Title: RNA-seq reveals a diminished acclimation response to the combined effects of ocean acidification and elevated seawater temperature in *Pagothenia borchgrevinki*

Running Head: cellular homeostasis in a dual-stressor environment

Authors: Troy J. Huth² and Sean P. Place^{1*}

Address:¹Sonoma State University

Department of Biology

Rohnert Park, CA USA 94928

²University of South Carolina

Department of Biological Sciences

Columbia, SC USA 29208

hutht@email.sc.edu

***Corresponding Author:** Sean P. Place

ph: 1+ (707) 664-3054

fax: 1+ (707) 664-4046

email: places@sonoma.edu

1 **Abstract**

2 *Purpose* – The IPCC has reasserted the strong influence of anthropogenic CO₂ contributions on
3 global climate change and highlighted the polar-regions as ahgihly vulnerable. With these
4 predictions the cold adapted faunaendemic to the Southern Ocean, which is dominated by fishes
5 of the sub-order Notothenioidei, will face considerable challenges in the near future. Recent
6 physiological studies have demonstrated that the synergistic stressors of elevated temperature
7 and ocean acidificationhave a considerable, although variable, impact on notothenioid fishes.

8 The present study explored the transcriptomic response of *Pagothenia borchgrevinki* to increased
9 temperatures and *p*CO₂after 7, 28 and 56 days of acclimation. We compared this response to
10 short term studies assessing heat stress alone and foretell the potential impacts of these stressors
11 on *P. borchgrevinki*'sability to survive a changing Southern Ocean.

12 *Results* – *P. borchgrevinkidid* demonstrate a coordinated stress response to the dual-stressor
13 condition, and even indicated that some level of inducible heat shock response may be conserved
14 in this notothenioid species. However, the stress response of *P. borchgrevinki*was considerably
15 less robust than that observed previously in the closely related notothenioid,*Trematomus*
16 *bernacchii*, and varied considerably when compared across different acclimation time-points.
17 Furthermore, the molecular response of these fish under multiple stressors displayed distinct
18 differences compared to their response to short term heat stress alone.

19 *Conclusions* – When exposed to increased sea surface temperatures, combined with ocean
20 acidification, *P. borchgrevinki* demonstrated a coordinated stress response that has already
21 peaked by 7 days of acclimation and quickly diminished over time. However, this response is
22 less dramatic than other closely related notothenioids under identical conditions, supporting

- 1 previous research suggesting that this notothenioid species is less sensitive to environmental
- 2 variation.
- 3 **Key Words:** ocean acidification, thermal stress, cellular stress response, notothenioid, gene
- 4 expression

Background

In its Fifth Assessment, the Intergovernmental Panel on Climate Change (IPCC) confirmed that human influence on the global climate system is clear and growing, with average land and sea surface temperatures already exhibiting a warming of approximately 0.85 °C over the period from 1880 to 2012 (Pachauri *et al.*, 2014). While the IPCC anticipates that these changes will have severe, pervasive, and irreversible impacts on all ecosystems; the polar environments, including the Southern Ocean surrounding Antarctica, may be affected to a greater extent by global climate change (Pachauri *et al.*, 2014). Though the Southern Ocean has recently experienced a degree of warming (Gille, 2002), it still represents an extremely cold and oceanographically stable environment with fauna that have adapted to this unique environment (Eastman, 1991, 1993; Ritchie *et al.*, 1997; Rogers *et al.*, 2007). Due to its unique and extreme nature, there is no foreseeable similar environment to which its endemic fauna can migrate if the Southern Ocean undergoes significant change at a rapid pace. Thus, this fauna will likely have to adapt to the changing environment or face potentially precipitous declines.

The notothenioids, an endemic sub-order of perciform fishes found in the Southern Ocean, have been thoroughly studied on a physiological level with a particular focus on the impact of heat stress in these fish (Bilyk & DeVries, 2011; Bilyk *et al.*, 2012; Davison *et al.*, 1990; Forester *et al.*, 1987; Pörtner *et al.*, 2005; Robinson & Davison, 2008; Sleadd *et al.*, 2014; Somero & DeVries, 1967). Several genomic-based studies analyzing thermal stress in notothenioids have also been more recently conducted, elucidating the transcriptomic response to one of the potential stressors brought about by global climate change (Bilyk & Cheng, 2014; Huth & Place, 2013). These transcriptomic studies, along with several physiological studies performed on notothenioid fishes, suggest many of these fishes have retained at least some capacity to

acclimate to warmer oceans (Bilyk & DeVries, 2011; Bilyk et al., 2012; Franklin *et al.*, 2007; Robinson & Davison, 2008a; Robinson & Davison, 2008b; Robinson *et al.*, 2010; Seebacher *et al.*, 2005). However, prior results from a variety of marine organisms have indicated that the variation of multiple environmental stressors simultaneously may have a greater effect than the sum of individual stressors, potentially reducing their capacity to respond (O'Donnell *et al.*, 2009; Rosa & Seibel, 2008; Schulte, 2007). Some more recent studies on notothenioid fishes have begun to investigate the physiological effects of multiple stressors on their physiology and have identified similar synergistic effects when climate change related stressors are co-varied (Enzor *et al.*, 2013; Enzor & Place, 2014; Enzor & Place, in review). Furthermore, in one of the first studies to assess the genome-wide response of a notothenioid fish to multiple stressors, we noted the effects of these synergistic stressors resulted in a prolonged cellular stress response in *Trematomus bernacchii* that may reduce the acclimation capacity of this benthic species (Huth & Place, in review). With the understanding that assessing a species vulnerability to environmental change requires the consideration of multiple variables (Gutt et al., 2014; Hofmann & Todgham, 2010) and the assessment of multiple co-regulated processes (Hofmann & Todgham, 2010; Place *et al.*, 2008), it is clear that further inquiry into the effects of these synergistic stressors on notothenioids is necessary.

Application of transcriptome-wide differential gene expression analyses allows us to examine any number of physiological pathways separately and in conjunction, and thus further our understanding of the physiological plasticity of notothenioids to conditions brought about by global climate change (Bilyk & Cheng, 2014; Huth & Place, 2013, Huth and Place, 2016). To this end, we utilized RNA sequencing (RNA-seq) to study the molecular effects of the synergistic stressors of elevated temperature and ocean acidification (OA) on the

1 cryopelagicnotothenioid, *Pagothenia borchgrevinki*, with the goal of assessing the potential
2 ramifications of global climate change as forecasted by the IPCC (Pachauri et al., 2014). In doing
3 so, we were also able to compare the longer-term effects of synergistic stressors against the acute
4 effects of heat stress in *P. borchgrevinki* (Bilyk & Cheng, 2014) to assess the additional impacts
5 of duration and multiple stressors, as well as assess the overall physiological plasticity of *P.*
6 *borchgrevinki* under these conditions.

7 **Results and Discussion**

8 *i. Sequencing and quality control*- Paired end sequencing of 150bp paired-end reads of all 23
9 individual samples (derived from one IlluminaHiSeq 2500 Rapid Run lane) yielded an average
10 of 28,930,437 paired-endreads (s.d. = 5,166,515) per sample. After aggressive quality control to
11 ensure high quality input data for assembly, where reads exhibiting overall low quality and reads
12 orphaned from their pairs were eliminated, an average of 66.4% of input reads remained (s.d. =
13 1.28%) Resulting quality controlled samples possessed an average of 19,238,626 paired end
14 reads (s.d. = 3,549,213) for input into the assembly.

15 Single end sequencing of 100bp reads of all 23 individual samples on two IlluminaHiSeq
16 2500 Rapid Run lanes yielded an average of 25,850,311 single reads (s.d. = 4,609,844). Quality
17 control removed single reads that exhibited overall low quality, however, read quality was
18 generally high with an average of 89.5% of reads surviving the quality control screening (s.d. =
19 0.14%). Following quality control the individual samples possessed an average of 23,203,167
20 single end reads (s.d. = 4,139,946). Although this depth of sequencing is below the suggested
21 threshold for rare transcript identification and analysis, it is thought to be sufficient for the
22 analysis of commonly expressed genes that underlie a majority of the physiological responses we
23 aimed to capture in this study (Sims *et al.*, 2014). Given the rapidly diminishing payoff

1 associated with sequencing depths above ~ 10M reads/ sample (Vijay *et al.*, 2013) we opted to
2 sacrifice total read depth in order to increase biological replication and achieve a better
3 representation of the variation among individuals within a population.

4 *ii. Reference transcriptome assembly and annotation* – Following *de novo* assembly the
5 transcriptome consisted of 474,450 transcripts (contigs) and 276,892 ‘genes’ (unique gene
6 products including all related transcripts) with a median transcript length of 484bp, mean
7 transcript length of 1050bp and N50 of 3356bp (Supplementary Fig. 1). Following transcript
8 compacting to eliminate redundant sequences, 278,744 transcripts and 205,132 genes remained
9 (median = 410bp, mean = 743bp, N50 = 1746bp). Lastly, RSEM filtering applying a minimum
10 expression threshold of FPKM ≥ 1 to remove transcripts with very low expression produced a
11 final reference transcriptome containing 83,869 transcripts and 46,176 genes with a median
12 transcript length of 1002bp, mean transcript length of 1533bp and N50 of 2590bp (Table 1).

13 BLASTx searches yielded hits for 59,012 transcripts (70.4%), which represented 28,729 hits
14 on the gene level (62.2%, Table 2). Gene Ontology annotation yielded a total of 38,497
15 transcripts (65.2%) with assigned GO Terms, representing 19,577 GO annotated genes (68.1%,
16 Table 2).

17 *iii. Transcriptomic comparisons* – *De novo* assemblies of other teleost fish sequenced with
18 similar technology, assembled with the Trinity *de novo* assembler, and pruned in a similar
19 manner generated equivalent results to the current transcriptome with 44,990 genes in
20 *Oncorhynchus mykiss* (Pierson, Lamers, Flik, & Mayer-Gostan, 2004), and 96,641 transcripts and
21 54,429 genes from the head kidney of *Trematodus bernacchii* (Gerdolet *et al.*, 2015).

22 Additionally, comparisons to a previously assembled transcriptome of *P. borchgrevinki* from
23 Bilyk and Cheng (2013) demonstrated a high degree of similarity between the two

transcriptomes. The previous transcriptome assembly conducted by Bilyk and Cheng (2013) for *P. borchgrevinki* contained 39,758 contigs in the final pruned transcriptome, similar to the 46,176 genes found in the current transcriptome assembly. Direct comparisons of sequence homology (BLAST cutoff = $10E-3$) using the current reference transcriptome as query sequences against a database composed of the contigs from Bilyk and Cheng (2013) resulted in 70,915 transcripts with a BLAST result or a 84.6% match rate. GO Slim comparisons further confirmed the high degree of homology between these two transcriptomes, with both transcriptomes demonstrating similar distributions of the GO terms for biological process, cellular component, and molecular function categories (Fig.1). The small differences found within the BLAST results and GO distribution are likely attributable to tissue specific differences in gene expression. The current reference transcriptome was assembled solely from RNA extracted from gill tissue, whereas the previously published *P. borchgrevinki* transcriptome was assembled from RNA extracted from gill, liver, brain, white muscle, spleen, head kidney, gill and lens (Bilyk & Cheng, 2013).

iv. Transcriptome level differential gene expression analysis – Mappings of the trimmed reads to the reference transcriptome yielded an average of 21,009,072 mapped reads per sample (s.d. = 3,691,533) representing an average mapping rate of 90.5% (s.d. = 0.5%).

The overall trends observed in the gene expression patterns of *P. borchgrevinki* were similar to the trends previously observed in a closely related notothenioid, *T. bernacchii* (Huth & Place, 2016). However, the overall magnitude of change observed in *P. borchgrevinki* was much lower than that previously observed in *T. bernacchii* (Huth & Place, 2016). Of the roughly 46,000 gene products in our transcriptome, we identified a total of 3,872 differentially expressed (DE) gene products demonstrating at least a two-fold change in expression ($FDR \leq 0.05$) in fish acclimated to the dual-stressor treatment relative to control fish. The greatest number of differentially

expressed genes (923) was observed in fish acclimated to the dual-stressor treatments for 7d relative to 7d control fish, of which, 665 genes were up-regulated while 258 were down-regulated in the gills of these fish. This was nearly 50% lower than the total number of DE genes identified in *P. borchgrevinkia* after 4 days of acclimation to an acute thermal stress alone (Bilyk & Cheng, 2014). The number of DE genes observed in dual-stressor acclimated fish relative to control fish at the 28d time point fell to 372 (221 up-regulated and 151 down-regulated) and then rose again at the 56d time point which exhibited 606 DE genes in the dual-stressor acclimated fish relative to control specimens (220 up-regulated and 386 down-regulated).

Pairwise comparison of gene expression profiles across treatments and time-points indicated a high level of correlation between individuals within the same acclimation group which suggests the dual-stressor treatment had a significant impact on the physiology of the fish over both short and long time scales (Fig. 2). However, a high level of correlation was not observed between the dual-stressor treatments at different time-points (Fig. 2). This indicated that the gene level response of *P. borchgrevinkia* to the effects of elevated sea surface temperature and OA changes significantly over time.

v. Transcriptome-wide gene ontology analysis – Comparison of GO terms within the broad molecular function category revealed increases in genes responsible for ATP binding, metal ion binding, protein binding, DNA transcription factor activity, zinc ion binding and chemokine activity for fish in the 7d dual-stressor acclimation group relative to control fish from the same time point (Supplemental Fig. 2). Additionally, the gill tissue of fish in the 7d dual-stressor acclimation group demonstrated relatively high levels of gene up-regulation in the biological processes: chemotaxis, immune response, protein ubiquitination, pyrimidine metabolism, regulation of transcription, and signal transduction relative to control fish

(Supplemental Fig. 3). Interestingly, despite observing nearly one thousand genes that displayed differential expression between the control and dual-stressor groups at the 7d time point, Fisher's exact tests did not reveal any over-represented GO terms at any treatment or time-point (FDR < 0.05). These findings differed substantially from those for *T. bernacchii* specimens acclimated to the same conditions (Huth & Place, 2016) as well as specimens of *P. borchgrevinki* acclimated to a 2d and 4d thermal stress alone (Bilyk & Cheng, 2014). On the surface, these results may be an indication that the major cellular response to the dual-stressors has already passed, which would suggest the acclimation period is much shorter in this notothenioid species. Alternatively, the additional stress associated with OA may have resulted in a more muted transcriptional response as severe hypercapnia has been shown dampen physiological responses and result in metabolic depression (Langenbuch & Pörtner, 2003; Pörtner & Langenbuch 2005; Pörtner *et al.* 1997; Strobel *et al.* 2012). As such, the data could be evidence of changes in protein turn-over strategies or acid-base regulation not observable at the level of the transcript.

In addition to the Gene Ontology analysis, we also performed a second transcriptome wide analysis using the KEGG pathway mapping tool (Kyoto Encyclopedia of Genes and Genomes, Kanehisa *et al.*, 2014). This pathway analysis largely supported the results of the GO analysis, indicating a number of differentially expressed genes involved in signaling cascades primarily associated with inflammatory responses (Table 3). These pathways included Toll-like receptor signaling pathways (8 genes), RIG-I –like receptor signaling pathways (8 genes), the p38/ JNK MAP kinase signaling pathways (6 genes), NOD-like receptor signaling pathways (5 genes) and the JAK/STAT signaling pathways (5 genes). In addition to these related signaling pathways, KEGG mapping of differentially expressed genes highlighted a number of genes with related functions associated with regulation of apoptosis (4 genes), protein processing in the ER (6

genes) and genes involved in metabolic processes such as glycolysis (5 genes) and oxidative phosphorylation (5 genes)(Supplementary Table 1).

Organisms experiencing environmental stress can often display two conserved responses, a rapid, transient response known as the cellular stress response (CSR) and a more permanent response termed the cellular homeostasis response (CHR) (Kültz, 2005). Given the differences in gene expression patterns observed within fish acclimated to the dual-stressor treatments for varying amounts of time, it is likely the DE genes observed in fish acclimated to both stresses for 7d compared to 28d and 56d acclimated fish from the dual-stressor treatments highlights the transition from the CSR to the CHR, and are representative of both the immediate and long-term adjustments necessary to cope with these environmental conditions. As such, we performed a more detailed analysis of gene pathways involved in several biological processes generally associated with cellular homeostasis and response to stress (Almroth *et al.*, 2015; Bilyk *et al.*, 2012; Enzor & Place, 2014; Enzor *et al.*, 2013; Gerdol *et al.*, 2015; Huth & Place, 2013; Kültz, 2005; Robinson & Davison, 2008a).

Pathway Specific Responses

i. Immune system processes – Of all the gene ontology categories considered, immune responses and stress responses displayed the greatest number of DE genes, although the total number of DE expressed genes in any GO category were a fraction of that observed in other species.

For the GO Slim category immune system process (GO:0002376), 44 of the 471 genes associated with immune response in the reference transcriptome were differentially regulated in the fish acclimated to both stressors compared to the control fish ($FDR \leq 0.05$) for at least one time-point (Fig.3, Supplementary Table 2). Fish in the 7d dual-stressor group demonstrated the largest number of DE genes relative to control fish at the same time point with 31 total, of which

28 are up-regulated and 3 down-regulated. This strong initial response undergoes a substantial reduction with 9 differentially expressed genes in the dual-stress acclimated fish at the 28d time-point (8 up-regulated, 1 down-regulated) and only 5 at 56d (4 up-regulated, 1 down-regulated) relative to control fish samples at the same time points.

The initial up-regulation of immune system related genes is consistent with a broader suite of changes associated with the cellular stress response and is highlighted by the up-regulation of a diverse family of chemokines (Supplementary Table 2). The chemokine super family plays a central role in recruiting various elements of the immune system including monocytes, macrophages, t-cells, neutrophils, and natural killer (NK) cells (Cole et al., 1998; Robertson, 2010; Weber et al., 2011; Wuyts et al., 1997). By the 28d time-point we no longer observed an up-regulation of any of these chemokines in fish from the dual-stressor treatment, but did observe a large-scale up-regulation of SAMHD1 in these fish, which may play a role in mediating pro-inflammatory responses to TNF-alpha signaling.

While it is possible that the immune response observed in fish acclimated to the dual-stressor treatment is in response to a latent infection that is able to manifest due to the effects of elevated temperature and $p\text{CO}_2$ on *P. borchgrevinkii*, the inflammation response observed here is more likely associated with intracellular damage to macromolecules. *P. borchgrevinkii* has been shown to exhibit a significant increase in oxidative damage after a 7d acclimation to elevated temperature and $p\text{CO}_2$ (Enzor & Place, 2014). Furthermore, the nuclear transcription factor κB (NF- κB) has been implicated in the transcriptional up-regulation of inflammatory genes in response to oxidants or changes in cellular oxidation-reduction status (Hennet et al., 1993; Schulze-Osthoff et al., 1992). Thus, the more likely scenario is that wide spread cellular damage and remodeling are activating these pathways.

1 *ii. Response to stress* – In teleost fish, environmental perturbation of cellular homeostasis is often
2 marked by a massive transcriptional up-regulation of genes associated with a conserved cellular
3 stress response (CSR)(Buckley *et al.*, 2006; Gracey *et al.*, 2001; Jeffries *et al.*, 2014; Podrabsky
4 & Somero, 2004; Tomalty *et al.*, 2015). Although *P. borchgrevinkii* displayed a clear stress
5 response at the level of the transcript, this response was significantly muted in comparison to
6 other fish species. Of the 696 total genes associated with the GO category response to stress (GO:
7 0006950) found in the reference transcriptome, only 30 transcripts displayed significant changes
8 in expression ($FDR \leq 0.05$) in at least one of the dual-stressor treatments relative to control fish
9 (Fig.3, Supplementary Table 3). In contrast, a closely related species, *T. bernacchii*, showed
10 nearly four-times as many DE genes in this GO category after a 7d acclimation to the same
11 treatment (Huth & Place, 2016).

12 Typically the teleost heat shock response involves 3 major HSP families: HSP90, HSP70 and
13 the small HSPs (Basu *et al.*, 2002). However, notothenioids have consistently demonstrated the
14 lack of an inducible heat shock response (Bilyk & Cheng, 2014; Buckley *et al.*, 2004; Buckley &
15 Somero, 2009; Huth & Place, 2013; Place & Hofmann, 2005; Place *et al.* 2004). Even more
16 striking, significant down-regulation of HSPs has been observed in *P. borchgrevinkii* when
17 exposed to thermal stress alone for 2d and 4d(Bilyk & Cheng, 2014) and *T. bernacchii* after
18 28d(Huth & Place, 2013). Overall, the lack of significant changes in mRNA levels held true for
19 the majority of HSP families annotated in our transcriptome, including HSP70. However, in the
20 first known deviation from this apparent lack of an inducible HSR in an archetypal notothenioid,
21 we observed a significant up-regulation of two contigs encoding HSP90 α (4.9 and 6.4-fold) in fish
22 acclimated to the dual-stressor treatment for 7d. This was a highly unexpected finding given the
23 number of pre-existing studies that have not observed stress inducible changes in this molecular

1 chaperone in notothenioid fishes (Bilyk & Cheng, 2014; Buckley & Somero, 2009; Hofmann *et*
2 *al.*, 2001; Huth & Place 2013). Further analysis of the expression patterns of all HSP90 contigs
3 represented in the annotated transcriptome found 4 of the total 6 HSP90 contigs displayed a 2-
4 fold or greater increase in transcript abundance in fish acclimated to the dual-stressor relative to
5 control fish at the 7d timepoint. The remaining 2 contigs displayed 1.2-1.4 fold changes. Of the 4
6 contigs displaying a ≥ 2 -fold increase, only 2 contigs met the minimum FDR cutoff of ≤ 0.05 ,
7 with the other two ranging from 0.051 – 0.2. Thus, although the change in expression fails to
8 approach anything close to the robust induction seen in temperate species (Buckley *et al.*, 2004),
9 it appears the increase in HSP90 transcript observed here in *P. borchgrevinkii* is not likely a
10 sequencing artifact. By 28d of acclimation, this up-regulation of HSP90 α is reversed, and in fact,
11 all 6 representative contigs display down-regulation in fish exposed to the dual-stressor relative
12 to control fish, with changes in expression ranging from -1.1 to -3.5-fold. However, of the six
13 annotated contigs, only a single HSP90 α contig displayed a FDR below 0.05 and with at least a 2-
14 fold change in expression relative to control fish (-3.4-fold down-regulation, FDR = 0.004) while
15 a second contig displayed a -3.5-fold down-regulation with a FDR = 0.051.

16 The initial up-regulation of HSP90 α at the 7d time-point contrasts with HSP90 α expression
17 levels of *P. borchgrevinkii* found in Bilyk and Cheng (2014) where HSP90 α was found to be
18 down-regulated 3.2-3.9-fold after acclimation to 4 °C for 4d. Given the differences in treatment
19 (single vs. dual-stressors), acclimation time (4d vs 7d) and tissue (liver vs gill), it is difficult to
20 postulate what underlies these contrasting expression results. However, HSP90 is thought to
21 support the proper folding of large protein complexes primarily involved in signal transduction
22 (Younget *al.*, 2001). In addition, HSP90 plays a critical regulatory role in the NOD-like receptor
23 (NLR) signaling pathway involved in inflammatory responses to reactive oxygen

species, stabilizing NALP3 in the cell, which in turn activates the processing of pro-inflammatory cytokines by caspase 1 and the downstream integration of signals with the MAP kinase pathways (Fig. 4) (Mayoret *et al.*, 2007; da Silva Correia *et al.*, 2007). Thus, the observed prevalence of signaling cascades highlighted in the KEGG analysis, especially the NLR signaling pathway in particular, may provide a potential link to the mechanisms underlying the unexpected change in HSP90 expression when inflammatory responses are prolonged.

iii. Cell proliferation and death— Under severe stress, a slowing of cell proliferation can frequently be observed, presumably to ensure sufficient time for DNA repair to proceed and potentially conserve cellular resources. Such a slowing, in addition to a reduction in translation, was observed in *P. borchgrevinkia* after 4d of thermal stress (Bilyk & Cheng, 2014). Upon extending the acclimation period to 7d, we observed signs that this slowdown had persisted in *P. borchgrevinkia* when acclimated to dual-stressors. Several suppressors of cytokine signaling (SOCS), which inhibit the JAK/STAT and growth factor signaling pathways (Cooney, 2002), are up-regulated (+3.3-fold, +3.0-fold, +3.3-fold) in the 7d dual-stressor treatment relative to control fish (Supplementary Table 4). The pathway analysis using KEGG maps further highlights the negative regulation of cell proliferation with the activation of multiple genes implicated in the p38/JNK MAP kinase signaling pathways that target downstream effectors of cell proliferation, inflammation and apoptosis (Supplemental Table 1). This trend is not found in the 28d time-point, and is reversed in the 56d time-point with all three SOCS genes being down-regulated (-2.4-fold, -2.3-fold, -2.1-fold) in fish experiencing both stresses relative to control fish (Supplementary Table 4).

Additionally, fish acclimated to the dual-stressor treatment for 7d demonstrated a strong up-regulation of genes regulating cell death (10) that continued to be elevated into the 28d time-

point (4) before reversing at 56d (5) (Fig. 3, Supplementary Table 5). By 28d *P. borchgrevinki* appears to be undergoing elevated rates of apoptosis (GO:0008219) with the up-regulation of a number of genes encoding caspase-8 (+20.6-fold, +3.8-fold, +2.5-fold, +2.0-fold), which is thought to be a cellular tipping point in apoptosis initiation (Kruidering & Evan, 2000). These changes at the level of the transcriptome coincide with measurements of cellular damage in these same fish. We have previously reported levels of protein damage spike within the first 7d of acclimation to the dual-stressor treatment but return to basal levels by 56d (Enzor & Place, 2014).

iv. Carbohydrate and lipid metabolism – Given the significant energetic costs often associated with the CSR, increased metabolic capacity and mobilization of energy stores frequently accompanies the up-regulation of stress response genes (Buckley *et al.*, 2006; Gracey *et al.*, 2001; Hennet *et al.*, 1993; Jeffries *et al.*, 2014; Logan & Somero, 2011; Podrabsky & Somero, 2004; Schulze-Osthoff *et al.*, 1992, 1993; Tomalty *et al.*, 2015). Nevertheless, *P. borchgrevinki* displayed little change in metabolic pathways despite displaying elevated rates of oxygen consumption after acclimation for 28d (Enzor *et al.*, 2013) and 56d (Enzor and Place, in review). The analysis of significant changes in mRNA expression for genes associated with the GO Slim category lipid metabolic process (GO: 0006629) (Fig.3, Supplementary Table 6) yielded a total of 589 genes associated with this term, 13 of which were significantly differentially expressed ($FDR \leq 0.05$) at one or more time-points in fish acclimated to the dual-stressor treatment relative to control fish at the same time point. Fish in the 7d dual-stressor time-point showed the up-regulation of 4 genes involved in lipid metabolism (cis-aconitate decarboxylase, +5.0-fold; endothelial lipase, LIPG, +1.9-fold; cholesterol 25-hydroxylase, CH25H, +1.8-fold; long-chain acyl- synthetase, ACSL, +1.4-fold). Aside from ACSL, which continues to be up-regulated into the 28d time-point (+2.3-fold), the majority of the changes in

lipid metabolism are reversed in the 28d or 56d acclimation groups, suggesting an early energetic cost that quickly returns to basal levels (Fig.3, Supplementary Table 6). This is supported by physiological data collected on these same specimens. Analysis of lipid levels in the liver as well as whole organism condition indices revealed no significant changes in overall fish condition for *P. borchgrevinkii* over the 56d acclimation period (Enzor & Place, in review).

As seen with lipid metabolism, only 14 of the 546 genes associated with the GO Slim category carbohydrate metabolic process (GO:0005975) were found to be DE ($FDR \leq 0.05$) at one or more time-points in fish acclimated to the dual-stressor treatment relative to control fish (Fig. 3, Supplementary Table 7). Most notably was the observed up-regulation of enzymes involved in simple sugar metabolism (phosphomannomutase, PMM2, +2.0-fold; fructose-bisphosphate aldolase, ALDOA, +3.1-fold; acetyl-coA, +2.1-fold) at the 28d time-point. At 56d glycolytic pathways still demonstrate some signs of increased activity with the 9-fold up-regulation of beta-enolase-like isoform 1 (ENO1).

Conclusion

In line with previous single stressor studies, we have demonstrated that despite lacking a fully functional heat shock response, *P. borchgrevinkii* does demonstrate a coordinated and consistent response to dual-stressors. Although exhibiting many of the hallmarks of the classical cellular stress response, this response appears to be diminished in magnitude compared to other species. We further noted that while previous studies indicated that the short term effects of heat stress in *P. borchgrevinkii* resulted in the general slowing of cell cycle progression and protein biosynthesis, the longer time scales observed in this study indicated that this is likely just a temporary effect.

Together, the GO and KEGG analyses point to a rapid onset of pro-inflammatory responses in the gills of *P. borchgrevinki* that likely peaks between 7-14 days as these signals are no longer observed in the 28d samples. This extended inflammatory response may have resulted in perhaps the most striking finding of this study, the up-regulation of the molecular chaperone HSP90. The inflammatory response is closely followed by a wave of apoptosis peaking around day 28 of acclimation to the dual-stressor. By day 56 these signals have been largely reversed, potentially indicating the completion of cellular remodeling and transition to a more stable, long-term homeostatic state consistent with the CHR.

Even though the muted response to elevated temperature and ocean acidification could be viewed as a reduced capacity to respond, the whole of the data presented here, along with several earlier studies investigating individual biochemical and physiological metrics, suggests *P. borchgrevinki* may simply exhibit a lower sensitivity to environmental variation. Our finding that *P. borchgrevinki* appears capable of up-regulating one of the major HSP protein families further supports this idea. To our knowledge, this is the first study to observe elevated expression of any of the major HSPs in an archetypal notothenioid and highlights the importance of using longer-term acclimations in combination with co-variation of multiple stressors in studies assessing the physiological plasticity of polar organisms.

Methods

Collection of fish: Specimens of *P. borchgrevinki* were collected in McMurdo Sound, Antarctica from September through December, 2012. Fish were caught using hook and line through 10-inch holes drilled through the sea ice and transported back to McMurdo Station in aerated coolers where they were housed in a flow-through aquaria maintained at ambient seawater temperature (-1.5°C). Fish closely examined for signs of X-cell infection and then tank-

1 acclimated under ambient conditions for one week prior to being placed in experimental tanks.
2 Any fish displaying visible signs of X-cell infection were excluded from the acclimation
3 experiment described below (this is a primary reason for the control treatment having a n=3 fish
4 for 56d time point). All procedures were conducted in accordance with the Animal Welfare Act
5 and were approved by the University of South Carolina Institutional Animal Care and Use
6 Committee (ACUP protocol # 100377).

7 *Experimental design:* We used four, 1240 L experimental tanks to assess the combined
8 effects of elevated temperature and $p\text{CO}_2$ on *P. borchgrevinki*. Our two experimental treatments
9 consisted of a control tank which was held near ambient conditions for McMurdo Sound (-1°C
10 and $430\ \mu\text{atm}$, Matson *et al.*, 2011) and a high temperature + high $p\text{CO}_2$ treatment ($+4^\circ\text{C}/1000$
11 μatm) that encompassa sub-lethal temperature tolerated by *P. borchgrevinki* for extended periods
12 of time and near future $p\text{CO}_2$ levels predicted by the IPCC. Fish were placed in experimental
13 tanks and acclimated for a total of 56 days. Over the course of the 56-day acclimation period we
14 observed no mortality in any of the treatments. Five fish per treatment were removed at 7d, 28d,
15 and 56d time-points (n=3 fish for the 56d control group), after which fish were sacrificed and gill
16 tissues were collected and immediately flash-frozen in liquid nitrogen. Although we recognize a
17 fully replicated experimental design is ideal to exclude tank effects as a possible confounding
18 factor, the constraints of working in Antarctica prevented us from using this approach. However,
19 our previous analyses show no tank effect when treatments were alternated between tanks across
20 multiple seasons (Enzor *et al.*, 2013; Enzor & Place, 2014).

21 *Manipulation of seawater conditions:* Temperature and $p\text{CO}_2$ levels were manipulated within the
22 experimental treatment tanks using a $p\text{CO}_2$ generation system first described by Fangueet *al.*
23 (2010) and adapted for use with large-scale applications and combined withthermostated

1 titanium heaters [Process Technology, Brookfield CT, USA](Enzor *et al.*, 2013). Atmospheric
2 air was pumped through drying columns (filled with drierite) to remove moisture, and air was
3 scrubbed of CO₂ using columns filled with Sodasorb. Pure CO₂ and CO₂-free air were then
4 blended using digital mass flow controllers and bubbled into header tanks that were continuously
5 replenished with ambient seawater using venturri injectors, which in turn fed into experimental
6 treatment tanks.

7 Temperature, pH (total scale), salinity, total alkalinity (TA) and oxygen saturation were
8 measured daily from both incoming seawater as well as experimental treatment tanks (Table 4).
9 For *p*CO₂ analysis, we followed the SOP as described in the Best Practices Guide (Gattuso *et al.*, 2010)
10 for the spectrophotometric determination of pH using m-cresol purple and
11 measurement of total alkalinity via acid titration using a computer-controlled T50 Titrator
12 (Mettler Toledo, Columbus, OH, USA) . Temperature was measured with a calibrated digital
13 thermocouple (Omega Engineering Inc., Stamford, CT, USA) and salinity was measured using a
14 YSI 3100 Conductivity meter (Yellow Springs, OH, USA). CO₂calc(Robbinset *et al.*, 2010), using
15 the constants of Mehrbach *et al.* (1973) as refit by Dickson & Millero (1987), was used to
16 calculate all other carbonate parameters. Oxygen saturation was recorded using a galvanic
17 oxygen probe (Loligo Systems, Denmark). Mean values ($\pm$ s.d.) of temperature ($^{\circ}$ C) and *p*CO₂
18 (μ atm) over the course of the experiment were first reported in Enzor and Place (2014).
19 Additionally, treatment tanks were sampled daily for the presence of ammonia, nitrite and
20 nitrates, with no significant increase in waste products noted over the course of the experiment
21 (data not shown).

22 *Tissue collection and RNA extraction:* To obtain individual gill-specific expression profiles, we
23 separately indexed and sequenced RNA samples from gill tissue that had been collected from

1 fish acclimated to the two experimental treatments described above (n=5 fish per treatment, n=3
2 in 56d control) for 7 days, 28 days and 56 days. Immediately after euthanizing the fish, tissues
3 were excised in a -2 °C environmental chamber, flash frozen in liquid nitrogen, and shipped back
4 to our home institution on dry ice where they were stored at -80 °C until used. Total RNA from
5 approximately 100 mg of frozen tissue was extracted using TRIzol (Invitrogen) following the
6 manufacturer's recommendations. The RNA was further cleaned by re-suspending in 0.1 ml of
7 RNase/ DNase-free water and adding 0.3 ml of 6 M guanidine HCl and 0.2 ml of 100%
8 ethylalcohol (EtOH). The entire volume was loaded onto a spin column (Ambion) and
9 centrifuged for 1 min at $12,000 \times g$ at 4 °C. Flow-through was discarded, and filters were washed
10 twice with 0.2 ml 80% EtOH. RNA was eluted off of the filters twice with 0.1 ml of DEPC-
11 treated water. RNA was precipitated by the addition of 0.1 vol of 3 M sodium acetate (pH 5.0)
12 and 2.5 vol of 100% EtOH, mixed by inversion of tubes and placed at -80 °C for 1 h. After this
13 period, tubes were centrifuged at $12,000 \times g$ for 20 min at 4°C. Pellets were washed twice with
14 80% EtOH and re-suspended in 30 µl of RNase/ DNase-free water. Lastly, RNA was DNase
15 treated at 30 °C for 10 min.

16 Total RNA from n=5 fish (n=3 in 56d control) within an acclimation treatment was
17 submitted to the Vaccine and Gene Therapy Institute (VGTI) Florida for quality assessment and
18 determination of specific concentration using an Agilent 2100 BioAnalyzer. From the original
19 samples, the 4 highest quality replicates (n=3 in 56d control) from each treatment and time point
20 were selected for cluster generation using the Illumina® TruSeq RNA Sample Prep v2
21 Hsprotocol and sequencing via an Illumina® HiSeq 2500 Rapid Run initialized for both paired-
22 end 150bp reads and single-end 100bp reads.

1 *Quality control* - Raw reads from each of the twenty-three samples were processed using
2 Trimmomatic (version Trimmomatic-0.32) (Bolger *et al.*, 2014). For both paired-end and single-
3 end reads Illumina® TruSeq RNA Sample Prep v2 HS adapters were removed as well as any
4 bases on the end of the reads with a PHRED33 score of < 20 or any portion of the read that did
5 not average at least a PHRED33 score of > 20 across a minimum span of 4bp. Paired-end reads
6 with a length < 100 were removed, as well as any reads that were orphaned during the quality
7 control process; whereas single-end reads with a length < 75 were removed (paired-end
8 Trimmomatic parameter input: VGTI_Adapters_TrueSeq2-PEMultiplex.fa:2:30:10 LEADING:20
9 TRAILING:20 SLIDINGWINDOW:4:20 MINLEN:100; single-end Trimmomatic parameter
10 input: VGTI_Adapters_TrueSeq2-PEMultiplex.fa:2:30:10 LEADING:20 TRAILING:20
11 SLIDINGWINDOW:4:20 MINLEN:75).

12 *Reference transcriptome assembly, annotation and comparison* – All paired-end reads surviving
13 quality control were used as input in the Trinity *de novo* assembly utility (version trinityrnaseq-
14 2.0.3) (Grabherr *et al.*, 2011). The Trinity package efficiently recovers full-length transcripts and
15 spliced isoforms across a range of expression levels, with less artificially constructed transcripts
16 than other assembly utilities (Zhao *et al.*, 2011). Assembly was conducted utilizing the Trinity's
17 default parameters with read normalization (N=50). Following assembly raw transcripts were
18 compacted using CD-HIT-EST (version cd-hit-v4.6.1-2012-08-27) (Fuet *et al.*, 2012) with a
19 sequence similarity requirement of 95% to eliminate redundant transcript sequences. Then the
20 single ends reads were mapped to the compacted transcripts utilizing Bowtie2 (Langmead &
21 Salzberg, 2012) with default parameters; after which RNA-Seq by Expectation-Maximization
22 ("RSEM" version 1.2.18) (Li & Dewey, 2011) was conducted to generate calculated fragments
23 per kilobase million (FPKM) values for each compacted transcript. Compacted transcripts were

1 then filtered with only those possessing $\text{FPKM} \geq 1$ being retained for the final reference
2 transcriptome.

3 Transcripts from the final reference transcriptome were used as query sequences in BLASTx
4 (version: ncbi-blast-2.2.25+) (Camacho *et al.*, 2009) searches with a minimum confidence value
5 of $10\text{E-}6$ required for annotation against the NCBI nr (non-redundant) database (version May
6 2015). BLASTx was implemented in a massively parallel manner utilizing the resources of the
7 Data Intensive Academic Grid (DIAG). After BLASTx results were obtained, the transcript
8 sequences and the corresponding BLASTx results were input into the BLAST2GO utility
9 (Conesa *et al.*, 2005) for further annotation including GO Mapping, GO Annotation, Enzyme
10 Code Annotation, and InterproScan(Jones *et al.*, 2014). GO Slim (Gene Consortium, 2014)
11 annotations were further generated for broad transcriptome wide comparisons.

12 Comparisons to an existing *P. borchgrevink* transcriptome were conducted utilizing the
13 transcripts of the current transcriptome as query sequences in BLASTn against the transcripts of
14 the transcriptome from Bilyk and Cheng (2013) as the database with a threshold cutoff of $10\text{E-}3$.
15 *Differential Gene Expression Analysis:* Using raw mapping counts from Bowtie2 (Langmead &
16 Salzberg, 2012), RSEM(Li & Dewey, 2011) analyses were conducted to generate estimated read-
17 count (count) and FPKM counts for each sample at the transcript and gene level. The Trinity
18 pipeline (Trinity version trinityrnaseq-2.0.3) (Broad Institute and the Hebrew University of
19 Jerusalem, 2014) was used to aggregate these counts into master matrices for import into the R
20 statistical package (R Core Team, 2013). Before import, the samples were grouped by time-point
21 and treatment similarity to conduct pairwise analyses of the effect of the dual-stressor treatment
22 over time as compared to the control. Empirical analysis of digital gene expression data in R
23 (“edgeR” version 3.4.2) was implemented to conduct differential gene expression analyses

(Robinson *et al.*, 2010). Dispersion values were calculated using the replicate groups and exact tests utilizing a negative binomial distribution with a cutoff false discovery rate (FDR) of 0.05 were used to identify differentially expressed transcripts and genes.

A sample-level differential expression heat map was generated from the differential gene expression analyses resulting from edgeR using the Trinity pipeline. Within the BLAST2GO graphical interface package (Conesa *et al.*, 2005) Fisher's Exact tests ($FDR \leq 0.05$) were conducted for differentially expressed transcripts of the dual-stressor treatment to identify over-represented gene ontology terms within the up- and down-regulated transcripts within each treatment group in general. Using custom Python scripts: GO Slim, BLAST annotation and expression data files were combined, and GO categories and genes of interest were extracted for further analysis.

KEGG mapping analyses: Gene IDs corresponding to putative homologues in *Daniorerio* were obtained for all transcripts in the annotated transcriptome of *P. borchgrevinkii* that displayed differential expression in at least one of the three acclimation time points. These gene IDs were then used as search identifiers in the KEGG Search&Colorpathway mapping tool and mapped against the *D. rerio* KEGG pathway database to highlight cellular pathways highly represented in the dataset.

1 Tables

	Transcript Level	Gene Level
# Sequences	83,869	46,176
N50 (bp)	2,590	2,771
Median Length (bp)	1,002	998
Mean Length (bp)	1,533	1,589
Total Transcriptome Length (bp)	128,610,174	73,397,004

2 *Table 1: Pagothenia borchgrevinkitranscriptome assembly statistics:* Statistics for the filtered *de*
3 *novo* assembly were computed on the transcript and gene level. The # of sequences on the
4 transcript level represent all surviving contiguous sequences, the # of sequences on the gene
5 level represent the number of putative genes surviving based upon groupings of homologous
6 transcripts.

7

	Transcript Level	Gene Level
Total	83,869	46,176
GO Annotation	38,497	19,577
Blast Only	20,515	9,152
No Annotation Result	24,857	17,447

8 *Table 2: Pagothenia borchgrevinkitranscriptome annotation statistics:* Statistics for the filtered
9 *de novo* assembly were computed on the transcript and gene level. The # of sequences of the
10 transcript level represent all surviving contiguous sequences, the # of sequences on the gene
11 level represent the number of putative genes surviving based upon groupings of homologous
12 transcripts.

13

7d up-regulated pathways	DE genes	7d down-regulated pathways	DE genes
Metabolic pathways	17	Exosome	8
Toll-like receptor signaling pathway	8	Protein phosphatases	5
MAPK signaling pathway	6	Transcription factors	5
Protein processing in ER	6	Chromosome	5
RIG-I-like receptor signaling pathway	6	Cellular antigens	3
AGE-RAGE signaling pathway	5	Peptidases	2
Cytosolic DNA-sensing pathway	5	Transporters	2
NOD-like receptor signaling pathway	5	DNA repair and recombination	2
Cytokine-cytokine receptor interaction	4	Mitochondrial biogenesis	2
Apoptosis	4	Messenger RNA biogenesis	2

1

28d up-regulated pathways	DE genes	28d down-regulated pathways	DE genes
Metabolic pathways	4	Metabolic pathways	2
Fructose and Manose Metabolism	2	Terpenoid biosynthesis	2
Glycolysis/ Gluconeogenesis	2	SNAREs	1
Pyruvate metabolism	1	Cell adhesion molecules	1
Oxidative phosphorylation	1	AGE-RAGE signaling pathway	1
TOLL-like receptor signaling pathway	1	Steroid hormone biosynthesis	1
p53 signaling pathway	1	MAPK signaling pathway	1
NOD-like receptor signaling pathway	1	Purine metabolism	1
Pentose phosphate pathway	1	Calcium signaling	1
Apoptosis	1	Fatty acid metabolism	1

2

56d up-regulated pathways	DE genes	56d down-regulated pathways	DE genes
Metabolic pathways	11	Metabolic pathways	7
Purine metabolism	4	Steroid biosynthesis	3
Oxidative phosphorylation	3	RNA transport	3
Spliceosome	2	Apoptosis	2
Steroid hormone biosynthesis	2	p53 signaling pathway	2
Calcium signaling pathway	2	RNA degradation	1
Regulation of actin skeleton	2	GnRH signaling pathway	1
Glycolysis/ gluconeogenesis	1	Glycolysis/ gluconeogenesis	1
Glycosphingolipid biosynthesis	1	Tyrosine metabolism	1
Nicotinate and nicotinamide metabolism	1	Calcium signaling pathway	1

Table 3: Enriched KEGG pathways in dual-stressor acclimated fish. The top 10 pathways identified in the *Daniorerio* KEGG database using the pathway mapper tool. Numbers in the colored columns represent the total number of differentially expressed genes (DE) with a ≥ 2.0 -fold increase (yellow) or decrease (blue) in expression relative to control fish from the same time point (FDR ≤ 0.05).

	Total Alkalinity ($\mu\text{mol/kg sol'n}$)	pH _T	DO	pCO ₂ (μatm)	Temperature (°C)
Incoming Seawater	2343.187 (± 8.410)	8.012 (± 0.021)	91.89 (± 3.786)	427.66 (± 23.97)	-1.03 (± 0.152)
Low Temperature + Low pCO₂	2345.713 (± 8.622)	8.001 (± 0.020)	93.80 (± 2.465)	432.04 (± 22.50)	-0.707 (± 0.153)
High Temperature + High pCO₂	2350.513 (± 8.403)	7.659 (± 0.027)	94.14 (± 2.20)	1053.44 (± 71.87)	4.03 (± 0.316)

Table 4: Seawater parameters collected over the course of the experiment. Temperature, pH (total scale), salinity, total alkalinity (TA) and oxygen saturation (DO) were measured daily from

- 1 both incoming seawater as well as experimental treatment tanks using published standard
- 2 operating procedures. These measured values were then used to calculate $p\text{CO}_2$ using CO2 Calc.
- 3 Values are reported as the mean ($\pm s.d.$) over the course of the 56d experiment.

1 **Figure Legends**

2 *Figure 1 – P. borchgrevinkitranscriptome level GO comparison:* A comparison of the GO term
3 distribution for the broad category of molecular function is provided for the previous *P.*
4 *borchgrevinkitranscriptome* assembly and annotation conducted by Bilyk and Cheng (2014) and
5 the current transcriptome assembly and annotation. To provide a more direct comparison, the
6 data bars represent the proportion of terms assigned to the specific GO term listed along the x-
7 axis out of all GO terms within that broad category.

8 *Figure 2 – Sample level gene expression correlation matrix:* The correlation matrix demonstrates
9 the level of transcriptome wide gene expression correlation from 0-1, with 0 indicating no
10 correlation and 1 indicating an identical expression profile. The current correlation matrix is
11 indexed between 0.5 and 0.9 with black representing a correlation of 0.5 and yellow representing
12 a correlation of 0.9. Cluster dendrograms are provided to demonstrate the relationships between
13 the expression profiles of each individual sample.

14 *Figure 3 – Pathway expression summary:* The number of differentially expressed genes ($FDR \leq$
15 0.05) for the biological process gene ontology categories: response to stress (GO:0006950),
16 immune system process (GO:0002376), homeostatic process (GO:0042592), carbohydrate
17 metabolic process (GO:0005975), lipid metabolic process (GO:0006629), cell proliferation
18 (GO:0008283) and cell death (GO:0008219) are shown for each dual-stressor treatment as
19 compared to the control for the 7d, 28d and 56d time-points. A bar above the black line indicates
20 the number of up-regulated genes within that GO category, whereas the bar below the black lines
21 indicates the number of down-regulated gene within that GO category.

22 *Figure 4 – Integration of the inflammatory signaling response:* KEGG pathway analysis
23 highlighted a number of conserved cell signaling cascades that produce an integrated

inflammatory/ immune response among vertebrates and may provide insight into the unexpected changes in HSP90 expression. Among the pathways that appeared to be enriched for differentially expressed genes in *T. bernacchii* when acclimated to the dual-stressor treatment include the Toll-like receptor signaling pathway (TLR), NOD-like receptor signaling pathway (NLR) and the MAP kinase p38/ JNK signaling pathway (MAPK) whose simplified signaling cascades are represented in the grey boxes. Oxidative damage from may directly activate the NLR by recruiting HSP90, an inhibitor of NALP3. ROS may also indirectly activate the TLR pathway via production of damage-associated molecular pattern molecules (DAMPs). Pro-inflammatory cytokines activated by the TRL and NRL pathways converge on the MAPK pathway, resulting activation of pro-apoptotic signals and negative feedback loops. Colored boxes represent genes annotated in the transcriptome of *T. bernacchii* that are up-regulated (yellow), down-regulated (blue), or displayed no change in expression (green).

Availability of Supporting Data

The raw data were deposited at the NCBI Sequence Read Archive under the Bioproject accession number PRJNA294774.

List of Abbreviations

bp – base pair

CHR – cellular homeostasis response

CSR – cellular stress response

DE – differential expression

ER – endoplasmic reticulum

- 1 FDR – false discovery rate
- 2 FPKM – fragments per kilobase million
- 3 GO – Gene Ontology
- 4 IPCC – International Panel on Climate Change
- 5 $p\text{CO}_2$ – partial pressure carbon dioxide
- 6 RNA-Seq – RNA sequencing
- 7 RSEM – RNA-Seq by Expectation-Maximization
- 8 SST – sea surface temperature
- 9 UPR – unfolded protein response

10

11

12

13

14 **Authors Contributions**

15 SP designed the research and performed the experiments. TH prepared samples for sequencing,
16 processed the data, and analyzed the data. TH and SP wrote the article. All authors read and
17 approved the final manuscript.

18

19 **Description of Additional Files**

20 *Supplementary Table 1 – Enriched pathways highlighted by KEGG mapping analysis:* Gene IDs
21 corresponding to homologous genes in *Daniorerio* were obtained for all transcripts in the
22 annotated transcriptome of *P. borchgrevinki* that displayed differential expression in at least one
23 of the three acclimation time points. These gene IDs were then used as search identifiers in the

1 KEGG Search&Color Pathway mapping tool (Kyoto Encyclopedia of Genes and Genomes,
2 Kanehisa *et al.*, 2014) and mapped against the *D. rerio* KEGG Pathway database. The top ten
3 pathways with the highest number of genes mapping to a specific cellular function are shown.

4 *Supplementary Table 2 – Immune system process:* The table provides all differentially expressed
5 genes exceeding the confidence threshold ($FDR \leq 0.05$) that are associated with the GO Term
6 immune system process (GO:0002376) at 7d, 28d and 56d ($n = 4$). The table includes the gene
7 sequence name from the reference transcriptome, the BLAST result description, the sequence
8 length, the number of BLAST hits to that gene sequence, the minimum e-value of the BLAST
9 hits, the mean similarity, the # of GO terms associated with that sequence, the list of those GO
10 terms, the Enzyme Codes associated with that sequence, InterproScan results associated with that
11 sequence, the log2 adjusted fold change in expression, the log2 counts per million (CPM) of the
12 reads mapped to that sequence, and the p-value and FDR reflecting the confidence in the
13 displayed differential expression of that gene.

14 *Supplementary Table 3 – Response to stress:* The table provides all differentially expressed
15 genes exceeding the confidence threshold ($FDR \leq 0.05$) that are associated with the GO Term
16 response to stress (GO:0006950) at 7d, 28d and 56d ($n = 4$). The table includes the gene
17 sequence name from the reference transcriptome, the BLAST result description, the sequence
18 length, the number of BLAST hits to that gene sequence, the minimum e-value of the BLAST
19 hits, the mean similarity, the # of GO terms associated with that sequence, the list of those GO
20 terms, the Enzyme Codes associated with that sequence, InterproScan results associated with that
21 sequence, the log2 adjusted fold change in expression, the log2 counts per million (CPM) of the
22 reads mapped to that sequence, and the p-value and FDR reflecting the confidence in the
23 displayed differential expression of that gene.

Supplementary Table 4 – Cell proliferation: The table provides all differentially expressed genes exceeding the confidence threshold ($FDR \leq 0.05$) that are associated with the GO Term cell proliferation (GO:0008283) at 7d, 28d and 56d ($n = 4$). The table includes the gene sequence name from the reference transcriptome, the BLAST result description, the sequence length, the number of BLAST hits to that gene sequence, the minimum e-value of the BLAST hits, the mean similarity, the # of GO terms associated with that sequence, the list of those GO terms, the Enzyme Codes associated with that sequence, InterproScan results associated with that sequence, the log2 adjusted fold change in expression, the log2 counts per million (CPM) of the reads mapped to that sequence, and the p-value and FDR reflecting the confidence in the displayed differential expression of that gene.

Supplementary Table 5 – Cell death: The table provides all differentially expressed genes exceeding the confidence threshold ($FDR \leq 0.05$) that are associated with the GO Term cell death (GO:0008219) at 7d, 28d and 56d ($n = 4$). The table includes the gene sequence name from the reference transcriptome, the BLAST result description, the sequence length, the number of BLAST hits to that gene sequence, the minimum e-value of the BLAST hits, the mean similarity, the # of GO terms associated with that sequence, the list of those GO terms, the Enzyme Codes associated with that sequence, InterproScan results associated with that sequence, the log2 adjusted fold change in expression, the log2 counts per million (CPM) of the reads mapped to that sequence, and the p-value and FDR reflecting the confidence in the displayed differential expression of that gene.

Supplementary Table 6 – Lipid metabolic process: The table provides all differentially expressed genes exceeding the confidence threshold ($FDR \leq 0.05$) that are associated with the GO Term lipid metabolic process (GO:0006629) at 7d, 28d and 56d ($n = 4$). The table includes the gene

sequence name from the reference transcriptome, the BLAST result description, the sequence length, the number of BLAST hits to that gene sequence, the minimum e-value of the BLAST hits, the mean similarity, the # of GO terms associated with that sequence, the list of those GO terms, the Enzyme Codes associated with that sequence, InterproScan results associated with that sequence, the log₂ adjusted fold change in expression, the log₂ counts per million (CPM) of the reads mapped to that sequence, and the p-value and FDR reflecting the confidence in the displayed differential expression of that gene.

Supplementary Table 7 – Carbohydrate metabolic process: The table provides all differentially expressed genes exceeding the confidence threshold ($FDR \leq 0.05$) that are associated with the GO Term carbohydrate metabolic process (GO:0005975) at 7d, 28d and 56d (n = 4). The table includes the gene sequence name from the reference transcriptome, the BLAST result description, the sequence length, the number of BLAST hits to that gene sequence, the minimum e-value of the BLAST hits, the mean similarity, the # of GO terms associated with that sequence, the list of those GO terms, the Enzyme Codes associated with that sequence, InterproScan results associated with that sequence, the log₂adjusted fold change in expression, the log₂ counts per million (CPM) of the reads mapped to that sequence, and the p-value and FDR reflecting the confidence in the displayed differential expression of that gene.

Supplementary Figure 1 – Transcriptome transcript size distribution: The length distribution (log_e adjusted) of transcripts with a minimum length of 200bp resulting from *de novo* assembly (grey), following CD-HIT-EST transcript clustering with a percent identity requirement of 95% (blue) and transcript filtering after RSEM requiring an expression level of at least 1.0 FPKM (yellow).

Supplemental Figure 2 – GO molecular function expression: Molecular function gene ontology terms with more than 5 differentially expressed genes per category ($FDR \leq 0.05$) across all time-points are shown for each time-point. Yellow bars indicate the number of up-regulated genes associated with that GO term and blue bars indicate the number of down-regulated genes associated with that GO term.

Supplemental Figure 3 – GO biological process expression: Biological process gene ontology terms with more than 5 differentially expressed genes per category ($FDR \leq 0.05$) across all time-points are shown for each time-point. Yellow bars indicate the number of up-regulated genes associated with that GO term and blue bars indicate the number of down-regulated genes associated with that GO term.

Acknowledgements

There are many people who assisted us during the course of this project. In particular, we thank Dr. Jeff Dudycha, Dr. Mackenzie Zippay, Evan Hunter and Allison “Sandwich” Barden for their help collecting specimens and with seawater chemistry measurements. We would also like to thank the United States Antarctic Program, Lockheed Martin Antarctic Support Contract, and the countless number of USAP support personnel for their tireless logistical and field support while at McMurdo Station.

This work used the computational resources of the Data Intensive Academic Grid (DIAG), National Science Foundation funded MRI-R2 project #DBI-0959894.

This research was supported by National Science Foundation (NSF) Grants PLR-1040495 and PLR-1447291 to SPP.

References

- Almroth, B. C., N. Asker, B. Wassmur, M. Rosengren, F. Jutfelt, A. Gräns, K. Sundell, et al. (2015). Warmer water temperature results in oxidative damage in an Antarctic fish, the bald notothen. *Journal of Experimental Marine Biology and Ecology* 468:130-137.
- Basu, N., Todgham, a. E., Ackerman, P. a., Bibeau, M. R., Nakano, K., Schulte, P. M., & Iwama, G. K. (2002). Heat shock protein genes and their functional significance in fish. *Gene*, 295(2), 173–183. doi:10.1016/S0378-1119(02)00687-X
- Bilyk, K. T., & Cheng, C. H. C. (2013). Model of gene expression in extreme cold - reference transcriptome for the high-Antarctic cryopelagic notothenioid fish *Pagothenia borchgrevinki*. *BMC Genomics*, 14(1), 634. doi:10.1186/1471-2164-14-634
- Bilyk, K. T., & Cheng, C. H. C. (2014). RNA-seq analyses of cellular responses to elevated body temperature in the high Antarctic cryopelagic nototheniid fish *Pagothenia borchgrevinki*. *Marine Genomics*, 18, 163–171. doi:10.1016/j.margen.2014.06.006
- Bilyk, K. T., & DeVries, A. L. (2011). Heat tolerance and its plasticity in Antarctic fishes. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, 158(4), 382–390. doi:10.1016/j.cbpa.2010.12.010
- Bilyk, K. T., Evans, C. W., & DeVries, A. L. (2012). Heat hardening in Antarctic notothenioid fishes. *Polar Biology*, 35(9), 1447–1451. doi:10.1007/s00300-012-1189-0
- Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics (Oxford, England)*. doi:10.1093/bioinformatics/btu170
- Broad Institute and the Hebrew University of Jerusalem. (2014). Identification and Analysis of Differentially Expressed Trinity Genes and Transcripts. Retrieved from http://trinityrnaseq.sourceforge.net/analysis/diff_expression_analysis.html
- Buckley, B. a, Gracey, A. Y., & Somero, G. N. (2006). The cellular response to heat stress in the goby *Gillichthys mirabilis*: a cDNA microarray and protein-level analysis. *The Journal of Experimental Biology*, 209(Pt 14), 2660–2677. doi:10.1242/jeb.02292
- Buckley, B. a, Place, S. P., & Hofmann, G. E. (2004). Regulation of heat shock genes in isolated hepatocytes from an Antarctic fish, *Trematomus bernacchii*. *The Journal of Experimental Biology*, 207(Pt 21), 3649–3656. doi:10.1242/jeb.01219

1 Buckley, B. a., & Somero, G. N. (2009). CDNA microarray analysis reveals the capacity of the
2 cold-adapted Antarctic fish *Trematomus bernacchii* to alter gene expression in response to
3 heat stress. *Polar Biology*, 32(3), 403–415. doi:10.1007/s00300-008-0533-x

4 Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T.
5 L. (2009). BLAST+: architecture and applications. *BMC Bioinformatics*, 10, 421.
6 doi:10.1186/1471-2105-10-421

7 Carney Almroth, B., Asker, N., Wassmur, B., Rosengren, M., Jutfelt, F., Gräns, A., ... Sturve, J.
8 (2015). Warmer water temperature results in oxidative damage in an Antarctic fish, the bald
9 notothen. *Journal of Experimental Marine Biology and Ecology*, 468, 130–137.
10 doi:10.1016/j.jembe.2015.02.018

11 Cole, K. E., Strick, C. a, Paradis, T. J., Ogborne, K. T., Loetscher, M., Gladue, R. P., ... Neote,
12 K. (1998). Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR
13 CXC chemokine with potent activity on activated T cells through selective high affinity
14 binding to CXCR3. *The Journal of Experimental Medicine*, 187(12), 2009–2021.
15 doi:10.1084/jem.187.12.2009

16 Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., & Robles, M. (2005). Blast2GO:
17 a universal tool for annotation, visualization and analysis in functional genomics research.
18 *Bioinformatics (Oxford, England)*, 21(18), 3674–6. doi:10.1093/bioinformatics/bti610

19 Cooney, R. N. (2002). JAK / STAT PATHWAY, 17(2), 83–90.

20 da Silva Correia, J., Miranda, Y., Leonard, N., Ulevitch, R. (2007) SGT1 is essential for Nod1
21 activation. *Proc. Natl. Acad. Sci. USA* 104, 6764– 6769.

22 Data Intensive Academic Grid. (n.d.). University of Maryland School of Medicine. Retrieved
23 from <http://diagcomputing.org/>

24 Davison, W., Franklin, C. E., & Carey, P. W. (1990). Oxygen uptake in the Antarctic teleost
25 *Pagothenia borchgrevinki* . Limitations imposed by X-cell gill disease. *Fish Physiology and*
26 *Biochemistry*, 8(1), 69–77.

27 Dickson, A. G., & Millero, F. J. (1987). A comparison of the equilibrium constants for the
28 dissociation of carbonic acid in seawater media. *Deep Sea Research Part A. Oceanographic*
29 *Research Papers*, 34(10), 1733–1743.

30 Eastman, J. (1991). Evolution and Diversification of Antarctic Notothenioid Fishes. *American*
31 *Zoologist*, 31(1), 93–109.

- 1 Eastman, J. (1993). *Antarctic fish biology: evolution in a unique environment*. Academic Press,
2 Inc.
- 3 Enzor, L. a, Zippay, M. L., & Place, S. P. (2013). High latitude fish in a high CO2 world:
4 Synergistic effects of elevated temperature and carbon dioxide on the metabolic rates of
5 Antarctic notothenioids. *Comparative Biochemistry and Physiology. Part A, Molecular &*
6 *Integrative Physiology*, 164(1), 154–61. doi:10.1016/j.cbpa.2012.07.016
- 7 Enzor, L. A., & Place, S. P. (2014). Is warmer better? Decreased oxidative damage in
8 notothenioid fish after long-term acclimation to multiple stressors. *Journal of Experimental*
9 *Biology*, 217(18), 3301–3310. doi:10.1242/jeb.108431
- 10 Enzor, L. A. & Place, S. P. The effects of elevated temperature and ocean acidification on the
11 metabolic pathways of notothenioid fish. *Conserv. Physiol. (in review)*.
- 12 Fangue, N. a., O'Donnell, M. J., Sewell, M. a., Matson, P. G., MacPherson, A. C., & Hofmann,
13 G. E. (2010). A laboratory-based, experimental system for the study of ocean acidification
14 effects on marine invertebrate larvae. *Limnology and Oceanography: Methods*, 8, 441–452.
15 doi:10.4319/lom.2010.8.441
- 16 Forester, M. E., Franklin, C. E., H, T. H., & Daviso, W. (1987). The aerobic scope of Antarctic
17 fish. *Pagothenia borchgrevinki* and it significance for metabolic cold adaptation. *Polar*
18 *Biology*, 8, 155–159.
- 19 Franklin, C. E., Davison, W., & Seebacher, F. (2007). Antarctic fish can compensate for rising
20 temperatures: thermal acclimation of cardiac performance in *Pagothenia borchgrevinki*. *The*
21 *Journal of Experimental Biology*, 210(Pt 17), 3068–3074. doi:10.1242/jeb.003137
- 22 Fu, L., Niu, B., Zhu, Z., Wu, S., & Li, W. (2012). CD-HIT: accelerated for clustering the next-
23 generation sequencing data. *Bioinformatics (Oxford, England)*, 28(23), 3150–2.
24 doi:10.1093/bioinformatics/bts565
- 25 Gene, T., Consortium, O., Gene, T., & Go, O. (2014). Gene Ontology Consortium: going
26 forward. *Nucleic Acids Research*, 43(D1), D1049–D1056. doi:10.1093/nar/gku1179
- 27 Gerdol, M., Buonocore, F., Scapigliati, G., & Pallavicini, A. (2015). Marine Genomics Analysis
28 and characterization of the head kidney transcriptome from the Antarctic fi sh *Trematomus*
29 *bernacchii* (Teleostea , Notothenioidea): A source for immune relevant genes. *Marine*
30 *Genomics*, 2014–2016. doi:10.1016/j.margen.2014.12.005
- 31 Gille, S. T. (2002). Warming of the Southern Ocean since the 1950s. *Science (New York, N.Y.)*,
32 295(5558), 1275–1277. doi:10.1126/science.1065863

- 1 Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. a, Amit, I., ... Regev, A.
2 (2011). Full-length transcriptome assembly from RNA-Seq data without a reference
3 genome. *Nature Biotechnology*, 29(7), 644–52. doi:10.1038/nbt.1883
- 4 Gracey, a Y., Troll, J. V, & Somero, G. N. (2001). Hypoxia-induced gene expression profiling in
5 the euryoxic fish *Gillichthys mirabilis*. *Proceedings of the National Academy of Sciences of*
6 *the United States of America*, 98(4), 1993–1998. doi:10.1073/pnas.98.4.1993
- 7 Gattuso, J-P, Kunshan Gao, Kitack Lee, B. R. and K. G. S. (2010). Guide to best practices for
8 ocean acidification research and data reporting. *Sciences-New York*.
- 9 Gutt, J., Bertler, N., Bracegirdle, T. J., Buschmann, A., Comiso, J., Hosie, G., ... Xavier, J. C.
10 (2014). The Southern Ocean ecosystem under multiple climate change stresses - an
11 integrated circumpolar assessment. *Global Change Biology*, n/a–n/a. doi:10.1111/gcb.12794
- 12 Hennes, T., Richter, C., & Peterhans, E. (1993). Tumour necrosis factor- α induces
13 superoxide anion generation in mitochondria of L929 cells. *The Biochemical Journal*, 289 (
14 Pt 2)(1 993), 587–592.
- 15 Hofmann, G. E., & Todgham, A. E. (2010). Living in the now: physiological mechanisms to
16 tolerate a rapidly changing environment. *Annual Review of Physiology*, 72, 127–145.
17 doi:10.1146/annurev-physiol-021909-135900
- 18 Huth, T. J., & Place, S. P. (2016). Transcriptome wide analyses reveal a sustained cellular stress
19 response in the gill tissue of *Trematomus bernacchii* after acclimation to multiple stressors.
20 *BMC Genomics (in press)*.
- 21 Huth, T. J., & Place, S. P. (2013). De novo assembly and characterization of tissue specific
22 transcriptomes in the emerald notothen, *Trematomus bernacchii*. *BMC Genomics*, 14, 805.
23 doi:10.1186/1471-2164-14-805
- 24 Jeffries, K. M., Hinch, S. G., Sierocinski, T., Pavlidis, P., & Miller, K. M. (2014).
25 Transcriptomic responses to high water temperature in two species of Pacific salmon.
26 *Evolutionary Applications*, 7(2), 286–300. doi:10.1111/eva.12119
- 27 Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., ... Hunter, S. (2014).
28 InterProScan 5: genome-scale protein function classification. *Bioinformatics (Oxford,*
29 *England)*, 30(9), 1236–40. doi:10.1093/bioinformatics/btu031
- 30 Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M. & Tanabe, M. (2014) Data,
31 information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Res.*,
32 42, D199–D205.

- 1 Kruidering, M., & Evan, G. I. (2000). Caspase-8 in apoptosis: the beginning of “the end”?
- 2 *IUBMB Life*, 50(2), 85–90. doi:10.1080/713803693
- 3 Kültz, D. (2005). Molecular and evolutionary basis of the cellular stress response. *Annual*
- 4 *Review of Physiology*, 67(1), 225–257. doi:10.1146/annurev.physiol.67.040403.103635
- 5 Langenbuch, M. & Pörtner, H.O. (2003). Energy budget of hepatocytes from Antarctic fish
- 6 (*Pachycara brachycephalum* and *Lepidonotothen kempfi*) as a function of ambient CO₂: pH-
- 7 dependent limitations of cellular protein biosynthesis? *Journal of Experimental Biology*, 206,
- 8 3895-3903. doi:10.1242/jeb.00620.
- 9 Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature*
- 10 *Methods*, 9(4), 357–9. doi:10.1038/nmeth.1923
- 11 Li, B., & Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data
- 12 with or without a reference genome. *BMC Bioinformatics*, 12, 323. doi:10.1186/1471-2105-
- 13 12-323
- 14 Logan, C. A., & Somero, G. N. (2011). Effects of thermal acclimation on transcriptional
- 15 responses to acute heat stress in the eurythermal fish *Gillichthys mirabilis* (Cooper).
- 16 *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*,
- 17 300(6), R1373–R1383. doi:10.1152/ajpregu.00689.2010
- 18 Matson, P. G., Martz, T. R., & Hofmann, G. E. (2011). High-frequency observations of pH
- 19 under Antarctic sea ice in the southern Ross Sea. *Antarctic Science*, 23 (6), 607–613.
- 20
- 21 Mayor, A., Martinon, F., De Smedt, T., Petrilli, V., Tschopp, J. (2007) A crucial function of
- 22 SGT1 and HSP90 in inflammasome activity links mammalian and plant innate immune
- 23 responses. *Nat. Immunol.* 8, 497– 503.
- 24
- 25 Mehrbach, C., Culberson, C. H., Hawley, J. E., & Pytkowicz, R. M. (1973). Measurement of the
- 26 apparent dissociation constants of carbonic acid in seawater at atmospheric pressure.
- 27 *Limnology and Oceanography*, 18(6), 897–907. doi:10.4319/lo.1973.18.6.0897
- 28 O'Donnell, M. J., Hammond, L. M., & Hofmann, G. E. (2009). Predicted impact of ocean
- 29 acidification on a marine invertebrate: Elevated CO₂ alters response to thermal stress in sea
- 30 urchin larvae. *Marine Biology*, 156(3), 439–446. doi:10.1007/s00227-008-1097-6
- 31 Pachauri, R. K., Meyer, L., Van Ypersele, J.-P., Brinkman, S., Van Kesteren, L., Leprince-
- 32 Ringuet, N., & Van Boxtmeer, F. (2014). *Climate Change 2014 Synthesis Report The Core*
- 33 *Writing Team Core Writing Team Technical Support Unit for the Synthesis Report. Russian*

1 *Federation*), Hoesung Lee (Republic of Korea) Scott B. Power (Australia) N.H.
2 Ravindranath (India).

3 Pierson, P. M., Lamers, a., Flik, G., & Mayer-Gostan, N. (2004). The stress axis, stanniocalcin,
4 and ion balance in rainbow trout. *General and Comparative Endocrinology*, 137, 263–271.
5 doi:10.1016/j.ygcen.2004.03.010

6 Place, S. P., & Hofmann, G. E. (2005). Constitutive expression of a stress-inducible heat shock
7 protein gene, hsp70, in phylogenetically distant Antarctic fish. *Polar Biology*, 28(4), 261–
8 267. doi:10.1007/s00300-004-0697-y

9 Place, S. P., O'Donnell, M. J., & Hofmann, G. E. (2008). Gene expression in the intertidal
10 mussel *Mytilus californianus*: Physiological response to environmental factors on a
11 biogeographic scale. *Marine Ecology Progress Series*, 356, 1–14. doi:10.3354/meps07354

12 Place, S. P., Zippay, M. L., & Hofmann, G. E. (2004). Constitutive roles for inducible genes:
13 evidence for the alteration in expression of the inducible hsp70 gene in Antarctic
14 notothenioid fishes. *American Journal of Physiology. Regulatory, Integrative and*
15 *Comparative Physiology*, 287(2), R429–R436. doi:10.1152/ajpregu.00223.2004

16 Podrabsky, J. E., & Somero, G. N. (2004). Changes in gene expression associated with
17 acclimation to constant temperatures and fluctuating daily temperatures in an annual
18 killifish *Austrofundulus limnaeus*. *The Journal of Experimental Biology*, 207(Pt 13), 2237–
19 2254. doi:10.1242/jeb.01016

20 Pörtner, H.O. & Langenbuch, M. (2005). Synergistic effects of temperature extremes, hypoxia,
21 and increases in CO₂ on marine animals: From Earth history to global change. *Journal of*
22 *geophysical research*, 110, c09s10, doi:10.1029/2004jc002561,

23 Pörtner, H.O., REIPSCHLÄGER, A. & HEISLER, N. (1997). Acid–base regulation, metabolism
24 and energetics in *sipunculus nudus* as a function of ambient carbon dioxide level. *Journal of*
25 *Experimental Biology*, 201, 43–55

26 Pörtner, H. O., Lucassen, M., & Storch, D. (2005). Metabolic Biochemistry: Its Role in Thermal
27 Tolerance and in the Capacities of Physiological and Ecological Function. *Fish Physiology*.
28 doi:10.1016/S1546-5098(04)22003-9

29 R Core Team. (2013). R: A language and environment for statistical computing. Vienna,
30 Austria.: R Foundation for Statistical Computing,. Retrieved from URL [http://www.R-](http://www.R-project.org/)
31 [project.org/](http://www.R-project.org/)

- 1 Ritchie, P. a, Lavoué, S., & Lecointre, G. (1997). Molecular phylogenetics and the evolution of
2 antarctic notothenioid fishes. *Comparative Biochemistry and Physiology. Part A,*
3 *Physiology*, 118(4), 1009–25. Retrieved from
4 <http://www.ncbi.nlm.nih.gov/pubmed/9505416>
- 5 Robbins, L. L., Hansen, M. E., Kleypas, J. a, & Meylan, S. C. (2010). CO2calc - a user-friendly
6 seawater carbon calculator for Windows, Mac OS X and iOS (iPhone). *U.S. Geological*
7 *Survey Open-File Report 2010-1280*, 17.
- 8 Robertson, M. J. (2010). Role of chemokines in the biology of natural killer cells. *Current Topics*
9 *in Microbiology and Immunology*, 341(1), 37–58. doi:10.1007/82-2010-20
- 10 Robinson, E., & Davison, W. (2008)a. Antarctic fish can survive prolonged exposure to elevated
11 temperatures. *Journal of Fish Biology*, 73(7), 1676–1689. doi:10.1111/j.1095-
12 8649.2008.02041.x
- 13 Robinson, E., & Davison, W. (2008)b. The Antarctic notothenioid fish *Pagothenia borchgrevinki*
14 is thermally flexible: Acclimation changes oxygen consumption. *Polar Biology*, 31(3), 317–
15 326. doi:10.1007/s00300-007-0361-4
- 16 Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: a Bioconductor package for
17 differential expression analysis of digital gene expression data. *Bioinformatics (Oxford,*
18 *England)*, 26(1), 139–40. doi:10.1093/bioinformatics/btp616
- 19 Rogers, A. D., Murphy, E. J., Johnston, N. M., & Clarke, A. (2007). Introduction. Antarctic
20 ecology: from genes to ecosystems. Part 2. Evolution, diversity and functional ecology.
21 *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*,
22 362(1488), 2187–9. doi:10.1098/rstb.2007.2135
- 23 Rosa, R., & Seibel, B. a. (2008). Synergistic effects of climate-related variables suggest future
24 physiological impairment in a top oceanic predator. *Proceedings of the National Academy*
25 *of Sciences of the United States of America*, 105(52), 20776–20780.
26 doi:10.1073/pnas.0806886105
- 27 Schulte, P. M. (2007). Responses to environmental stressors in an estuarine fish: Interacting
28 stressors and the impacts of local adaptation. *Journal of Thermal Biology*, 32(3), 152–161.
29 doi:10.1016/j.jtherbio.2007.01.012
- 30 Schulze-Osthoff, K., Bakker, A. C., Vanhaesebroeck, B., Beyaert, R., Jacob, W. a., & Fiers, W.
31 (1992). Cytotoxic activity of tumor necrosis factor is mediated by early damage of
32 mitochondrial functions: Evidence for the involvement of mitochondrial radical generation.
33 *Journal of Biological Chemistry*, 267(8), 5317–5323.

- 1 Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G., & Fiers, W. (1993).
2 Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-
3 inductive effects of TNF. *The EMBO Journal*, 12(8), 3095–3104.
- 4 Seebacher, F., Davison, W., Lowe, C. J., & Franklin, C. E. (2005). A falsification of the thermal
5 specialization paradigm: compensation for elevated temperatures in Antarctic fishes.
6 *Biology Letters*, 1(2), 151–154. doi:10.1098/rsbl.2004.0280
- 7 Sims, D., Sudbery, I., Illott, N. E., Heger, A., & Ponting, C. P. (2014). Sequencing depth and
8 coverage: key considerations in genomic analyses. *Nature Reviews Genetics*, 15(2), 121-
9 132. doi:10.1038/nrg3642
- 10 Sleadd, I. M., Lee, M., Hassumani, D. O., Stecyk, T. M. a, Zeitz, O. K., & Buckley, B. A.
11 (2014). Sub-lethal heat stress causes apoptosis in an Antarctic fish that lacks an inducible
12 heat shock response. *Journal of Thermal Biology*, 44(1), 119–125.
13 doi:10.1016/j.jtherbio.2014.06.007
- 14 Somero, G. N., & DeVries, A. L. (1967). Temperature tolerance of some Antarctic fishes.
15 *Science*, 156, 257–258.
- 16 Strobel, A., Bennecke1, S., Leo, E.,Mintenbeck, K.,Pörtner, H.O. &Mark, C.F.(2012). Metabolic
17 shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2 .
18 *Frontiers in Zoology*,9(28), 1-15.
- 19 Tomalty, K. M. H., Meek, M. H., Stephens, M. R., Rincon, G., Fangue, N. a., May, B. P., &
20 Baerwald, M. R. (2015). Transcriptional Response to Acute Thermal Exposure in Juvenile
21 Chinook Salmon Determined by RNAseq. *G3: Genes/Genomes/Genetics*,
22 5(July), 1335–1349. doi:10.1534/g3.115.017699
- 23 Vijay N, Poelstra JW, Künstner A, Wolf JBW. 2013. Challenges and strategies in transcriptome
24 assembly and differential gene expression quantification. A comprehensive in silico
25 assessment of RNA-seq experiments. *Mol Ecol* 22: 620–634
- 26 Weber, C., Weber, K. S. C., Klier, C., Gu, S., Wank, R., Horuk, R., ... Dc, W. (2011).
27 Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of
28 monocytes and T H 1-like / CD45RO + T cells Specialized roles of the chemokine receptors
29 CCR1 and CCR5 in the recruitment of monocytes and T H 1-like / CD45RO 2 T cells,
30 97(4), 1144–1146. doi:10.1182/blood.V97.4.1144
- 31 Wuyts, A., Van Osselaer, N., Haelens, A., Samson, I., Herdewijn, P., Ben-Baruch, A., ... Van
32 Damme, J. (1997). Characterization of synthetic human granulocyte chemotactic protein 2:

- 1 Usage of chemokine receptors CXCR1 and CXCR2 and in vivo inflammatory properties.
2 *Biochemistry*, 36(9), 2716–2723. doi:10.1021/bi961999z
- 3 Young, J. C., Moarefi, I., & Ulrich Hartl, F. (2001). Hsp90: A specialized but essential protein-
4 folding tool. *Journal of Cell Biology*, 154(2), 267–273. doi:10.1083/jcb.200104079
- 5 Zhao, Q.-Y., Wang, Y., Kong, Y.-M., Luo, D., Li, X., & Hao, P. (2011). Optimizing de novo
6 transcriptome assembly from short-read RNA-Seq data: a comparative study. *BMC*
7 *Bioinformatics*, 12(Suppl 14), S2. doi:10.1186/1471-2105-12-S14-S2

Figure 1

Proportion of GO Terms

Figure 2 SIMILARITY

Figure 3

Figure 4