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RESEARCH ARTICLE

Is warmer better? Decreased oxidative damage in notothenioid fish after long-term acclimation to multiple stressors

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ABSTRACT

Antarctic fish of the suborder Notothenioidei have evolved several unique adaptations to deal with subzero temperatures. However, these adaptations may come with physiological trade-offs, such as an increased susceptibility to oxidative damage. As such, the expected environmental perturbations brought on by global climate change have the potential to significantly increase the level of oxidative stress and cellular damage in these endemic fish. Previous single stressor studies of the notothenioids have shown they possess the capacity to acclimate to increased temperatures, but the cellular-level effects remain largely unknown. Additionally, there is little information on the ability of Antarctic fish to respond to ecologically relevant environmental changes where multiple variables change concomitantly. We have examined the potential synergistic effects that increased temperature and $\dot{P}_{CO_2}$ have on the level of protein damage in *Trematomus bernacchii*, *Pagothenia borchgrevinki* and *Trematomus newnesi*, and combined these measurements with changes in total enzymatic activity of catalase (CAT) and superoxide dismutase (SOD) in order to gauge tissue-specific changes in antioxidant capacity. Our findings indicate that total SOD and CAT activity levels displayed only small changes across treatments and tissues. Short-term acclimation to decreased seawater pH and increased temperature resulted in significant increases in oxidative damage. Surprisingly, despite no significant change in antioxidant capacity, cellular damage returned to near-basal levels, and significantly decreased in *T. bernacchii*, after long-term acclimation. Overall, these data suggest that notothenioid fish currently maintain the antioxidant capacity necessary to offset predicted future ocean conditions, but it remains unclear whether this capacity comes with physiological trade-offs.

KEY WORDS: Ocean acidification, Oxidative damage, Superoxide dismutase, Thermal stress, Notothenioid, Global climate change

INTRODUCTION

The highly endemic fishes of the suborder Notothenioidei represent the dominant perciform fish fauna and a significant portion of the biomass found in the Southern Ocean (Eastman, 1993). Contributing to their endemism, notothenioids have evolved under extreme cold conditions for several million years and, as such, several unique cellular and physiological alterations have arisen, such as the evolution of anti-freeze proteins (DeVries, 1969), the functional loss of heme proteins (O'Brien and Sidell, 2000; Sidell and O'Brien, 2006) and the constant expression of inducible heat shock proteins (Hofmann et al., 2000; Place et al., 2004). Additionally, evolution in this extreme cold environment has led to several unique metabolic adaptations including increased mitochondrial density and the utilization of lipids as a primary energy source (Lin et al., 1974; Clarke et al., 1984; Johnston et al., 1998; Pörtner et al., 2005). These adaptations have been suggested to potentially result in an increased susceptibility to reactive oxygen species (ROS) (Abele and Puntarulo, 2004), which can be further exacerbated by environmental perturbations associated with global climate change, such as changes in temperature and pH (Cadenas, 1989; Lesser, 2006). If left un-checked, or allowed to form faster than they can be detoxified, ROS can cause significant damage to proteins, DNA and lipids (Abele and Puntarulo, 2004; Lesser, 2006) and may represent a significant energetic cost to basal cellular maintenance in Antarctic fish.

The effects of increased temperature on Antarctic fish have been well documented (Somero and DeVries, 1967; Forster et al., 1987; Davison et al., 1990; Pörtner, 2008; Robinson and Davison, 2008a; Robinson and Davison, 2008b). At the level of the whole organism, an early study from Somero and DeVries (Somero and DeVries, 1967) illustrated a narrow thermal window exists in which notothenioids can survive; however, more recent studies have shown that notothenioid fishes have retained the capacity to acclimate to increased temperatures (Seebacher et al., 2005; Franklin et al., 2007; Robinson and Davison, 2008a; Robinson and Davison, 2008b; Bilyk and DeVries, 2011; Bilyk et al., 2012). While these studies provide evidence that Antarctic fish are capable of acclimating to an increase in temperature, there is little information about the cost of acclimation on a cellular level. Of the studies that have examined the effects of temperature on oxidative damage in Antarctic fish, most have focused primarily on the hemoglobinless fish from the family Channicthyidae, with only three red-blooded notothens represented, *Gobionotothen gibberifrons*, *Notothenia rossii* and *Notothenia coriiceps* (Beers and Sidell, 2011; Mueller et al., 2011; Mueller et al., 2012; Machado et al., 2014). Moreover, with the exception of the study by Machado et al. (Machado et al., 2014), which examined liver tissues of fishes acclimated up to 6 days, these studies utilized isolated mitochondria from fish exposed to their critical thermal maximum for 2 h. To date, little information exists with respect to tissue-specific levels of cellular damage in red-blooded notothenioids, especially when considering potential differences in chronic versus acute exposures.

In addition to elevated sea surface temperature (SST), a growing body of literature has focused on the impacts ocean acidification will have on marine teleosts; however, the majority of these studies have focused primarily on tropical or temperate species (Melzner et al., 2009; Munday et al., 2009a; Munday et al., 2009b; Munday et al., 2010; Munday et al., 2011; Esbaugh et al., 2012; Heuer et al., 2012; Nowicki et al., 2012; Bignami et al., 2013; Chivers et al., 2014; Munday et al., 2014; Murray et al., 2014; Pope et al., 2014). Despite projections that indicate changes in SST and partial pressure of CO_2 ($\dot{P}_{CO_2}$) in seawater will impact higher latitudes faster and to a greater

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List of symbols and abbreviations

CAT	catalase
PBS	phosphate-buffered saline
$\dot{P}_{CO_2}$	partial pressure of CO_2
RMR	resting metabolic rate
ROS	reactive oxygen species
SOD	superoxide dismutase
SST	sea surface temperature
TBS	Tris-buffered saline

extent than temperate regions (Walther et al., 2002; Orr et al., 2005; Turner et al., 2005; McNeil and Matear, 2008; Fabry et al., 2008; Fabry et al., 2009; Halpern et al., 2008; McNeil et al., 2010; Mathis et al., 2011a; Mathis et al., 2011b), only a handful of studies have examined the effects of ecologically relevant increases in seawater $\dot{P}_{CO_2}$ on high latitude marine teleosts (Hurst et al., 2012; Strobel et al., 2012; Enzor et al., 2013; Strobel et al., 2013a; Strobel et al., 2013b). Similar to impacts seen with elevated temperature, ocean acidification may also perturb oxidative stress in marine teleosts (Murphy, 2009; Tomanek et al., 2011) and may even exacerbate the detrimental effects of reactive oxygen species at the cellular level (Ezraty et al., 2011).

Currently, the potential synergistic effects of increased temperature and increased $\dot{P}_{CO_2}$ in fish have only been examined in a small number of studies (Pankhurst and Munday, 2011; Nowicki et al., 2012; Strobel et al., 2012; Enzor et al., 2013; Strobel et al., 2013a; Strobel et al., 2013b; Gräns et al., 2014). We have previously shown that increased temperature and $\dot{P}_{CO_2}$ levels result in a rapid increase in resting metabolic rates in several species of Antarctic fish (Enzor et al., 2013). This rapid increase in respiration has the potential to negatively impact the organism through elevated production of ROS (Lesser, 2006). Therefore, given the known impacts of temperature and elevated $\dot{P}_{CO_2}$ on metabolic rates in these fish, combined with the potential increased susceptibility of Antarctic fish to ROS production, we set out to test the hypothesis that Antarctic fish will experience a significant increase in oxidative stress and cellular damage caused by acclimation to a multi-stressor scenario of increased temperature and $\dot{P}_{CO_2}$. We first investigated the amount of oxidative damage by quantifying the level of protein carbonyls from gill and liver tissue isolated from three notothenioid fish, *Trematomus bernacchii* (Boulenger, 1902), *Trematomus newnesi* (Boulenger, 1902) and *Pagothenia borchgrevinki* (Boulenger, 1902) after long-term acclimation to both single- and multi-stressor treatments. While little information is available on the cellular-level effects of temperature on *T. newnesi*, both *T. bernacchii* and *P. borchgrevinki* have previously shown a decreased capacity to respond to thermal stress at the cellular level (Hofmann et al., 2000; Place et al., 2004). However, it is unknown whether the reduced capacity to respond to thermal insults extends to secondary stressors associated with increased temperature such as ROS production. Therefore, we also assessed whether acclimation resulted in an increase in key antioxidant defense mechanisms, which are ubiquitous among vertebrates (Lesser, 2006). To this end, we further quantified changes in total catalase (CAT) and superoxide dismutase (SOD) enzymatic activity to discern whether these species are capable of compensating for the additional oxidative stress that likely occurs when fish are acclimated to a multi-stressor scenario.

RESULTS

Protein carbonyl protein concentration

Trematomus bernacchii

Overall, both elevated temperature and $\dot{P}_{CO_2}$ had a significant effect on the level of oxidative damage to cells. Both gill and liver tissues showed main-level effects of temperature, $\dot{P}_{CO_2}$ and time (P<0.001; Fig. 1A,B). Additionally, both tissues displayed a significant three-way interaction between time, temperature and $\dot{P}_{CO_2}$ (P<0.001). Short-term acclimation resulted in elevated protein carbonyl levels, suggesting an initial increase in cell damage. Yet, over a long-term exposure, protein carbonyl levels either returned to control values or significantly decreased, which may be an indicator of physiological compensation (Fig. 1A,B). Across acclimation groups, we observed a tissue-specific shift in the effects of elevated temperature and $\dot{P}_{CO_2}$ after 28 days of acclimation. In the gill tissue of fish sampled from the single-stressor acclimation groups, the level of protein carbonyl formation returned to control levels (Fig. 1A). In the combined treatment of elevated temperature and $\dot{P}_{CO_2}$, we observed a more rapid decline in the level of oxidatively damaged proteins in the gill tissue (Fig. 1A). By 56 days, the level of oxidative damage in gill tissue was significantly lower in all treatments compared with control levels (Fig. 1A). Conversely, protein carbonyl levels continued to increase in liver tissues isolated from fish in all three stress treatments from the 28-day acclimation group (Fig. 1B). For fish acclimated for 56 days to the single-stressor treatments, protein carbonyl levels were no longer distinguishable from control fish; however, the level of oxidative damage in liver tissues sampled from fish in the multi-stressor treatment remained slightly elevated (Fig. 1B).

The interaction plots generated for *T. bernacchii* gill tissue showed a significant interaction between temperature and $\dot{P}_{CO_2}$ for each acclimation group, and suggest that this interaction is slightly antagonistic (supplementary material Fig. S1A,C,E). Alternatively, only the 28- and 56-day acclimation groups displayed a significant interaction between temperature and $\dot{P}_{CO_2}$ in the liver, and this interaction appears to shift from antagonistic at day 28 to synergistic by day 56 (supplementary material Fig. S1B,D,F).

Pagothenia borchgrevinki

Overall, protein carbonyl levels measured from gill and liver tissues from *P. borchgrevinki* showed a trend similar to that of tissues analyzed from *T. bernacchii*. Both gill and liver tissues from *P. borchgrevinki* showed a main-level effect of time, temperature and $\dot{P}_{CO_2}$ (P<0.001; Fig. 1C,D) as well as a three-way interaction between the three treatments (gill, P<0.001 and liver, P=0.018). Within the 7-day acclimation group we observed a marked increase in protein carbonyl formation followed by a general decline over time. Unlike the gill tissues from *T. bernacchii*, protein carbonyl levels in *P. borchgrevinki* gill tissue remained elevated above control levels after acclimation to the multi-stressor treatment for 28 days (Fig. 1C). By 56 days, levels of oxidatively damaged proteins had returned to near control values in all treatments (Fig. 1D).

While the general trends of protein carbonyl formation are similar between tissues from *T. bernacchii* and *P. borchgrevinki*, the interaction plots are markedly different. Significant interactions between temperature and $\dot{P}_{CO_2}$ were only observed on two occasions: the gill tissue from the 7-day acclimation group and the liver tissue for the 56-day acclimation group (supplementary material Fig. S2). While the gill tissue interaction appears additive and the liver tissue interaction slightly antagonistic, all other interaction plots appeared additive or synergistic in nature, although these interactions are not statistically significant (supplementary material Fig. S2).

Trematomus newnesi

Both liver and gill tissues showed main-level effects of time, temperature and $\dot{P}_{CO_2}$ in *T. newnesi* (P<0.001; Fig. 3). Additionally,

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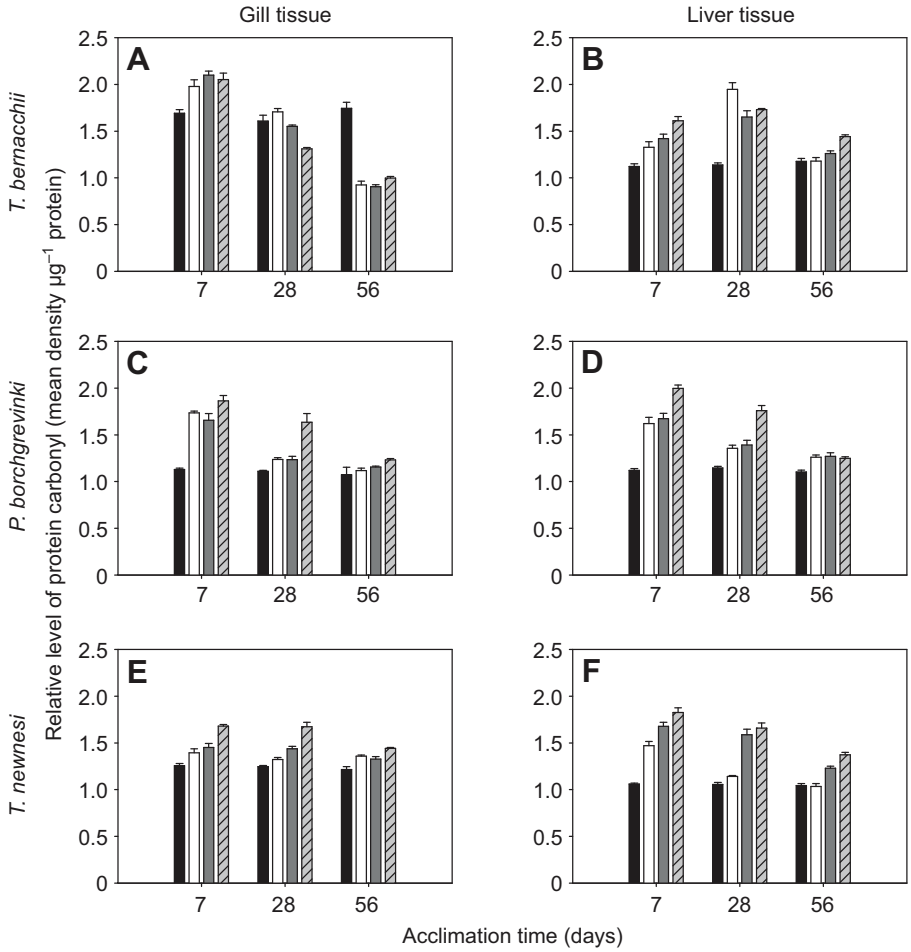

Fig. 1. Protein carbonyl concentration (mean ± s.e.m.) of gill and liver tissues. (A,B) *Trematomus bernacchii*, (C,D) *Pagothenia borchgrevinki* and (E,F) *Trematomus newnesi* acclimated at 7, 28 and 42/56 days to treatments of ambient conditions (low temperature + low $\dot{P}_{CO_2}$; black bars), low temperature + high $\dot{P}_{CO_2}$ (white bars), high temperature + low $\dot{P}_{CO_2}$ (dark grey bars) and high temperature + high $\dot{P}_{CO_2}$ (light grey bars with crosshatches).

liver tissues showed a three-way interaction between time, temperature and $\dot{P}_{CO_2}$ (P=0.002). Like the other fish species examined, protein carbonyl levels in gill and liver tissues from *T. newnesi* increased within 7 days of acclimation to all stress treatments (Fig. 1E,F). In gill tissues there was a decline in oxidatively damaged proteins in fish acclimated to high $\dot{P}_{CO_2}$ alone after 28 days; however, in the high temperature treatments the level of oxidative damage remained elevated above control levels (Fig. 1E). By 42 days of acclimation to the treatments, levels of protein carbonyl formation remained slightly elevated above control values, especially in the multi-stressor treatment (Fig. 1E). Similar to the trends seen in the gill tissue of *T. newnesi*, liver tissues from 28-day acclimated fish showed a reduction in protein carbonyl levels in fish acclimated to the low temperature + high $\dot{P}_{CO_2}$ treatment while levels of oxidative damage remained significantly elevated in fish from the two high temperature treatments (Fig. 1F). By 42 days, protein carbonyl formation declined in the high temperature treatments compared with the 7-day acclimation group, but overall, oxidative damage remained significantly elevated above control values (Fig. 1F),

Unfortunately, because of logistical constraints, we were unable to obtain tissue samples from a 56-day acclimation group for *T. newnesi*. The general trends observed in fish acclimated for 42 days suggests protein carbonyl levels may continue to drop and eventually level off near control values; however, physiological compensation may be slower in this species. Counter to what was observed for the other species, the interaction plots generated for *T. newnesi* at each acclimation time point suggests that the relationship between temperature and $\dot{P}_{CO_2}$ is largely synergistic in nature, which may be contributing to the slower recovery observed in *T. newnesi* (supplementary material Fig. S3).

SOD activity

We monitored total SOD enzyme activity in order to measure the effects of elevated temperature and $\dot{P}_{CO_2}$ on the total antioxidant capacity of *T. bernacchii*, *P. borchgrevinki* and *T. newnesi*. Overall, we observed only small fluctuations in the total activity of SOD across all three species, with *T. bernacchii* gill and *T. newnesi* liver displaying the greatest variation across treatments (Fig. 2). Apart from *T. bernacchii* liver tissue, the range of enzyme activities was found to be highly similar among the three notothenioid species studied here (Fig. 2). Under no circumstances did we observe a significant interaction among the treatment factors (time, temperature and $\dot{P}_{CO_2}$) for any of the fish species. Furthermore, for *T. bernacchii* and *P. borchgrevinki*, three-way ANOVA revealed no significant main-level effects. In *T. newnesi*, there was no effect of either temperature or $\dot{P}_{CO_2}$ in either gill or liver tissues, although SOD activity in *T. newnesi* did display a significant main effect of time in both tissues in which the 28-day acclimation groups displayed a small increase in SOD activity across all treatments (P=0.007; Fig. 2).

CAT activity

As a second measure of changes in antioxidant capacity in these species, we also measured CAT activity in the gill and liver tissues of fish from each acclimation group.

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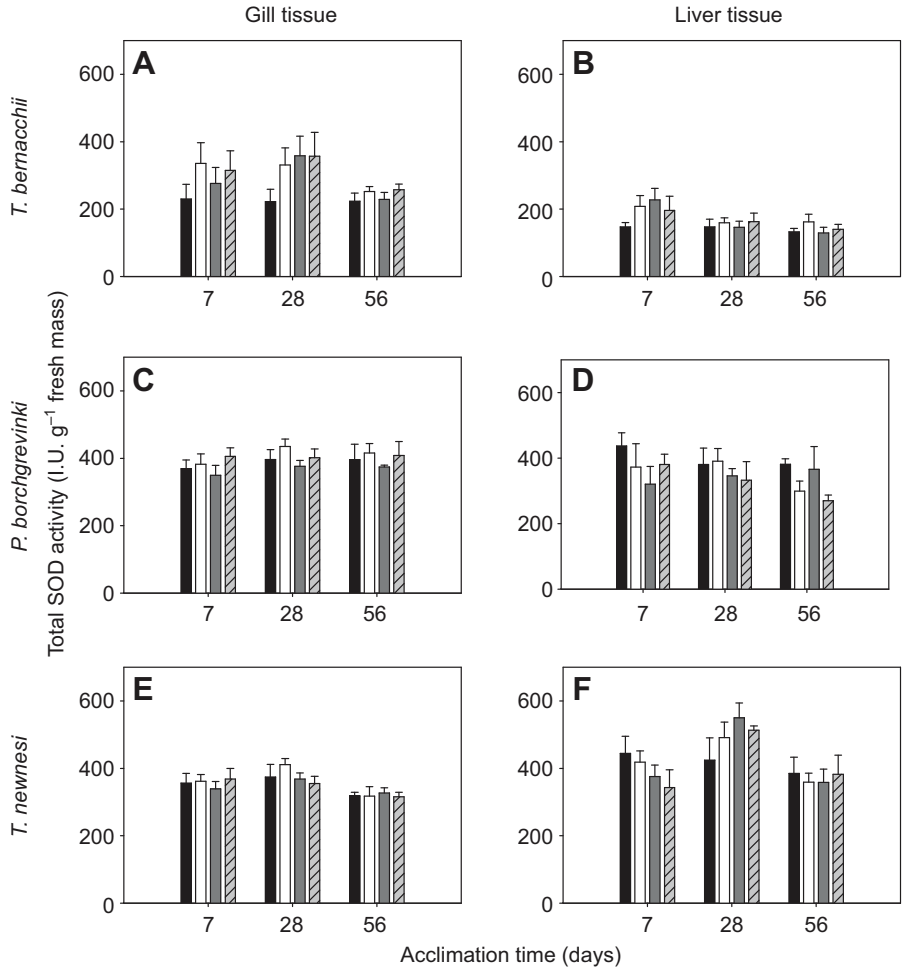

Fig. 2. Superoxide dismutase enzyme activity (mean ± s.e.m.) of gill and liver tissues. (A,B) *Trematomus bernacchii*, (C,D) *Pagothenia borchgrevinki* and (E,F) *Trematomus newnesi* acclimated at 7, 28 and 42/56 days to treatments of ambient conditions (low temperature + low $\dot{P}_{CO_2}$; black bars), low temperature + high $\dot{P}_{CO_2}$ (white bars), high temperature + low $\dot{P}_{CO_2}$ (dark grey bars) and high temperature + high $\dot{P}_{CO_2}$ (light grey bars with crosshatches).

CAT enzymes in *T. bernacchii* showed tissue-specific differences in activity, with liver tissues displaying activity levels roughly twice that of the gill tissue, although no main effects or interactions were detected (Fig. 3A,B). For *P. borchgrevinki*, we found no significant effect of temperature or $\dot{P}_{CO_2}$ in either gill or liver tissue across all acclimation groups (Fig. 3C,D). We did observe a main effect of time in gill tissue (P=0.016), and further analysis showed that 7-day acclimated fish were different from 56-day fish in the high temperature treatments (P=0.015; Fig. 3C).

CAT activity within the gill tissues collected from *T. newnesi* showed a main effect of temperature (P=0.033). This effect is noted when comparing the 28-day and 42-day acclimated fish, in which the CAT activity in fish acclimated to the high temperature treatments were lower when compared with the low temperature treatment levels (P=0.042; Fig. 3E). Liver tissues from *T. newnesi* showed the most variability over the course of the experiment and a main effect of time was seen (P<0.001; Fig. 3F). We also noted a highly tissue-specific response in *T. newnesi*, as CAT activity levels were significantly lower compared with control values after 7 days of acclimation to both single stressors as well as the multi-stressor treatment (Fig. 3F). Activity levels did increase from 7 to 28 days of acclimation across all three stress treatments; however, CAT activity never exceeded control values in fish exposed to any of the stressors (Fig. 3F).

DISCUSSION

The physiological adaptations that allowed notothenioids to flourish under extreme cold conditions may have also come with an inherent physiological cost in the form of susceptibility to oxidative stress (Abele and Puntarulo, 2004). Their increased mitochondrial densities as well as reliance upon lipid peroxidation as a sustained energy source may have pre-disposed these organisms to experience relatively high levels of oxidative damage compared with temperate fish species, as mitochondria are the primary site of ROS formation and β-oxidation of lipids also propagates ROS formation. If Antarctic fish have a pre-existing susceptibility to oxidative stress, then the predicted increases in oceanic temperatures coupled with ocean acidification in the polar regions could overwhelm their antioxidant capacity and significantly impact the health of notothenioid fish populations. Therefore, we attempted to assess the impacts these two potential stressors had on the antioxidant capacity of three Antarctic notothenioids, *T. bernacchii*, *P. borchgrevinki* and *T. newnesi*. Overall, our data revealed that all three species displayed non-significant changes in antioxidant capacity, and long-term acclimation to the experimental conditions actually led to a decrease in cellular damage for some fish. Below we provide a brief discussion of the observed changes and how they relate to previous studies. In doing so, we propose two competing interpretations of the potential physiological relevance of these observed changes.

Tissue-specific changes in antioxidant capacity

The acclimation conditions used in our study did in fact result in an initial elevation of oxidative damage across all three species, an indication that predicted changes for the Southern Ocean are capable of disrupting cellular homeostasis in these fish over short time scales. Although temperature increases have long been known to impact oxidative stress in organisms (Lesser, 2006), ocean

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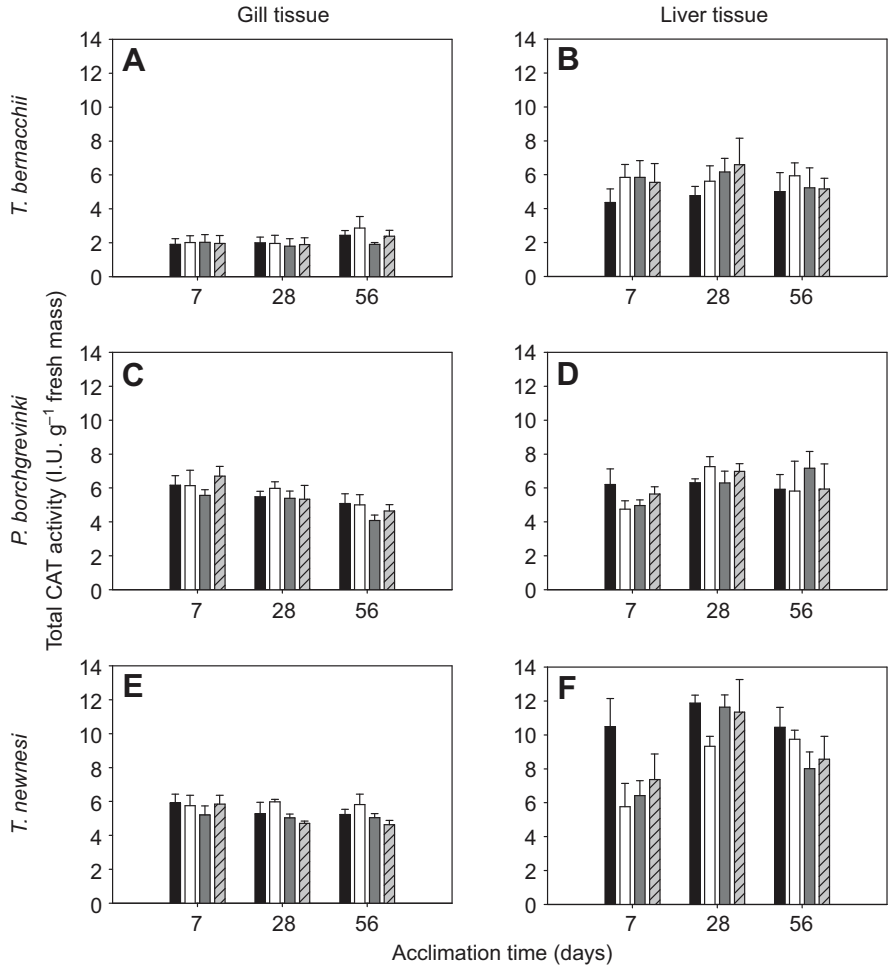

Fig. 3. Catalase enzyme activity (mean ± s.e.m.) of gill and liver tissues. (A,B) *Trematomus bernacchii*, (C,D) *Pagothenia borchgrevinki* and (E,F) *Trematomus newnesi* acclimated at 7, 28 and 42/56 days to treatments of ambient conditions (low temperature + low $\dot{P}_{CO_2}$; black bars), low temperature + high $\dot{P}_{CO_2}$ (white bars), high temperature + low $\dot{P}_{CO_2}$ (dark grey bars) and high temperature + high $\dot{P}_{CO_2}$ (light grey bars with crosshatches).

acidification has recently emerged as a potentially more pressing issue in the world's oceans, and to date, we still have a poor understanding of the cellular-level impact that ecologically relevant increases in seawater $\dot{P}_{CO_2}$ will have on cellular homeostasis. Increases in $\dot{P}_{CO_2}$ are specifically hypothesized to exacerbate oxidative stress by directly affecting mitochondrial function (Murphy, 2009; Tomanek et al., 2011), and recent studies have indeed shown that hypercapnia can negatively affect mitochondrial capacities in Antarctic fish (Strobel et al., 2012; Strobel et al., 2013b). We found that oxidative damage was most apparent within the first 7 days of acclimation to the treatment conditions, which coincides with a spike in the resting metabolic rate (RMR) of all three species under these same acclimation conditions (Enzor et al., 2013). However, after long-term acclimation, oxygen consumption rates began to decline across treatments in *T. bernacchii* and *P. borchgrevinki* (Enzor et al., 2013; L.A.E. and S.P.P., unpublished data), which also coincides with the observed changes in antioxidant activity and decrease in levels of protein damage reported in this study. We previously observed RMRs in *T. newnesi* acclimated to elevated temperature and $\dot{P}_{CO_2}$ remained significantly elevated even in fish acclimated up to 42 days. These data also correlate with our current findings that levels of protein damage in the multi-stressor treatment remained significantly higher compared with control fish over the same acclimation period (Enzor et al., 2013; L.A.E. and S.P.P., unpublished data). These data are strong indicators that the increased level of damaged protein is closely tied to metabolic production of ROS and that metabolic capacity will likely play a significant role in long-term adaptation to these predicted changes.

The observed lack of significant changes in activity of the two antioxidant enzymes examined in these fish illustrate similar trends when compared with previous studies, and may indicate that notothenioid fish maintain a sufficient level of antioxidant defence to compensate for the predicted climate change scenarios for the Southern Ocean. Similar to the findings in our study, Mueller and colleagues reported that SOD and CAT activity in the red-blooded notothenioid *Notothenia coriiceps* appeared relatively temperature insensitive (Mueller et al., 2012). Machado and colleagues also determined that exposure to elevated temperature in *N. coriiceps* and *N. rossii* did not elicit any changes in SOD or CAT activities (Machado et al., 2014). Furthermore, similar thermal responses have also been observed in non-notothenioid species with respect to SOD and CAT activity, with heart and liver tissues from cold-acclimated *Fundulus heteroclitus* and *Lepomis machrochirus* showing no increase in antioxidant activity levels (Grim et al., 2010).

Temporal changes in the oxidative damage response

By assessing the cellular response over a relatively long-term acclimation period, our data illustrate temporally different physiological responses within notothenioid fish. When the short-term acclimation responses are considered (~7 days), the lack of changes in SOD and CAT activity would suggest the cellular stress response was insufficient to offset ROS production in these fish, as we found a significant increase in protein damage after just 7 days of acclimation to all treatments. However, our data show that after an initial increase in protein carbonyl formation, levels of protein damage in all treatment groups returned close to basal levels or

significantly decreased after 56 days of acclimation in *T. bernacchii* and *P. borchgrevinki*. These long-term acclimations also highlight physiological differences among these closely related species, as this study and previous measurements of RMR suggest that *T. newnesi* in particular may require a significantly longer acclimation period to physiologically compensate for environmental perturbations (Enzor et al., 2013; L.A.E. and S.P.P., unpublished data).

In addition to providing insight into the basic cellular response of these endemic fish to global climate change, and perhaps more importantly, these data highlight the importance of considering multiple stressors over longer time scales. Recent studies by DeVries and colleagues suggest high-latitude fish have retained a level of thermal plasticity that is on par with temperate species despite evolution under a constant cold environment; however, the physiological response of the cold-adapted species were relatively slow to develop and would likely have gone undetected with short-term physiological measures (Bilyk et al., 2012; Bilyk and DeVries, 2011). Similarly, the cellular response we observed in these notothenioid species appears to be the result of long-term physiological adjustments as significant decreases in protein damage could not be detected before 2 months of acclimation in most cases. Furthermore, when the long-term acclimation responses are considered, the interpretation of the SOD and CAT activity shifts from potentially insufficient to a view that a robust response may not be necessary to alleviate the potential impacts global climate change may have on the physiological production of ROS in these fish. Taken together, these data indicate that functional changes at the cellular level may be absent, or at least too small to detect during acute exposures, and are suggestive that studies considering short-term cellular responses may be underestimating the adaptive capability of these stenothermal species.

Lastly, these long-term acclimations give us important insight into the tissue-level differences in susceptibility to ROS production. We found protein carbonyl formation in liver tissues remained elevated above those of control fish for a much longer period of time. Similar tissue-specific responses have been reported for other tissues that differ significantly in their functional capacity (Mueller et al., 2012), and it is likely that the temporal differences reported here are grounded in the functional importance of each tissue, as liver tissue is characterized by high metabolic activity and is a primary site for lipid peroxidation (Pörtner et al., 2005). While these studies give important insight into the likely drivers of tissue-level responses, they come up short when attempting to draw conclusions with respect to organism-level impacts. For instance, when considering only the short-term response of these notothenioids, we see tissue-specific responses that are negatively correlated and that would make extrapolation to a broader understanding of the whole organism response impossible. Alternatively, given the long-term considerations of these tissue-specific responses, we see that although levels of protein damage in the liver remained elevated even after 28 days across species, these levels also began to drop and most returned to the basal levels seen in control animals. Thus, the extended measurements allow us to extend these cellular-level data to the whole organism and generate the prediction that given enough time, the organism as a whole is fully capable of offsetting the effects of the stressors.

Predicting winners and losers in global climate change scenarios

In general, we found that acclimation to multiple stressors had no significant long-term negative effects on the accumulation of cellular damage in these fish. Furthermore, *T. bernacchii* in particular showed a substantial decrease in oxidative damage as measured by protein carbonyl formation. We argue that these findings can be interpreted in one of two ways. First, as our measurements of both SOD and CAT activity demonstrate little to no increase in antioxidant capacity over time, it seems these fish already maintain sufficient antioxidant capacity to offset the oxidative stress that cellular perturbations associated with global climate change are expected to produce. Second, given the significant decrease in protein damage seen in *T. bernacchii*, it seems some that Antarctic species may benefit at the cellular level from small increases in temperature in the sense that protein turnover may decrease over the long run and thus energy expenditure may be reduced after the initial cellular restructuring period.

The initial spike in oxidative damage observed in this study along with the increased metabolic rates previously observed within the first week of acclimation (Robinson and Davison, 2008a; Robinson and Davison, 2008b; Strobel et al., 2012; Enzor et al., 2013) may signal a surge in protein synthesis and turnover as the cellular environment is restructured. The slow decline of metabolic rates seen in previous studies, connected with the precipitous drop-off in damaged proteins seen in this study, may in turn signal that the initial energy expenditure has led to a more stable cellular environment. We now have data to support the idea that protein homeostasis in these fish may in fact become more stable at elevated temperatures. Our recent work has shown that the decrease in metabolic activity and protein carbonyl formation seen in *T. bernacchii* and *P. borchgrevinki* correlates with a three-fold reduction in the expression Hsc71, a molecular chaperone that plays an important role in maintaining protein homeostasis (Lindquist, 1986; Feder and Hofmann, 1999). This would suggest there is a significant reduction in the need for protein folding capacity in the cells of these fish at elevated temperature and, hence, a decrease in demand for ATP production (Huth and Place, 2013). In addition to changes seen at the cellular level, there is some evidence at the level of the whole organism that performance in these cold-adapted notothenioids may be optimal at elevated temperatures. Davison and colleagues have described similar effects of temperature on the RMR of *P. borchgrevinki* and also found that elevated temperature increases metabolic scope in these cold-adapted fish, with a maximal factorial scope occurring at +3°C (Seebacher et al., 2005; Lowe and Davison, 2006; Franklin et al., 2007; Robinson and Davison, 2008a; Robinson and Davison, 2008b). Thus, in the context of balancing energy expenditures and protecting metabolic scope, at least some Antarctic fish may perform better under future Southern Ocean conditions than previously predicted.

In contrast, an alternative interpretation of these data could reflect an entirely different scenario and the potential implications for climate change impacts on notothenioid fishes. As oxidative damage is directly related to changes in metabolic rate, the drop-off in damaged protein could be related to an overall decrease in ROS production as a secondary consequence resulting from energetic trade-offs necessary for acclimation to the experimental conditions. A growing body of literature has previously documented that increases in temperature and $\dot{P}_{CO_2}$ levels can negatively impact energetically costly processes such as protein synthesis in fish (Langenbuch and Pörtner, 2003; Abele and Puntarulo, 2004; Lesser, 2006; Vinagre et al., 2012). Growth rates in some fish appear to decrease under ocean acidification conditions (Foss et al., 2003; Ishimatsu et al., 2008), and these changes in growth rates may result as a consequence of energetic trade-offs associated with a notable increase in acid–base regulation in high $\dot{P}_{CO_2}$ environments (Esbaugh et al., 2012). Furthermore, even when fish display a capacity to compensate for ocean acidification at the cellular level,

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the combination of multiple stressors may eventually overwhelm the organism. For instance, Reid and colleagues discovered that juvenile rainbow trout, *Oncorhynchus mykiss*, displayed compensation at the cellular level for small amounts of environmental acidification (Reid et al., 1997). However, when faced with increases in temperature and $\dot{P}_{CO_2}$ simultaneously, this cellular level compensation came at the expense of growth potential in these fish as rates of protein synthesis and turnover were dramatically reduced (Reid et al., 1997). In a study assessing the impact of ecologically relevant increases in temperature and $\dot{P}_{CO_2}$ on reef fish, Munday and colleagues report that these two environmental parameters act synergistically to decrease the aerobic scope of tropical fish (Munday et al., 2009a). Furthermore, recent studies on the Antarctic fish *N. rossii* have shown that although these fish have a limited capacity to respond to ocean warming and ocean acidification, their response is highlighted by uncompensated mitochondrial enzyme activities that result in increased energy demands met largely by mobilizing energy stores in the liver, lending additional information to the supposition that ocean acidification may lead to long-term energetic impacts in notothenioid fishes (Strobel et al., 2013a; Strobel et al., 2013b). Together, these studies further suggest that physiological compensations identified at the cellular level could have significant impacts on performance at the level of the whole animal. Thus it is possible that the decrease in RMR previously reported in these fish is achieved through energetic trade-offs aimed at reducing the overall cellular oxygen demand while meeting the elevated costs of basal maintenance under these conditions.

When interpreted under this latter scenario, our data suggest that although physiologically capable of compensating for the environmental changes at the cellular level, these fish might still be vulnerable at the level of the whole organism as physiological trade-offs may have significant impacts on long-term growth and reproduction. As the exact interpretation of our findings cannot currently be discerned, further investigation is necessary to determine whether these fish experience energetic trade-offs as a result of the increased challenges to cellular homeostasis generally associated with global climate change.

MATERIALS AND METHODS

Collection of fish

Trematomus bernacchii, *Pagothenia borchgrevinki* and *Trematomus newnesi* were collected in McMurdo Sound, Antarctica, between October and December 2011 as well as September through December 2012. Fish were caught using hook and line through 10 inch (25.4 cm) 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 were tank-acclimated under ambient conditions for 1 week prior to being placed in experimental tanks. All fish were handled according to guidelines set forth by the University of South Carolina Institutional Animal Care and Use Committee.

Experimental design

We used four 1240 l experimental tanks to assess the combined effects of elevated temperature and $\dot{P}_{CO_2}$ on *T. bernacchii*, *P. borchgrevinki* and *T. newnesi*. Our four experimental treatments consisted of a control tank, which was held near ambient conditions (−1°C/430 µatm), a low temperature + high $\dot{P}_{CO_2}$ treatment (−1°C/1000 µatm), a high temperature + low $\dot{P}_{CO_2}$ treatment (+4°C/430 µatm), and a high temperature + high $\dot{P}_{CO_2}$ treatment (+4°C/1000 µatm). In 2011, fish were placed in experimental tanks and acclimated for 28 days. Four fish per treatment were removed at 7 and 28 days, after which fish were euthanized and tissues were collected and immediately flash-frozen in liquid nitrogen. In 2012, the acclimation experiment was repeated and fish were acclimated for 42–56 days. Five fish per treatment were sampled as described above at 7, 28 and either 42 days (*T. newnesi*) or 56 days (*T. bernacchii* and *P. borchgrevinki*). One-way ANOVA showed no significant differences inter-annually between fish sampled from the same treatment, so data obtained from the 2011 and 2012 seasons were combined for all analyses. In total, nine fish per treatment were analyzed from each species at the 7- and 28-day time points. For *T. newnesi*, five fish per treatment were analyzed at the 42-day time point and for *T. bernacchii* and *P. borchgrevinki*, five fish per treatment were analyzed at the 56-day time point. Although the constraints of working in Antarctica prevented us from utilizing a fully replicated tank design to control for tank effect, the treatment conditions established for each tank were alternated between the 2011 and 2012 field seasons.

Manipulation of seawater conditions

Temperature and $\dot{P}_{CO_2}$ levels were manipulated within the experimental treatment tanks using a $\dot{P}_{CO_2}$ generation system first described by Fangue et al. (Fangue et al., 2010), and adapted for use with large-scale applications, and combined with thermostated titanium heaters (Process Technology, Brookfield CT, USA) (Enzor et al., 2013). Atmospheric air was pumped through drying columns (filled with Drierite) to remove moisture, and air was scrubbed of CO_2 using columns filled with Sodasorb. Pure CO_2 and CO_2-free air were then blended using digital mass flow controllers and bubbled into header tanks that were continuously replenished with ambient seawater using Venturi injectors, which in turn fed into experimental treatment tanks.

Temperature, pH (total scale), salinity, total alkalinity and oxygen saturation were measured daily from both incoming seawater as well as experimental treatment tanks. For $\dot{P}_{CO_2}$ analysis, we followed the standard operating procedure as described in the Best Practices Guide (Riebesell et al., 2010) for the spectrophotometric determination of pH using m-Cresol Purple and measurement of total alkalinity via acid titration using a computer-controlled T50 Titrator (Mettler Toledo, Columbus, OH, USA) . Temperature was measured with a calibrated digital thermocouple (Omega Engineering Inc., Stamford, CT, USA) and salinity was measured using a YSI 3100 conductivity meter (Yellow Springs, OH, USA). The program CO2 calc (Robbins et al., 2010), using the constants of Mehrbach et al. (Mehrbach et al., 1973) as refit by Dickson and Millero (Dickson and Millero, 1987), was used to calculate all other carbonate parameters. Oxygen saturation was recorded using a galvanic oxygen probe (Loligo Systems, Denmark). Mean (±s.d.) values of temperature (°C) and $\dot{P}_{CO_2}$ (µatm) over the course of the experiment are reported in Table 1. Although inter-annual variation was apparent within the same treatments, one-way ANOVA showed no significant differences inter-annually between the same treatments. Additionally, treatment tanks were sampled daily for the presence of ammonia, nitrite and nitrates, with no significant increase in waste products noted over the course of the experiment (data not shown).

Measurement of protein carbonyls

Protein carbonyl formation was quantified using reagents and antibodies from a Cell BioLabs kit (STA-308) and a protocol described by Robinson et al. (Robinson et al., 1999). Proteins were extracted by homogenizing ~50 mg of gill or liver tissue in 500 µl of SDS homogenization buffer

Table 1. Mean (±s.d.) measurements of $\dot{P}_{CO_2}$ and temperature over the course of the 2011 and 2012 field seasons

Treatment	$\dot{P}_{CO_2}$ (µatm)	Temperature (°C)
2011 season		
Incoming seawater	417.15±12.26	−1.24±0.08
Low temperature + low $\dot{P}_{CO_2}$	438.82±16.08	−0.61±0.17
Low temperature + high $\dot{P}_{CO_2}$	953.89±50.38	−0.45±0.16
High temperature + low $\dot{P}_{CO_2}$	525.11±21.07	4.02±0.44
High temperature + high $\dot{P}_{CO_2}$	1026.66±9.03	4.22±0.56
2012 season		
Incoming seawater	427.66±23.97	−1.03±0.152
Low temperature + low $\dot{P}_{CO_2}$	432.04±22.50	−0.707±0.153
Low temperature + high $\dot{P}_{CO_2}$	1024.76±94.20	−0.578±0.150
High temperature + low $\dot{P}_{CO_2}$	525.16±22.41	3.86±0.484
High temperature + high $\dot{P}_{CO_2}$	1053.44±71.87	4.03±0.316

(50 mmol l^{-1} Tris-HCl, pH 6.8, 4% SDS, 1 mmol l^{-1} EDTA). Briefly, protein extracts (5 µg) were diluted in 100 µl of 1× Tris-buffered saline (TBS; 20 mmol l^{-1} Tris base, 500 mmol l^{-1} NaCl, pH 7.4), and immobilized directly onto a PVDF membrane using a Bio-Rad DotBlot apparatus (Hercules, CA, USA). A single protein extract taken from a non-experimental animal was used as a protein standard in order to normalize spot density across all blots. All samples, including the protein standard, were randomly loaded in duplicate. Immediately before immunoblotting the membrane, a derivatization step was carried out whereby each membrane was incubated with a 1× dinitrophenylhydrazine solution for 5 min. Membranes were incubated in primary antibody [1:25,000 in antibody dilutent solution (5% non-fat dry milk in 1.0% TBS/Tween 20)] overnight at 4°C, and secondary antibody solution (1:5000 in antibody dilutent solution) for 1 h at room temperature. Membranes were incubated for 5 min in West Pico Super Signal and exposed on X-ray film (Thermo Scientific). DotBlot images were scanned using a Fotodyne imaging system (Hartland, WI, USA) and densitometry was performed using ImageJ software. The mean density of the duplicate measures for each sample was normalized across blots by dividing the mean density of each sample by that of the protein standard.

Enzyme activity measurements

SOD

We utilized a SOD assay kit (19160-1KT-F, Sigma-Aldrich) to quantify the level of total SOD activity (SOD1, SOD2 and SOD3) in gill and liver tissues of fish acclimated to the four treatments for 7, 28 or 42/56 days. A cleared protein extract was prepared by homogenizing approximately 100 mg of tissue in 100 mmol l^{-1} phosphate buffer followed by centrifugation at 1500 ***g*** for 10 min and transferring the supernatant to a fresh microcentrifuge tube. The total SOD activity in 20 µl of the cleared protein extract was determined by measuring the conversion of Dojindo's water-soluble tetrazolium salt (WST-1) to a water-soluble formazan dye and measuring the subsequent change in absorbance at 450 nm using a 96-well plate reader (Bio-Tek, Synergy HT, Winooski, VT, USA). Activities were then normalized to units of SOD per gram wet tissue mass and are reported as I.U. g^{-1} fresh mass (mean ± s.e.m.). One unit of SOD was defined as the amount of enzyme needed for 50% inhibition of formazan dye.

CAT

We used a spectrophotometric method to quantify CAT activity in gill and liver tissue by measuring the change in absorbance at 240 nm resulting from the disappearance of H_2O_2 (Beers and Sizer, 1952). Total CAT activity in 5 µl of cleared protein extract (prepared as described above), was combined with 300 µl of H_2O_2-phosphate buffer solution [made by diluting 0.16 ml H_2O_2 (30% w/v) to 100 ml with a 67 mmol l^{-1} phosphate buffer solution, pH 7.0]. Samples were read in a UV-transparent 96-well plate (UV-Star, Greiner Bio-One, Monroe, NC, USA) using a 96-well UV-Vis plate reader (Bio-Tek, Synergy HT, Winooski, VT, USA). Plates were scanned every 47 s for 5 min, using the pathway correction of the plate reader. Total CAT activities were calculated using the following equation:

$$\text{Activity} = S\left(\frac{V}{\varepsilon}\right), \quad (1)$$

where S is the slope describing the rate of disappearance of H_2O_2, V is the volume of H_2O_2 phosphate buffer added to each well and ε is the micromolar extinction coefficient of H_2O_2 (0.0436 ml µmol^{-1} cm^{-1} at 240 nm). Lastly, activities were normalized by gram wet tissue mass and are reported as I.U. g^{-1} fresh mass (mean ± s.e.m.).

Statistics

A Shapiro–Wilk test indicated that all data from this study exhibited a normal distribution and homogeneous variance with the exception of the protein carbonyl data gathered from *T. bernacchii* (both tissues) and the gill tissues from *P. borchgrevinki*. In those instances in which the assumptions of normal distribution and homogeneous variance were not met, we initially used the non-parametric Kruskal–Wallis test, which indicated significance differences existed between our control fish and fish exposed to the stress treatments (P<0.05). As the non-parametric test allows us to draw no further insight into the interactions among treatments, and given that ANOVA is considered a robust statistical test even within data sets that exhibit small deviations from normality (Lunney, 1970), we proceeded with the analyses using a three-way ANOVA. To test for significant differences between fish acclimated to specific treatments along with interactions among treatment variables, we used three-way ANOVA with time (7, 28 and 42/56 days), temperature (−1 or 4°C) and $\dot{P}_{CO_2}$ treatment (400 or 1000 µatm) as independent variables. For those comparisons that showed a significant interaction (P<0.05), we ran Holm–Sidak multiple comparison tests and generated interaction profiles by plotting the least square means for temperature × $\dot{P}_{CO_2}$ at each experimental end point in order to visualize the nature of the interaction.

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.A.E. aided in the design and execution of the experiments, interpretation of the results, and drafting and revising of the manuscript. S.P.P. conceived the experiments and aided in the design and execution of the experiments, interpretation of the results, and drafting and revising of the manuscript.

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Supplementary material

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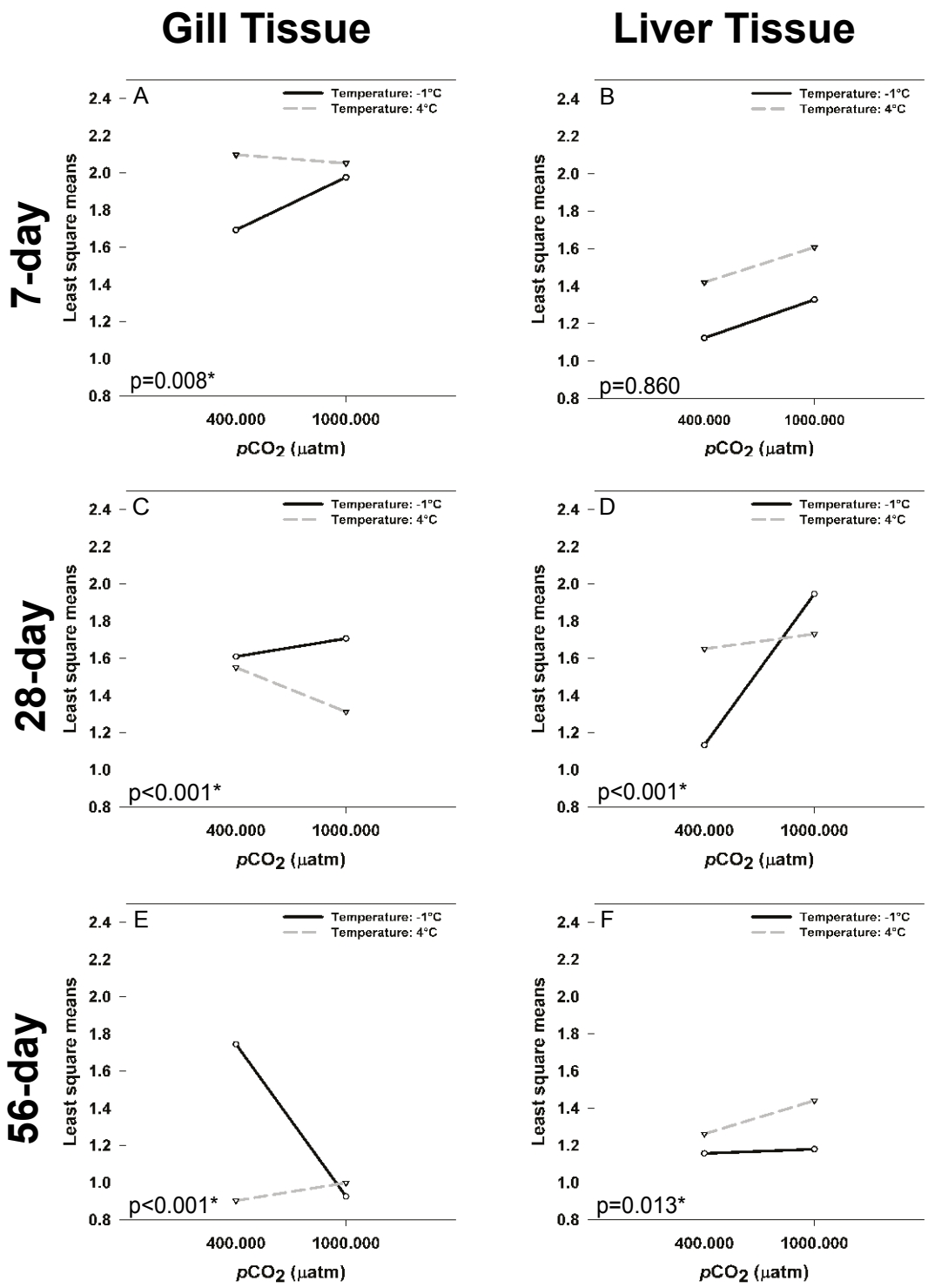

Fig. S1. Protein carbonyl interaction plots of *T. bernacchii* gill and liver tissues acclimated for 7 days (gill: A, liver: B), 28 days (gill: C, liver: D), and 56-days (gill: E, liver: F).

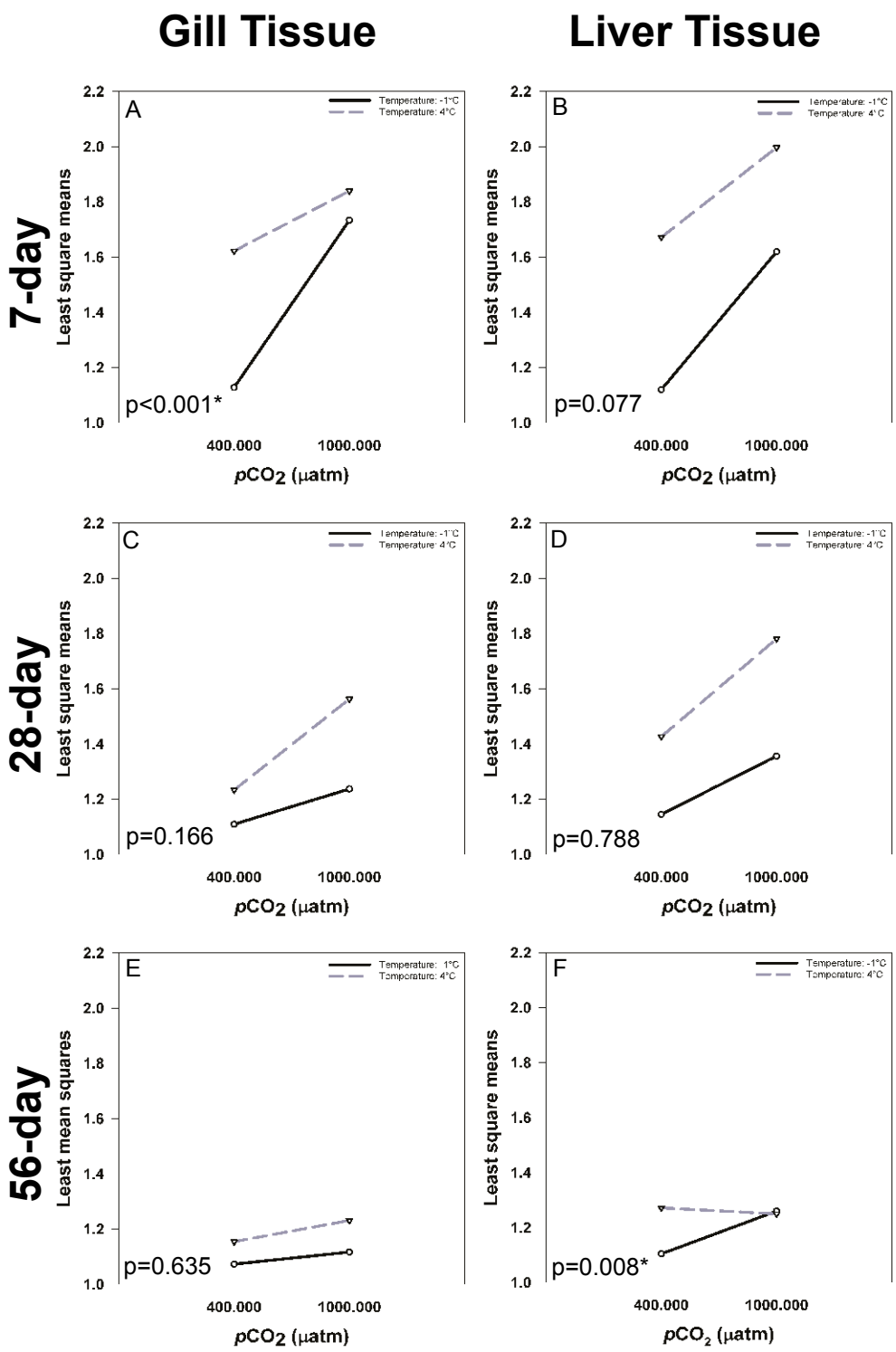

Fig. S2. Protein carbonyl interaction plots of *P. borchgrevinki* gill and liver tissues acclimated for 7 days (gill: A, liver: B), 28 days (gill: C, liver: D), and 56 days (gill: E, liver: F).

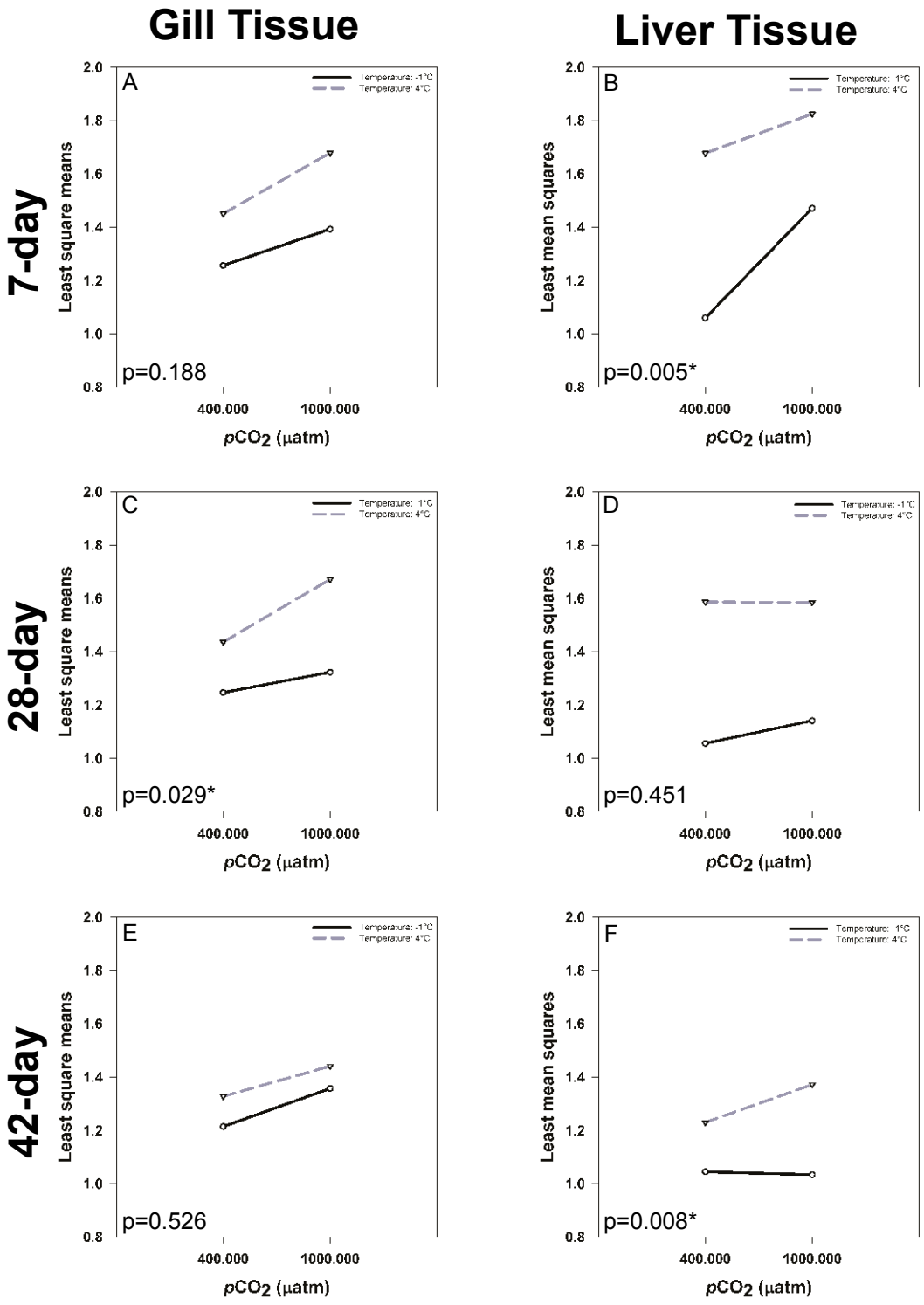

Fig. S3. Protein carbonyl interaction plots of *T. newnesi* gill and liver tissues acclimated for 7 days (gill: A, liver: B), 28 days (gill: C, liver: D), and 42 days (gill: E, liver: F).