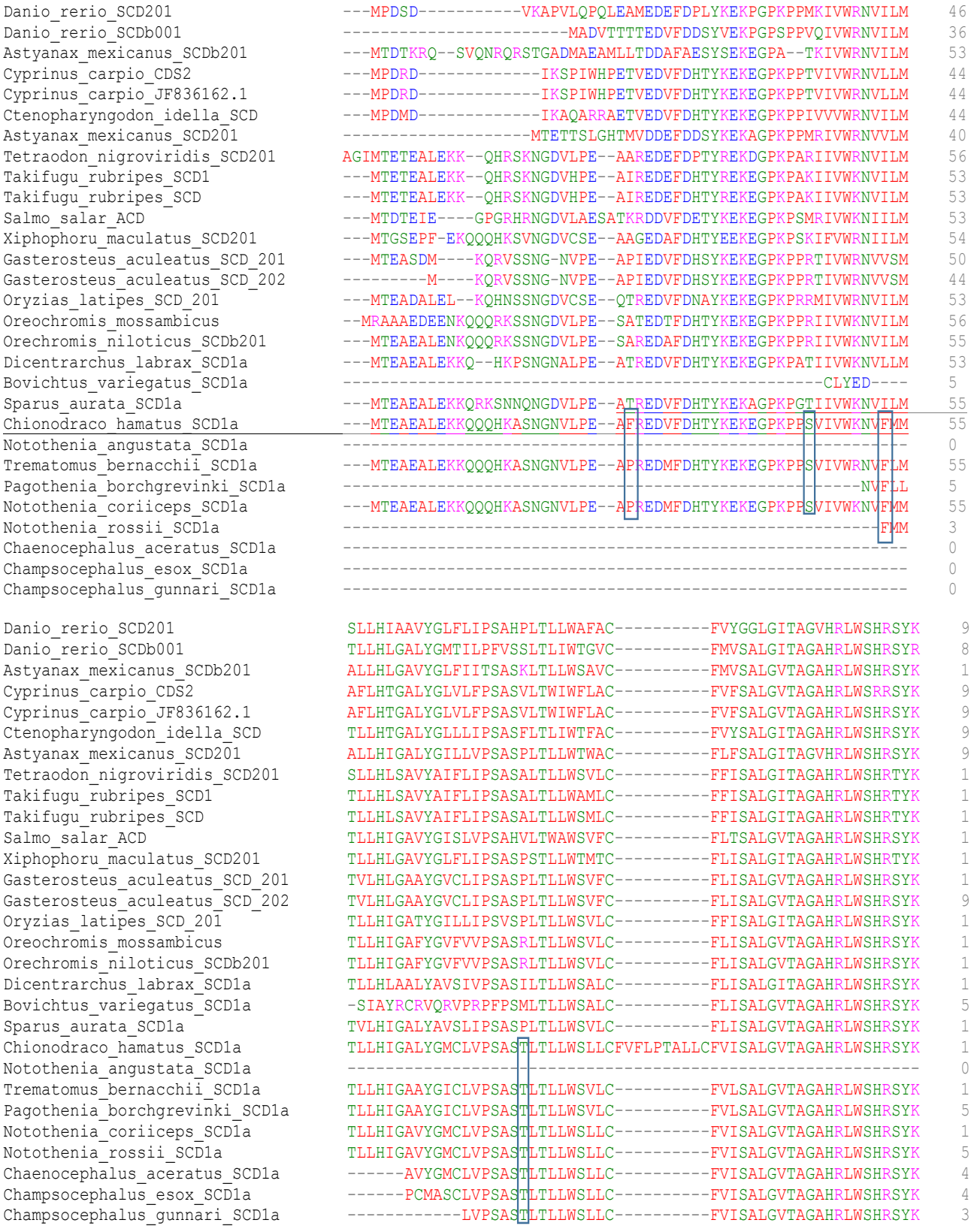
* Non-Antarctic sequences but having an Antarctic ancestry

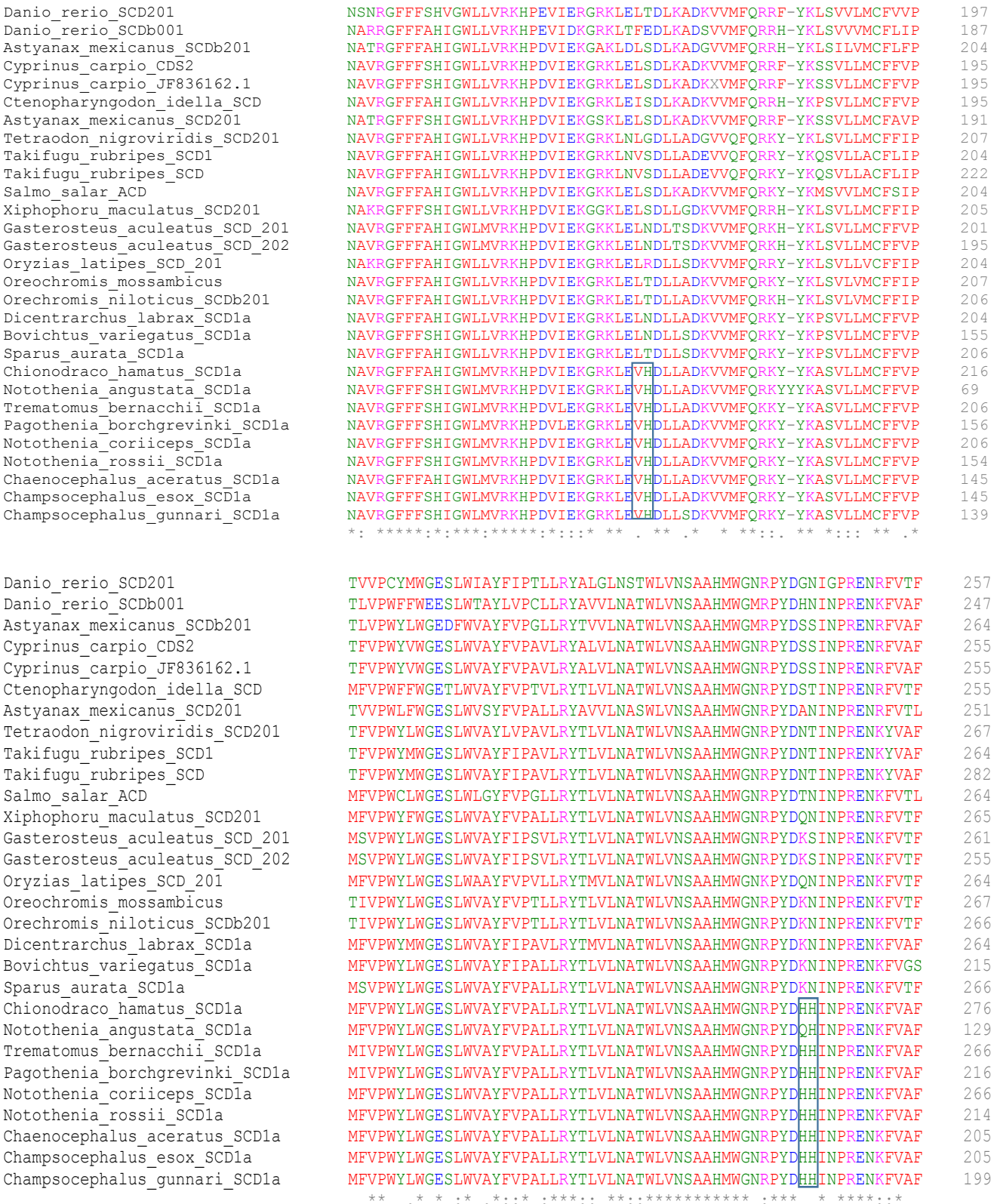
B.4 List of alignments used for the bioinformatics analysis and provided in the drop box link given below

Alignments in the drop box link are under embargo and will be replaced with accession number after deposit to GenBank

	Bioinformatic analysis	File Name
1	SCD phylogenetic analysis	SCD_phylogeny.fas
2	Test of positive selection SCD1a	SCD1a_selection.fas
3	Test of positive selection SCD1b	SCD1b_selection.fas
4	GA branch analysis	GA_branch.fas

B.5 Alignments of SCD1a isoform sequences from Antarctic and non-Antarctic fish species. Alignments specific for Antarctic fish and cold adapted notothenioid non-Antarctic fish have been boxed.




Danio_rerio_SCD201 NSNRGFFFSHVGWLLVRKHPEVIERGRKLELTDLKADKVVMFQRRF-YKLSVVLMCFVVP 197
Danio_rerio_SCDb001 NARRGFFFAHIGWLLVRKHPEVIDKGRKLTFEDLKADSVVMFQRRH-YKLSVVVMCFLIP 187
Astyanax_mexicanus_SCDb201 NATRGFFFAHIGWLLVRKHPDVIEKGAKLDLSDLKADGVVMFQRRH-YKLSILVMCFLFP 204
Cyprinus_carpio_CDS2 NAVRGFFFSHIGWLLVRKHPDVIEKGRKLELSDLKADKVVMFQRRF-YKSSVLLMCFFVP 195
Cyprinus_carpio_JF836162.1 NAVRGFFFSHIGWLLVRKHPDVIEKGRKLELSDLKADKXVMFQRRF-YKSSVLLMCFFVP 195
Ctenopharyngodon_idella_SCD NAVRGFFFAHIGWLLVRKHPDVIEKGRKLEISDLKADKVVMFQRRH-YKPSVLLMCFFVP 195
Astyanax_mexicanus_SCD201 NATRGFFFSHIGWLLVRKHPDVIEKGSKLELSDLKADKVVMFQRRF-YKSSVLLMCFAVP 191
Tetraodon_nigroviridis_SCD201 NAVRGFFFAHIGWLLVRKHPDVIEKGRKLNLGDLLADGVVQFQRKY-YKLSVLLMCFFIP 207
Takifugu_rubripes_SCD1 NAVRGFFFAHIGWLLVRKHPDVIEKGRKLNVSDLLADEVVQFQRRY-YKQSVLLACFLIP 204
Takifugu_rubripes_SCD NAVRGFFFAHIGWLLVRKHPDVIEKGRKLNVSDLLADEVVQFQRKY-YKQSVLLACFLIP 222
Salmo_salar_ACD NAVRGFFFAHIGWLLVRKHPDVIEKGKKLELSDLKADKVVMFQRKY-YKMSVVLMCFSIP 204
Xiphophoru_maculatus_SCD201 NAKRGFFFSHIGWLLVRKHPDVIEKGGKLELSDLLGDKVVMFQRRH-YKLSVLLMCFFIP 205
Gasterosteus_aculeatus_SCD_201 NAVRGFFFAHIGWLMVRKHPDVIEKGKKLELNDLTSDKVVMFQRKH-YKLSVLLMCFFVP 201
Gasterosteus_aculeatus_SCD_202 NAVRGFFFAHIGWLMVRKHPDVIEKGKKLELNDLTSDKVVMFQRKH-YKLSVLLMCFFVP 195
Oryzias_latipes_SCD_201 NAKRGFFFAHIGWLLVRKHPDVIEKGRKLELRDLLSDKVVMFQRRY-YKLSVLLVCFFIP 204
Oreochromis_mossambicus NAVRGFFFAHIGWLLVRKHPDVIEKGRKLELTDLLADKVVMFQRKY-YKLSVLVMCFFIP 207
Orechromis_niloticus_SCDb201 NAVRGFFFAHIGWLLVRKHPDVIEKGRKLELTDLLADKVVMFQRKH-YKLSVLVMCFFIP 206
Dicentrarchus_labrax_SCD1a NAVRGFFFAHIGWLLVRKHPDVIEKGRKLELNDLLADKVVMFQRKY-YKPSVLLMCFFVP 204
Bovichtus_variegatus_SCD1a NAVRGFFFSHIGWLLVRKHPDVIEKGRKLELNDLLSDKVVMFQRKY-YKPSVLLMCFFVP 155
Sparus_aurata_SCD1a NAVRGFFFAHIGWLLVRKHPDVIEKGRKLELTDLLSDKVVMFQRKY-YKPSVLLMCFFVP 206
Chionodraco_hamatus_SCD1a NAVRGFFFAHIGWLMVRKHPDVIEKGRKLEVHDLLADKVVMFQRKY-YKASVLLMCFFVP 216
Notothenia_angustata_SCD1a NAVRGFFFSHIGWLMVRKHPDVIEKGRKLEVHDLLADKVVMFQRKYYYKASVLLMCFFVP 69
Trematomus_bernacchii_SCD1a NAVRGFFFSHIGWLMVRKHPDVLEKGRKLEVHDLLADKVVMFQKKY-YKASVLLMCFFVP 206
Pagothenia_borchgrevinki_SCD1a NAVRGFFFSHIGWLMVRKHPDVLEKGRKLEVHDLLADKVVMFQKKY-YKASVLLMCFFVP 156
Notothenia_coriiceps_SCD1a NAVRGFFFSHIGWLMVRKHPDVIEKGRKLEVHDLLADKVVMFQRKY-YKASVLLMCFFVP 206
Notothenia_rossii_SCD1a NAVRGFFFSHIGWLMVRKHPDVIEKGRKLEVHDLLADKVVMFQRKY-YKASVLLMCFFVP 154
Chaenocephalus_aceratus_SCD1a NAVRGFFFAHIGWLMVRKHPDVIEKGRKLEVHDLLADKVVMFQRKY-YKASVLLMCFFVP 145
Champsocephalus_esox_SCD1a NAVRGFFFSHIGWLMVRKHPDVIEKGRKLEVHDLLADKVVMFQRKY-YKASVLLMCFFVP 145
Champsocephalus_gunnari_SCD1a NAVRGFFFSHIGWLMVRKHPDVIEKGRKLEVHDLLSDKVVMFQRKY-YKASVLLMCFFVP 139
Danio_rerio_SCD201 TVVPCYMWGESLWIAYFIPTLLRYALGLNSTWLVNSAAHMWGNRPYDGNIGPRENRFVTF 257
Danio_rerio_SCDb001 TLVPWFFWEESLWTAYLVPCLLRYAVVLNATWLVNSAAHMWGMRPYDHNINPRENKFVAF 247
Astyanax_mexicanus_SCDb201 TLVPWYLWGEDFWVAYFVPGLLRYTVVLNATWLVNSAAHMWGMRPYDSSINPRENRFVAF 264
Cyprinus_carpio_CDS2 TFVPWYVWGESLWVAYFVPAVLRYALVLNATWLVNSAAHMWGNRPYDSSINPRENRFVAF 255
Cyprinus_carpio_JF836162.1 TFVPWYVWGESLWVAYFVPAVLRYALVLNATWLVNSAAHMWGNRPYDSSINPRENRFVAF 255
Ctenopharyngodon_idella_SCD MFVPWFFWGETLWVAYFVPTVLRYTLVLNATWLVNSAAHMWGNRPYDSTINPRENRFVTF 255
Astyanax_mexicanus_SCD201 TVVPWLFWGESLWVSYFVPALLRYAVVLNASWLVNSAAHMWGNRPYDANINPRENRFVTL 251
Tetraodon_nigroviridis_SCD201 TFVPWYLWGESLWVAYLVPAVLRYTLVLNATWLVNSAAHMWGNRPYDNTINPRENKYVAF 267
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Takifugu_rubripes_SCD TFVPWYMWGESLWVAYFIPAVLRYTLVLNATWLVNSAAHMWGNRPYDNTINPRENKYVAF 282
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Xiphophoru_maculatus_SCD201 MFVPWYFWGESLWVAYFVPALLRYTLVLNATWLVNSAAHMWGNRPYDQNINPRENRFVTF 265
Gasterosteus_aculeatus_SCD_201 MSVPWYLWGESLWVAYFIPSVLRYTLVLNATWLVNSAAHMWGNRPYDKSINPRENKFVTF 261
Gasterosteus_aculeatus_SCD_202 MSVPWYLWGESLWVAYFIPSVLRYTLVLNATWLVNSAAHMWGNRPYDKSINPRENKFVTF 255
Oryzias_latipes_SCD_201 MFVPWYLWGESLWAAYFVPVLLRYTMVLNATWLVNSAAHMWGNKPYDQNINPRENKFVTF 264
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Orechromis_niloticus_SCDb201 TIVPWYLWGESLWVAYFVPTLLRYTLVLNATWLVNSAAHMWGNRPYDKNINPRENKFVTF 266
Dicentrarchus_labrax_SCD1a MFVPWYMWGESLWVAYFIPAVLRYTMVLNATWLVNSAAHMWGNRPYDKNINPRENKFVAF 264
Bovichtus_variegatus_SCD1a MFVPWYLWGESLWVAYFIPALLRYTLVLNATWLVNSAAHMWGNRPYDKNINPRENKFVGS 215
Sparus_aurata_SCD1a MSVPWYLWGESLWVAYFVPALLRYTLVLNATWLVNSAAHMWGNRPYDKNINPRENKFVTF 266
Chionodraco_hamatus_SCD1a MFVPWYLWGESLWVAYFVPALLRYTLVLNATWLVNSAAHMWGNRPYDHHINPRENKFVAF 276
Notothenia_angustata_SCD1a MFVPWYLWGESLWVAYFVPALLRYTLVLNATWLVNSAAHMWGNRPYDQHINPRENKFVAF 129
Trematomus_bernacchii_SCD1a MIVPWYLWGESLWVAYFVPALLRYTLVLNATWLVNSAAHMWGNRPYDHHINPRENKFVAF 266
Pagothenia_borchgrevinki_SCD1a MIVPWYLWGESLWVAYFVPALLRYTLVLNATWLVNSAAHMWGNRPYDHHINPRENKFVAF 216
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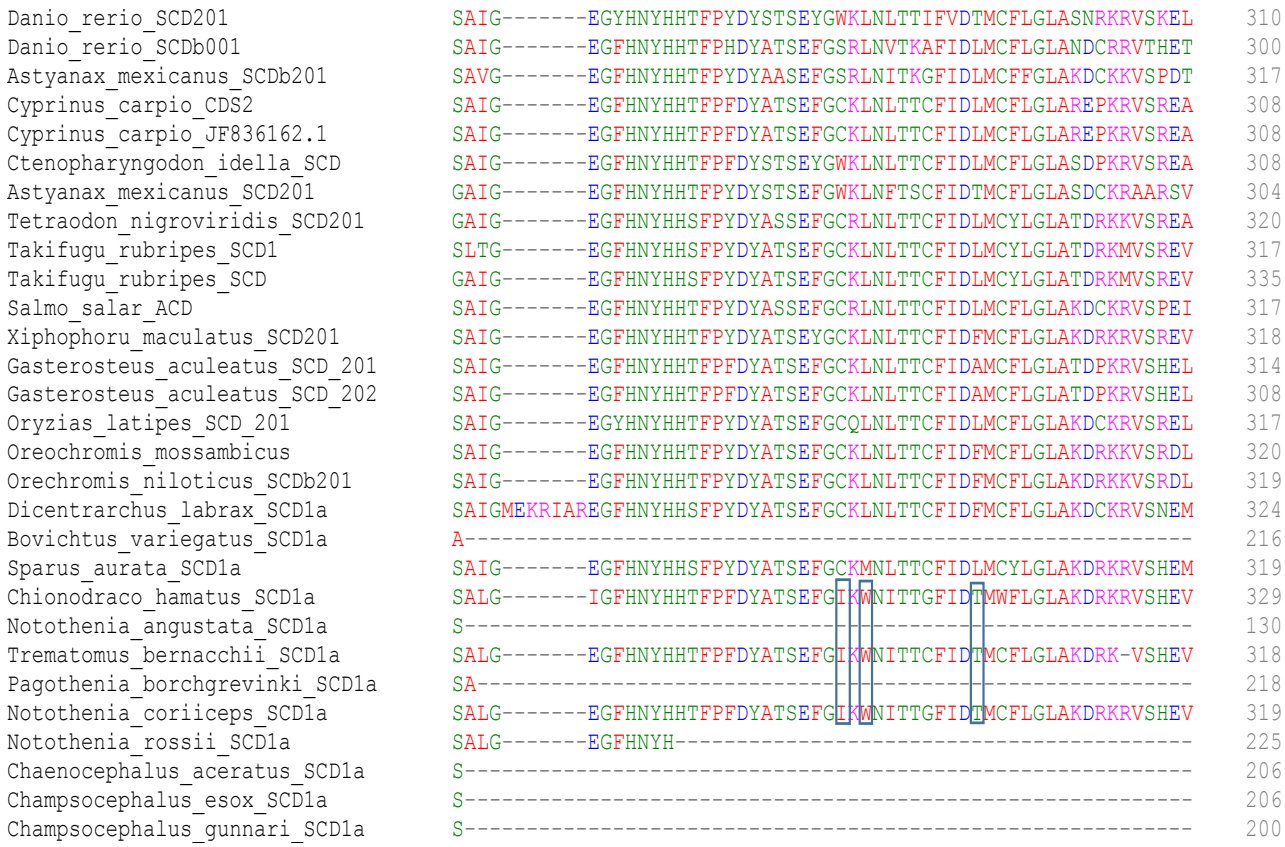
Champsocephalus_esox_SCD1a MFVPWYLWGESLWVAYFVPALLRYTLVLNATWLVNSAAHMWGNRPYDHHINPRENKFVAF 205
Champsocephalus_gunnari_SCD1a MFVPWYLWGESLWVAYFVPALLRYTLVLNATWLVNSAAHMWGNRPYDHHINPRENKFVAF 199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Cyprinus_carpio_CDS2 SAIG-------EGFHNYHHTFPFDYATSEFGCKLNLTTCFIDLMCFLGLAREPKRVSREA 308
Cyprinus_carpio_JF836162.1 SAIG-------EGFHNYHHTFPFDYATSEFGCKLNLTTCFIDLMCFLGLAREPKRVSREA 308
Ctenopharyngodon_idella_SCD SAIG-------EGFHNYHHTFPFDYSTSEYGWKLNLTTCFIDLMCFLGLASDPKRVSREA 308
Astyanax_mexicanus_SCD201 GAIG-------EGFHNYHHTFPYDYSTSEFGWKLNFTSCFIDTMCFLGLASDCKRAARSV 304
Tetraodon_nigroviridis_SCD201 GAIG-------EGFHNYHHSFPYDYASSEFGCRLNLTTCFIDLMCYLGLATDRKKVSREA 320
Takifugu_rubripes_SCD1 SLTG-------EGFHNYHHSFPYDYATSEFGCKLNLTTCFIDLMCYLGLATDRKMVSREV 317
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Salmo_salar_ACD SAIG-------EGFHNYHHTFPYDYASSEFGCRLNLTTCFIDLMCFLGLAKDCKRVSPEI 317
Xiphophoru_maculatus_SCD201 SAIG-------EGFHNYHHTFPYDYATSEYGCKLNLTTCFIDFMCFLGLAKDRKRVSREV 318
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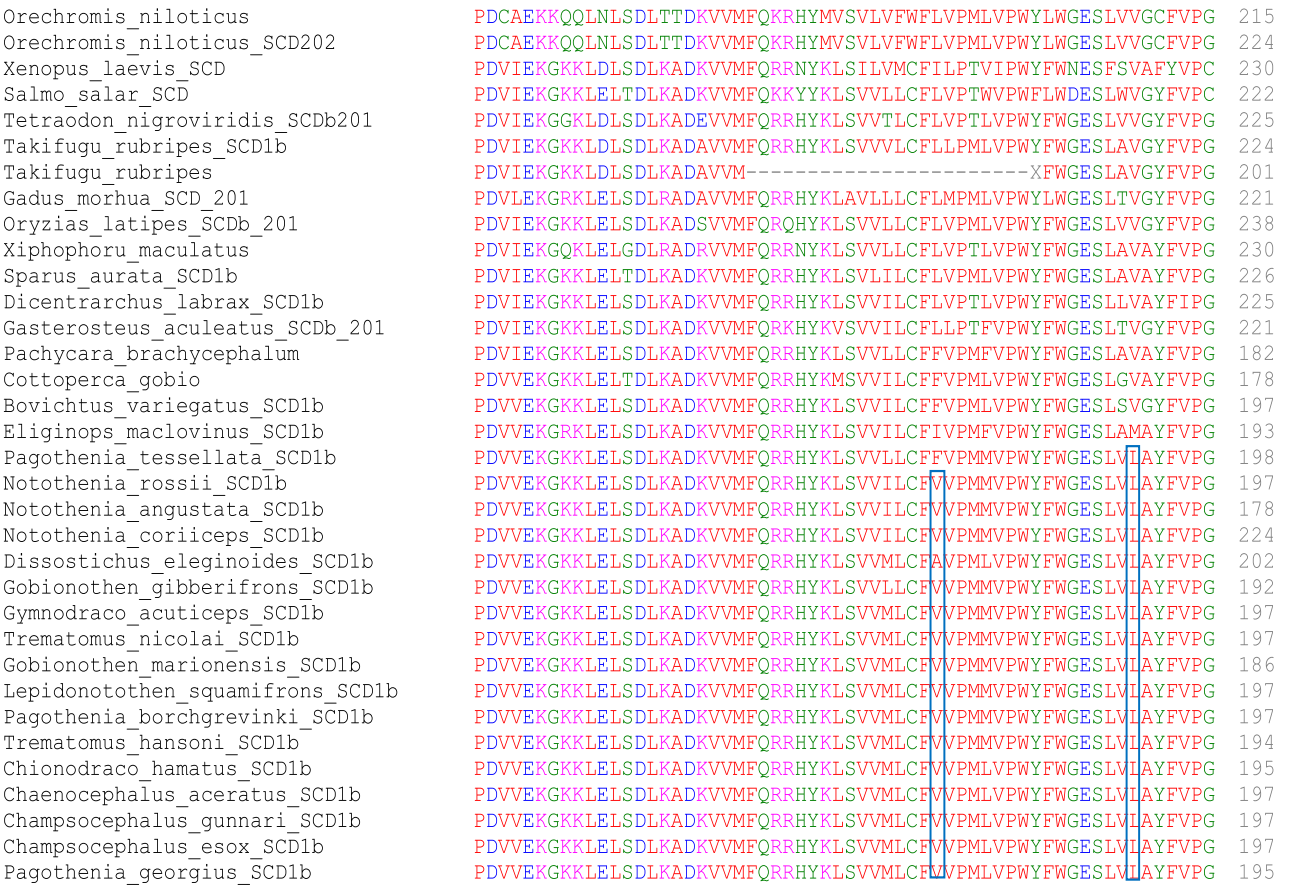
Gasterosteus_aculeatus_SCD_202 SAIG-------EGFHNYHHTFPFDYATSEFGCKLNLTTCFIDAMCFLGLATDPKRVSHEL 308
Oryzias_latipes_SCD_201 SAIG-------EGYHNYHHTFPYDYATSEFGCQLNLTTCFIDLMCFLGLAKDCKRVSREL 317
Oreochromis_mossambicus SAIG-------EGFHNYHHTFPYDYATSEFGCKLNLTTCFIDFMCFLGLAKDRKKVSRDL 320
Orechromis_niloticus_SCDb201 SAIG-------EGFHNYHHTFPYDYATSEFGCKLNLTTCFIDFMCFLGLAKDRKKVSRDL 319
Dicentrarchus_labrax_SCD1a SAIGMEKRIAREGFHNYHHSFPYDYATSEFGCKLNLTTCFIDFMCFLGLAKDCKRVSNEM 324
Bovichtus_variegatus_SCD1a A---------- 216
Sparus_aurata_SCD1a SAIG-------EGFHNYHHSFPYDYATSEFGCKMNLTTCFIDLMCYLGLAKDRKRVSHEM 319
Chionodraco_hamatus_SCD1a SALG-------IGFHNYHHTFPFDYATSEFGIKWNITTGFIDTMWFLGLAKDRKRVSHEV 329
Notothenia_angustata_SCD1a S---------- 130
Trematomus_bernacchii_SCD1a SALG-------EGFHNYHHTFPFDYATSEFGIKWNITTCFIDTMCFLGLAKDRK-VSHEV 318
Pagothenia_borchgrevinki_SCD1a SA---------- 218
Notothenia_coriiceps_SCD1a SALG-------EGFHNYHHTFPFDYATSEFGIKWNITTGFIDTMCFLGLAKDRKRVSHEV 319
Notothenia_rossii_SCD1a SALG-------EGFHNYH---------- 225
Chaenocephalus_aceratus_SCD1a S---------- 206
Champsocephalus_esox_SCD1a S---------- 206
Champsocephalus_gunnari_SCD1a S---------- 200

B.6 Alignments of SCD1b isoform sequences from Antarctic and non-Antarctic fish species. Alignments specific for Antarctic fish and cold adapted notothenioid non-Antarctic fish have been boxed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Orechromis_niloticus_SCD202 PDCAEKKQQLNLSDLTTDKVVMFQKRHYMVSVLVFWFLVPMLVPWYLWGESLVVGCFVPG 224
Xenopus_laevis_SCD PDVIEKGKKLDLSDLKADKVVMFQRRNYKLSILVMCFILPTVIPWYFWNESFSVAFYVPC 230
Salmo_salar_SCD PDVIEKGKKLELTDLKADKVVMFQKKYYKLSVVLLCFLVPTWVPWFLWDESLWVGYFVPC 222
Tetraodon_nigroviridis_SCDb201 PDVIEKGGKLDLSDLKADEVVMFQRRHYKLSVVTLCFLVPTLVPWYFWGESLVVGYFVPG 225
Takifugu_rubripes_SCD1b PDVIEKGKKLDLSDLKADAVVMFQRRHYKLSVVVLCFLLPMLVPWYFWGESLAVGYFVPG 224
Takifugu_rubripes PDVIEKGKKLDLSDLKADAVVM----------XFWGESLAVGYFVPG 201
Gadus_morhua_SCD_201 PDVLEKGRKLELSDLRADAVVMFQRRHYKLAVLLLCFLMPMLVPWYLWGESLTVGYFVPG 221
Oryzias_latipes_SCDb_201 PDVIEKGKKLELSDLKADSVVMFQRQHYKLSVVLLCFLVPMLVPWYFWGESLVVGYFVPG 238
Xiphophoru_maculatus PDVIEKGQKLELGDLRADRVVMFQRRNYKLSVVLLCFLVPTLVPWYFWGESLAVAYFVPG 230
Sparus_aurata_SCD1b PDVIEKGKKLELTDLKADKVVMFQRRHYKLSVLILCFLVPMLVPWYFWGESLAVAYFVPG 226
Dicentrarchus_labrax_SCD1b PDVIEKGKKLELSDLKADKVVMFQRRHYKLSVVILCFLVPTLVPWYFWGESLLVAYFIPG 225
Gasterosteus_aculeatus_SCDb_201 PDVIEKGKKLELSDLKADKVVMFQRKHYKVSVVILCFLLPTFVPWYFWGESLTVGYFVPG 221
Pachycara_brachycephalum PDVIEKGKKLELSDLKADKVVMFQRRHYKLSVVLLCFFVPMFVPWYFWGESLAVAYFVPG 182
Cottoperca_gobio PDVVEKGKKLELTDLKADKVVMFQRRHYKMSVVILCFFVPMLVPWYFWGESLGVAYFVPG 178
Bovichtus_variegatus_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVILCFFVPMLVPWYFWGESLSVGYFVPG 197
Eliginops_maclovinus_SCD1b PDVVEKGRKLELSDLKADKVVMFQRRHYKLSVVILCFIVPMFVPWYFWGESLAMAYFVPG 193
Pagothenia_tessellata_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVLLCFFVPMMVPWYFWGESLVLAYFVPG 198
Notothenia_rossii_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVILCFVVPMMVPWYFWGESLVLAYFVPG 197
Notothenia_angustata_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVILCFVVPMMVPWYFWGESLVLAYFVPG 178
Notothenia_coriiceps_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVILCFVVPMMVPWYFWGESLVLAYFVPG 224
Dissostichus_eleginoides_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVMLCFAVPMLVPWYFWGESLVLAYFVPG 202
Gobionothen_gibberifrons_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVLLCFVVPMMVPWYFWGESLVLAYFVPG 192
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Lepidonotothen_squamifrons_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVMLCFVVPMMVPWYFWGESLVLAYFVPG 197
Pagothenia_borchgrevinki_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVMLCFVVPMMVPWYFWGESLVLAYFVPG 197
Trematomus_hansoni_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVMLCFVVPMMVPWYFWGESLVLAYFVPG 194
Chionodraco_hamatus_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVMLCFVVPMLVPWYFWGESLVLAYFVPG 195
Chaenocephalus_aceratus_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVMLCFVVPMLVPWYFWGESLVLAYFVPG 197
Champsocephalus_gunnari_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVMLCFVVPMLVPWYFWGESLVLAYFVPG 197
Champsocephalus_esox_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVMLCFVVPMLVPWYFWGESLVLAYFVPG 197
Pagothenia_georgius_SCD1b PDVVEKGKKLELSDLKADKVVMFQRRHYKLSVVMLCFVVPMLVPWYFWGESLVLAYFVPG 195
** ** :*:* ** :* *** :* **: :. ::*


Orechromis_niloticus_ LLRYVALLNATWLVNSAAHMWGNRPYNKNINPRENKFVSFSAIGEGFHNYHHTFPCDYAA 275
Orechromis_niloticus_SCD202 LLRYVALLNATWLVNSAAHMWGNRPYNKNINPRENKFVSFSAIGEGFHNYHHTFPCDYAA 284
Xenopus_laevis_SCD LLRYALVLNATWLVNSAAHMYGNQPYDKTINPRENPLVAIGAIGEGFHNYHHTFPFDYSS 290
Salmo_salar_SCD LMRYALVLNATWLVNSAAHMWGNRPYDRNINPSENRFVAFSAIGEGFHNYHHTFPFDYAT 282
Tetraodon_nigroviridis_SCDb201 LLRYSVVLNASWLVNSAAHMWGNRPYDATINPRENPVVAFSAVGEGFHNYHHTFPFDYAT 285
Takifugu_rubripes_SCD1b LLRYSLILNATWLVNSAAHIWGNRPYDKTINPRENFMVALSAIGEGFHNYHHTFPFDYAT 284
Takifugu_rubripes LLRYSLILNATWLVNSAAHIWGNRPYDKTINPRENFMVALSAIGEGFHNYHHTFPFDYAT 261
Gadus_morhua_SCD_201 LMRYALVLNATWLVNSAAHMWGNRPYDQGINPRENRFVAFSAVGEGFHNYHHTFPFDYAT 281
Oryzias_latipes_SCDb_201 LLRYALVLNATWLVNSAAHMWGNRPYDKTINPRENIFVALSAIGEGFHNYHHTFPFDYAT 298
Xiphophoru_maculatus LLRYTVVLNASWLVNSAAHMWGNRPYDKTINPRENPLVAISAIGEGFHNYHHTFPFDYAT 290
Sparus_aurata_SCD1b LLRYTVMLNATWLVNSAAHIWGNRPYDKTINPRENALVALSAIGEGFHNYHHTFPFDYAT 286
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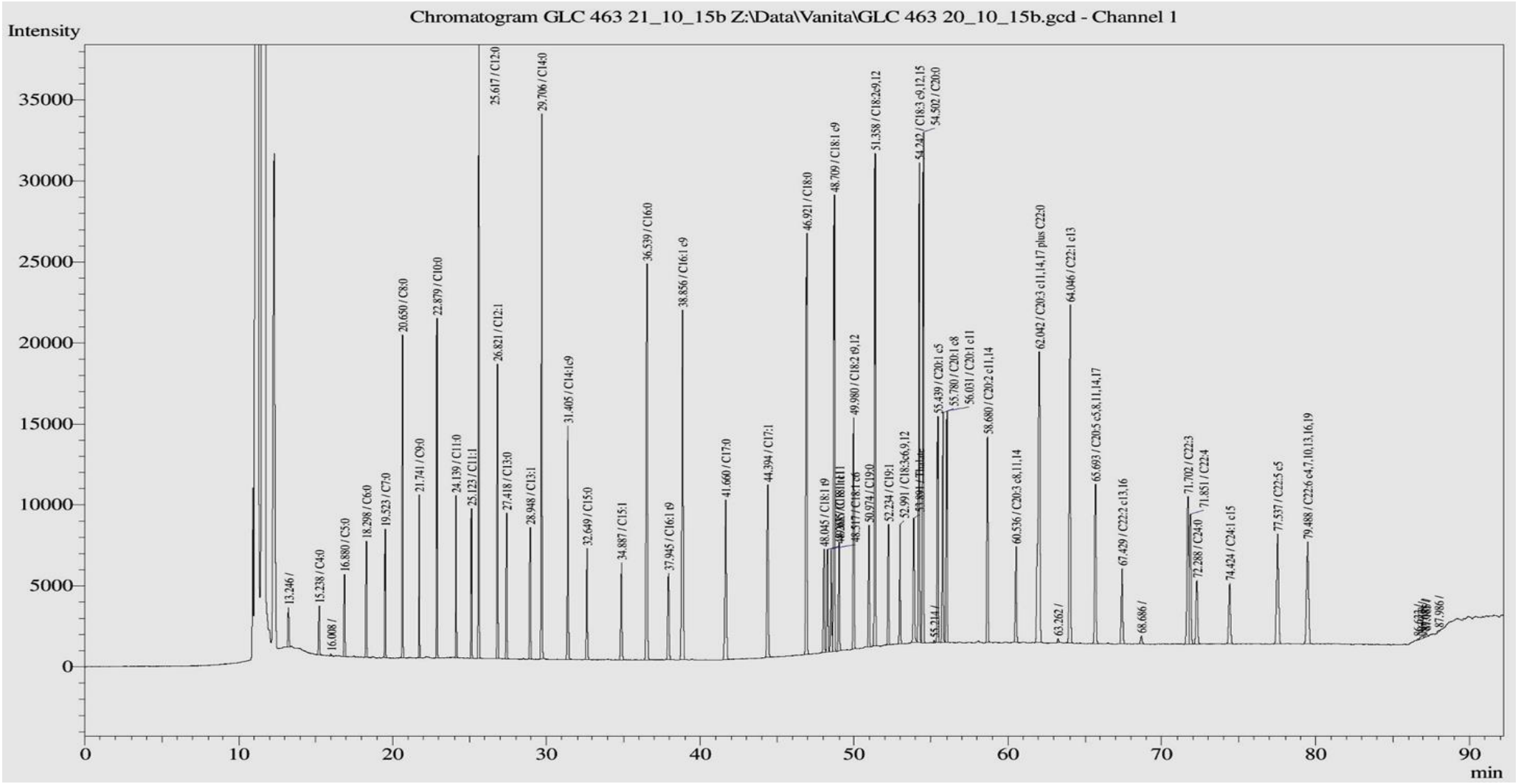
Gasterosteus_aculeatus_SCDb_201 LLRYAVMLNATWLVNSAAHLWGNRPYDKTINPRENRLVALSAIGEGFHNYHHTFPFDYAT 281
Pachycara_brachycephalum LLRYAVMLNAT---------- 193
Cottoperca_gobio LLRYAVMLNATWLVNSAAHIWGNRPYDKSI---------- 208
Bovichtus_variegatus_SCD1b LLRYAVMLNATWLVNSAAHIWGNRPYDKTINPRE---------- 231
Eliginops_maclovinus_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPRE---------- 227
Pagothenia_tessellata_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSI---------- 228
Notothenia_rossii_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKS---------- 226
Notothenia_angustata_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPRE---------- 212
Notothenia_coriiceps_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPRENPFVAFSAIGEGFHNYHHTFPFDYAT 284
Dissostichus_eleginoides_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDK---------- 230
Gobionothen_gibberifrons_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSI---------- 222
Gymnodraco_acuticeps_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPRE---------- 231
Trematomus_nicolai_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPRE---------- 231
Gobionothen_marionensis_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPR---------- 219
Lepidonotothen_squamifrons_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPRE---------- 231
Pagothenia_borchgrevinki_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPRE---------- 231
Trematomus_hansoni_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPY---------- 220
Chionodraco_hamatus_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPRENP---------- 231
Chaenocephalus_aceratus_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPRE---------- 231
Champsocephalus_gunnari_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPRE---------- 231
Champsocephalus_esox_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPRE---------- 231
Pagothenia_georgius_SCD1b LLRYAVMLNATWLVNSAAHLYGNRPYDKSINPR---------- 228
*:** :***:

Appendix C : Manufacturer details for chemicals used for the biochemical analysis

- Acetic acid- AJAX Finechem Pty Ltd
- Chloroform- Fisher Scientific-C/4966/17,HPLC grade
- Dichloromethane- Fisher Scientific-D151-4, HPLC grade
- Hexane- Sigma-Aldrich-270504. HPLC grade
- Methanol- Fisher Scientific-A452-4,-HPLC grade
- Potassium Chloride- BDH-101984l
- Potassium Hydroxide-Sigma Aldrich- P5958
- Tetrahydrofuran- Riedel-de Haën-34946
- Acetone-Fisher- FSBA949-4, HPLC grade.
- 2-Methoxyethanol- 284467, Sigma-Aldrich.

Appendix D Chromatogram of the standard GLC 463



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