Oxidative Stress and Biomarker Responses in the Atlantic Halibut After Long Term Exposure to Elevated CO₂ and a Range of Temperatures

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15 **Abstract:**

16 Oceans are warming and pH levels are decreasing as a consequence of increasing levels of
 17 dissolved CO₂ concentrations. The CO₂ emissions are predicted to be produce in greater and faster
 18 changes in the ocean than any other event in geological and historical records over the past 300
 19 million years. Marine organisms will need to respond to multiple stressors but the potential
 20 consequences of global change-related effects in fish are not fully understood. Since fish are
 21 affected by many biotic and abiotic environmental variables, including temperature and CO₂
 22 fluctuations, it is critical to investigate how these variables may affect physiological and
 23 biochemical processes. We investigated the effects of elevated CO₂ levels (pH of 8.0, which
 24 served as a control, or 7.6, which is predicted for the year 2100) combined with exposure to
 25 different temperatures (5, 10, 12, 14, 16, and 18 °C) in the Atlantic halibut (*Hippoglossus*
 26 *hippoglossus*) during a three month experiment. We assessed effects on antioxidant and
 27 cholinesterase enzymes (AChE and BChE), and CYP1A enzyme activities (EROD). The
 28 treatments resulted in oxidative stress, and damage was evident in the form of protein carbonyls
 29 which were consistently higher in the elevated CO₂-treated fish at all temperatures. Analyses of
 30 antioxidant enzymes did not show the same results, suggesting that the exposure to elevated CO₂
 31 increased ROS formation but not defences. The antioxidant defence system was insufficient, and
 32 the resulting oxidative damage could impact physiological function of the halibut on a cellular
 33 level.

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36 **Key words:** oxidative stress, carbon dioxide, ocean acidification, temperature, climate change,
 37 teleost fish, Atlantic halibut, *Hippoglossus hippoglossus*

38

39 Introduction

40 The release of carbon dioxide (CO₂) into the atmosphere is changing the ocean's chemistry at a
 41 pace never before seen. The oceans are becoming warmer and pH levels are decreasing as a
 42 consequence of increasing levels of dissolved CO₂ (Solomon et al., 2009; Steffen et al., 2015).
 43 The CO₂ emissions predicted for the coming centuries are expected to produce greater and faster
 44 changes in the oceans than any other event recorded in geological and historical records over the
 45 past 300 million years (Caldeira and Wickett, 2003). Since many marine animals have evolved to
 46 cope with changes within a certain range of temperature and CO₂ concentrations, climate change is
 47 expected to challenge their ability to function optimally at conditions outside of their scope of
 48 tolerance (Portner, 2010). In aquatic ectotherms such as fish, environmental temperature is a
 49 crucial variable since it has a direct effect on all biological processes, such as metabolism (Portner
 50 et al., 2006) and enzyme kinetics (Kavanau, 1950).

51 The effects of temperature changes and lower pH have been studied in marine animals and we are
 52 beginning to understand consequences and mechanisms involved (Gräns et al., 2014a; Gräns et al.,
 53 2014b). For example, recent studies have shown indications of oxidative stress in marine animals
 54 exposed to changes in temperature: long term exposure to increasing temperatures induces
 55 oxidative damage in lipids in rock fish (*Nothonei sp.*) (Klein et al., 2017); short-term elevation in
 56 temperature increases oxidative stress in Antarctic vertebrates and invertebrates (Abele and
 57 Puntarulo, 2004); low temperatures are reported to increase oxidative stress in gilthead sea bream
 58 (*Sparus aurata*) liver (Ibarz et al., 2010). The Antarctic fish species bald notothen (*Pagothenia*
 59 *borchgrevinki*) responded to acutely increased temperature with an increase in antioxidant
 60 defences while long-term temperature increase resulted in oxidative damage (Carney Almroth et
 61 al., 2015). Elevated CO₂ concentrations are also known to increase oxidative stress in cold water
 62 species: the great spider crab (*Hyas araneus*) showed up-regulation of genes associated with the

63 detoxification of H₂O₂ (ascorbate peroxidase, glutathione peroxidase) (Harms et al., 2014); Pacific
 64 oyster larvae (*Crassostrea gigas*) expressed higher levels of five of the six investigated antioxidant
 65 proteins (Tomanek et al., 2011a); and levels of the antioxidant protein glutaredoxin were up-
 66 regulated in the Sydney rock oyster (*Saccostrea glomerata*) exposed to elevated CO₂ levels
 67 (Thompson et al., 2015).

68 Fewer studies have exposed fish to multiple stressors. Gräns et al. (Gräns et al., 2014a) show that
 69 increased temperature and CO₂ affected growth in halibut, but did not determine mechanisms of
 70 these effects. Pementel et al (Pimentel et al., 2015) showed that the combined stress of OA and
 71 warmer temperatures resulted in the accumulation of peroxidative damage in a flatfish, and that
 72 early developmental stages are more susceptible to oxidative stress. The physiology of croaker
 73 fish (*Argyrosomus regius*) was found to be impacted by these factors as well, with some changes
 74 in oxidative stress parameters (Sampaio et al., 2018). These fish also demonstrated some ability to
 75 maintain physiological homeostasis in the face of a third stressor (mercury exposure), but the
 76 biochemical repercussions of the physiological responses were not fully understood and warrant
 77 further investigation.

78 Oxidative stress is commonly addressed in these studies since it is an essential physiological
 79 mechanism known to be affected by biotic and abiotic factors; it is a process initiated by the
 80 imbalance between the production of oxidants and their removal by antioxidants and antioxidant
 81 enzymes. Reactive oxidants including reactive oxygen species (ROS), are produced during
 82 normal cellular respiration in mitochondria, or leaked from enzymatic activity including the Phase
 83 I detoxification enzyme CYP1A, and are normally metabolized by antioxidants. This includes
 84 such enzymes as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxides or
 85 molecular antioxidants like glutathione (Ozcan and Ogun, 2015). ROS levels are important in
 86 homeostasis, and are a key regulator of biological processes, but ROS can also initiate oxidative

87 cascades causing severe cellular damage to proteins, lipids and DNA (Kohen and Nyska, 2002).
 88 Increased ROS production is a common consequence of metabolic and acid-base disturbances in
 89 animals (Tomanek, 2011a; Tomanek et al., 2011a) and also a common mechanism of toxicity
 90 (Valavanidis, 2006). Changes in the concentration of antioxidants or oxidative damage products
 91 are often used as indicators of environmental stress and pollutant exposure (Carney Almroth et al.,
 92 2008; Carney Almroth et al., 2005; Kohen and Nyska, 2002).

93 Hepatic function is associated with performance and maintenance of numerous physiological
 94 mechanisms, such as metabolism, degradation of endogenous compounds, and detoxification of
 95 various substances. Hence, hepatic tissue contains high levels of antioxidant enzymes and
 96 antioxidants and enzymes in the liver can be used to assess liver function. In addition, liver tissue
 97 is often target for analyses addressing environmental stressors: the measurement of
 98 ethoxyresorufin-*O*-deethylase (EROD) activity in fish liver is a well-established indicator of
 99 exposure to aromatic hydrocarbons (Förlin et al., 1994). However, induction of CYP1A is also
 100 closely related to detrimental effects such as apoptosis (Whyte et al., 2000) and may be influenced
 101 by a large number of biotic and abiotic factors, which in turn affects biotransformation processes
 102 (Rahman and Thomas, 2012; Whyte et al., 2000).

103 Changes in abiotic parameters in the environment are also know to affect the catalytic activity of
 104 acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Pfeifer et al., 2005; Pretti and
 105 Cognetti-Varriale, 2001), enzymes important hydrolysis of choline esters in central and peripheral
 106 nervous systems or in plasma and tissues. Inhibition of the catalytical activities of these enzymes
 107 often used as biomarkers for neurotoxic compounds including pyrethroids and organophosphorus
 108 insecticides in fish (Fulton and Key, 2009; Mushigeri and David, 2005). Both AChE and BChE
 109 have been indicated as being involved in immunity via modulation of the cholinergic anti-
 110 inflammatory pathway (Pohanka, 2014) and BChE is important in regulation of ghrelin, a peptide

hormone involved in regulation of appetite and growth hormone secretion (Brimijoin et al., 2016). Several studies have indicated that AChE and BChE are affected by warming temperatures as well as seawater pH in mussels (Pfeifer et al., 2005; Wu et al., 2016).

As the global climate changes, exposing organisms to environmental variations which are predicted to increase in the future, it is crucial to investigate how they may affect physiological processes (Parmesan and Yohe, 2003). Fish will be affected by a multitude of environmental variables, including both temperature and CO₂ variations. In order to investigate the effects of elevated CO₂, at levels predicted for the near future, in combination with different temperatures, we conducted an experiment exposing Atlantic halibut to a range of temperatures and water pH for three months. We analyzed the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione S-transferase (GST) and glutathione peroxidase (GPx), the levels of protein oxidation measured as protein carbonyls (PC), activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), and also the activity of the phase I detoxification enzyme CYP1a, measured as EROD activity.

Materials and Methods

Chemicals and reagents

7-ethoxyresorufin, glutathione reductase (GR), reduced and oxidized glutathione, 5,59-dithiobis (2-nitrobenzoic acid) (CDNB), 1-chloro-2,4-dinitrobenzene (DTNB), pyruvic acid, glucose-6-phosphate, oxidized and reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced nicotinamide adenine dinucleotide (NADH), butyrylthiocholine, acetylthiocholine iodine, 2,4-dinitrophenylhydrazine (DNPH), ethyl acetate, guanidine hydrochloride, digitonin and protease

inhibitor were obtained from Sigma Aldrich (St. Louis, MO, USA). Hydrogen peroxide is from Fluka (Buchs, Switzerland), ethylenediaminetetraacetic acid (EDTA) from Merck.

The fish model

The Atlantic Halibut (*Hippoglossus hippoglossus*) is a benthic marine fish widely distributed in the northern regions of the Atlantic Ocean and in parts of the Arctic Ocean (Haug, 1990). Juveniles stay in coastal areas of at depths of 20-60 m before migrating to more distant areas of both shallow and deep waters (Glover et al., 2006). The Atlantic Halibut was chosen as a model for our studies because of its large socio-economic and ecological importance, and its wide distribution in the Northern hemisphere, and the optimal and suboptimal temperatures for this species have been described (Gräns et al., 2014a). Since the Arctic marine biomes are warming twice as fast as the global average (Fossheim et al., 2015), and both coastal waters and deep-sea waters (with low water flow; (Cai et al., 2011)) are acidifying faster than marine open waters, the Atlantic halibut physiology and distribution are susceptible to impact.

Experimental setup and fish treatments

The experiment was part of a larger study and a detailed description of the set-up, fish exposure, water quality, data monitoring and sampling methods are available in previous publications (Bresolin de Souza et al., 2014a; Gräns et al., 2014a). Juvenile fish from both sexes (weight of 16.4 g +/- 0.2 g (SEM), from Fiskey's hatchery (Þorlákshöfn, Iceland) were exposed for 96 days to six different temperatures (5, 10, 12, 14, 16, and 18 °C). For each temperature treatment, two tanks were supplied with water at current seawater pH and two tanks with reduced pH. The CO₂ treatments represent the present day *p*CO₂ 400 µatm (~pH 8.0) and 1000 µatm (~pH 7.7), according to pH predictions for the end of this century (Gräns et al., 2014b; Solomon et al., 2007). The fish were distributed into twelve fish tanks (100 L), each supplied with aerated flow-through

seawater from header tanks (200 L), supplied in turn with flow-through seawater from 32 m depth (32.0 ± 0.14 ppt). Fish were kept under a 12 h:12 h light:dark photoperiod, and were fed once a day with 2.5 % of body mass with commercial fish feed. Water parameters were monitored daily in the header tanks and fish tanks. Temperature and salinity were continuously recorded.

Sampling

Fish were killed with a sharp blow to the head and liver samples were collected from eight fish from each treatment (total of 96 fish). Livers were divided into sub-fractions, frozen in liquid nitrogen, and stored at -80 °C prior to handling. Practices concerning methods of animal handling, exposure, and sampling were approved by the Animal ethical committee Gothenburg, Sweden, (ethical permits 221-2010 and 329-2010).

Sample preparation

Liver cytosol fractions were prepared according to (Forlin, 1980). Liver samples were homogenized (glass/Teflon) in 4 volumes of 0.1 M Na/K-PO₄ buffer containing 0.15 M KCl at pH 7.4. Homogenates were centrifuged in two steps, first at 10 000 g for 20 min at 4 °C, and then the supernatant was re-centrifuged for 105 000 g for 1 h at 4 °C. The supernatant (cytosolic fraction) was aliquoted and stored at -80 °C prior to analysis. The cytosolic fractions were used to determine the activities of the antioxidant enzymes. The pellets containing the microsomes were re-suspended in homogenizing buffer containing 20 % glycerol and stored at -80 °C prior to analysis.

For protein carbonyl analysis liver samples were homogenized in 4 volumes of 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 0.1 % digitonin, and a cocktail of anti-proteases (Sigma P8340). Samples were then centrifuged for 20 min at 10 000 g at 4 °C. Supernatants were

178 collected for use in DNPH reactions, and total protein content was determined according to Lowry
179 (Lowry et al., 1951).

180 ***Biochemical analysis***

181 Catalase (CAT, EC 1.11.1.6) activity was measured according to Cribb et al. (Cribb et al., 1989a)
182 using hydrogen peroxide as substrate. Glutathione S-transferase (GST, EC 2.5.1.18) activity was
183 measured according to Stephensen et al. (2002a) using CDNB as substrate. Superoxide dismutase
184 (SOD, EC 1.15.1.1) activity was measured using a SOD assay kit from Sigma Aldrich, according
185 to the manufacturer's instructions. Glutathione peroxidase (GPx, EC 1.11.1.9) activity was
186 measured by the method of Greenwald (Greenwald, 1985), modified by Stephensen et al.
187 (Stephensen et al., 2002b). Glutathione reductase (GR) activity was measured according to the
188 method described by Cribb (Cribb et al., 1989b). Protein carbonylation (PC) was measured via
189 reaction with DNPH followed by TCA precipitation as described previously (Levine et al., 1994;
190 Reznick and Packer, 1994). All assays were performed on a microplate reader (Molecular
191 Devices) at room temperature. Acetylcholinesterase (AChE, EC 3.1.1.7) activity was measured
192 according to a modification of the spectrophotometric method described by Ellman et al. (1961)
193 adapted to a microplate reader. Butyrylcholinesterase (BChE, EC 3.1.1.8) activity was measured
194 as described for AChE with butyrylthiocholine as substrate instead of acetylthiocholine iodine.
195 Ethoxyresorufin O-deethylase (EROD) activity was measured in the liver microsomal fraction
196 according to the method described by Förlin et al. (Förlin et al., 1994) using rhodamine as
197 standard. Total protein content in cytosol and microsomes was measured using the BCA kit from
198 Pierce, according to the manufacturer's protocol, using bovine serum albumin as standard.

199

200 ***Statistics***

201 Data was compared using two-way ANOVA ($\alpha = 0.05$) with temperature and pH as fixed factors.
 202 Tukey Post-Hoc test (95% confidence) was applied. Statistical analyses, figures and tables were
 203 prepared using GraphPad Prism 7.00, and probability for Type-I error was set to 5% for all tests.
 204 All data that did not display homogeneity of variance according to Levene's test, or normal
 205 distribution according to the Shapiro-Wilk test, were log-transformed prior to testing. T-tests were
 206 used to assess differences between duplicate aquaria; no differences were found so the samples
 207 both tanks within a treatment were pooled t for subsequent analyses.

208

209 **Results**

210 In the present study the oxidative stress indicators (SOD, CAT, GR, GST, GPx, and PC), esterases
 211 AChE and BChE, and phase I detoxification activity (EROD) were measured in liver of Atlantic
 212 halibut. Results indicate the occurrence of oxidative stress in the elevated CO₂-treated fish, though
 213 temperature also appears to play an important role in the balance of antioxidant homeostasis.
 214 Results are displayed in Figures 1, 2 and 3.

215 The elevated CO₂ treatment resulted in reduced activity of SOD, except at 18 °C where there was
 216 a shift in this enzyme activity (higher activity in the elevated CO₂ group). CAT activity was
 217 slightly higher in the elevated CO₂-treated group. Both enzymes showed some changes related to
 218 temperature: SOD and CAT correlate significantly ($p = 0.017$) with one another in control groups,
 219 but not in elevated CO₂-exposed fish. See Figure 1.

220 EROD activity and PC levels were consistently increased in the elevated CO₂-treated groups at all
 221 temperatures, showing a clear CO₂ effect. EROD activity (Fig. 3) was higher at lower
 222 temperatures and reduced with temperature increase in both control and elevated CO₂-treated
 223 groups, while PC levels (Fig. 2) increased from temperatures of 10 to 18 °C in both control and

224 elevated CO₂-treated groups. The activities of GR, GST, and GPx varied within temperature
 225 treatments with a trend towards increasing with temperature (Fig. 1).

226 Both AChE and BChE increased significantly with increasing temperature, and while BChE was
 227 not affected by elevated CO₂ levels, AChE was found to differ between these treatments. In
 228 addition, a significant interaction between temperature and CO₂-treatment was identified in results
 229 from AChE measurements.

230 The two-way ANOVA revealed significant overall effects of temperature (Wilks' Lambda =
 231 0.048, $F = 5.173$, $p < 0.001$), pH (WL = 0.35, $F = 10.96$, $p < 0.001$) and an interaction between the
 232 two (WL = 0.321, $F = 1.55$, $p < 0.021$) measured parameters. Values for each specific parameter
 233 are listed in Table 1.

234 **Table 1.** Results from statistical testing of measurements conducted in tissue from halibut
 235 experimentally exposed to different temperatures and carbon dioxide concentrations. Values from
 236 the two-way ANOVA and Tukey's post-hoc test investigating significant differences between the
 237 two treatment parameters as well as interactions between these two. Boldfaced text highlights
 238 significant differences. The total number of measurements (fish individuals) for each enzyme
 239 ranged from 72 to 77, and was 122 for protein carbonyls assay.

240

	Temperature		Elevated CO ₂		Temperature * Elevated CO ₂	
Parameter	F	p	F	p	F	p
Superoxide dismutase (SOD)	5.141	0.001	1.128	0.292	3.440	0.008
Catalase (CAT)	1.281	0.284	10.156	0.002	0.474	0.794
Glutathione reductase (GR)	12.423	0.000	1.697	0.198	1.783	0.130
Glutathione S-transferase (GST)	4.704	0.001	0.044	0.835	1.078	0.382
Glutathione peroxidase (GPx)	8.967	0.000	0.007	0.936	2.701	0.029
Ethoxyresorufin O-deethylase (EROD)	8.541	0.000	11.895	0.001	1.560	0.185
Acetylcholinesterase (AChE)	14.01	0.000	9.012	0.004	4.721	0.001
Butyrylcholinesterase (BChE)	16.07	0.000	3.324	0.072	2.127	0.071
Protein carbonylation (PC)	5.393	0.000	29.258	0.000	0.535	0.749

241

242

243 **Figure 1. Oxidative stress indicators measured in liver of Atlantic halibut at different**
 244 **temperatures.** From top left to right down: superoxide dismutase (SOD), catalase (CAT),
 245 glutathione reductase (GR), glutathione S-transferase (GST), glutathione peroxidase (GPx).
 246 Elevated CO₂-treated groups significantly different from their respective controls are indicated by
 247 *. Letters indicate significant differences between temperatures within the same CO₂ treatment.
 248 High and low caption letters indicate respectively control and elevated CO₂ values. Error bars
 249 presented as ± SEM and confidence interval of 95 %.

250

251 **Figure 2. Oxidative damage measured as protein carbonyls, in liver of Atlantic halibut at**
 252 **different temperatures.** Elevated CO₂-treated groups significantly different from their respective
 253 controls are indicated by *. Letters indicate significant differences between temperatures within
 254 the same CO₂ treatment. High and low caption letters indicate respectively control and elevated
 255 CO₂ values. Error bars presented as ± SEM and confidence interval of 95 %.

256

Figure 3. Biomarker responses, in liver of Atlantic halibut at different temperatures.

Elevated CO₂-treated groups significantly different from their respective controls are indicated by *. CYP1A activity measured as ethoxyresorufin O-deethylase (EROD), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Letters indicate significant differences between temperatures within the same CO₂ treatment. High and low caption letters indicate respectively control and elevated CO₂ values. Error bars presented as ± SEM and confidence interval of 95 %.

Discussion

This study addresses the effects of elevated CO₂ combined with different temperatures on biochemical responses in the Atlantic halibut. Our results are indicative of oxidative stress, which can result in damage macromolecules such as proteins (Pastore et al., 2003), as evident in increased protein carbonyl levels. Levels of oxidized proteins are often measured as protein carbonyls and these are frequently used as an indicator of oxidative stress (Carney Almroth et al., 2005; Dalle-Donne et al., 2003). Oxidation of proteins can lead to non-reversible conformational changes, which decrease enzyme activities and can result in protein degradation by proteases (Carney Almroth et al., 2005; Grune et al., 2004). We show that protein carbonyl levels in the elevated CO₂ fish are consistently higher at all temperatures, and protein carbonyl levels are elevated at both high and low temperature extremes (Fig. 2). However, the antioxidant enzymes does not show the same pattern, suggesting that the exposure to elevated CO₂ increased ROS formation, with consequent oxidative damage resulting from an insufficient antioxidant defense system.

279 The mechanisms underlying oxidative stress responses to ocean acidification (OA) are not fully
 280 understood, and three possible pathways have been described earlier in (Tomanek et al., 2011b),
 281 but induced oxidative stress and damage have been indicated as consequences of OA in several
 282 different groups of organisms (Hernroth et al., 2012; Kaniewska et al., 2012; Pimentel et al., 2015;
 283 Wood et al., 2016). We have previously shown that most of the Atlantic halibut used in our study
 284 were probably not experiencing acidosis or ionic homeostasis unbalance, since plasma lactate and
 285 ionic (K^+ , Na^+ , Ca^{++} , Cl^-) levels were the same in controls and elevated CO_2 fish after 96 days of
 286 exposure (Bresolin de Souza et al., 2016). However, since we did not measure pH in the plasma or
 287 the intracellular compartment, we cannot determine whether the effects we measure are directly
 288 related to mitochondrial function or ROS production. Previous studies show that exposure to
 289 increased levels of CO_2 can result in overall stress responses, changes in protein repair and
 290 degradation, as well as changes in the expression of hypoxia inducible factor 1 (HIF-1), a gene
 291 expressed in response to hypoxia stress (Dennis Iii et al., 2014). We hypothesize that the increased
 292 levels of protein carbonyls seen here may be related to the activation of hypoxia inducible factors
 293 (HIF-1) as well as the regulation of genes involved in oxygen transport and anaerobic energy
 294 production (Kassahn et al., 2009; Turrens, 2004). It has also been proposed that elevated CO_2
 295 exposure can affect the electron transport chain, increasing the production and release of ROS and
 296 other free radicals, (Dean et al., 1997; Tomanek, 2014a; Tomanek et al., 2011b).

297 The ideal growth temperature range for Icelandic Atlantic halibut juveniles (used in this study) is
 298 from 11 to 14 °C (Björnsson and Tryggvadóttir, 1996), while 18 °C is their upper tolerance limit
 299 and results in a reduced growth rate (Imsland, 2001; Langston et al., 2002). In overall, antioxidant
 300 enzyme activities did not increase linearly with increasing temperature, in agreement with
 301 previous studies in fish (Madeira et al., 2013; Vinagre et al., 2012). Oxidative stress-related
 302 enzymes increase in activity with increased temperature until reaching a peak, and after the

303 temperature peak the enzymes activity decreases. The temperature of this peak is related to the
304 thermal niche of the species and is species-specific (Gräns et al., 2014a).

305 Gräns et al. (2014a) studied effects of global climate change on Atlantic halibut: acclimation to
306 warmer temperatures resulted in increased aerobic scope and cardiac performance, an effect that
307 was even more pronounced by elevated CO₂ exposure. These effects were not reflected in growth
308 rate, which was slower at the warmest temperatures (16 and 18 °C), leading the authors to
309 conclude that oxygen uptake was not a limiting factor for growth. However, this study also found
310 no differences in oxygen consumption between fish kept at control or elevated CO₂ (pH 8.1 or 7.7
311 respectively), at any given temperature. Therefore, we can conclude that increased oxygen
312 consumption, potentially leading to an increase in ROS production, is not directly responsible for
313 the increase in levels of protein carbonyls seen in the elevated CO₂-treated fish in the current
314 study.

315 Our results also showed that activity of GR was lower at 12 and 14 °C in the elevated CO₂-treated
316 groups, which could possibly indicate an overconsumption of glutathione in other cellular
317 reactions. Glutathione is an important molecular antioxidant, and the regeneration of its reduced
318 form from oxidized molecules is catalyzed by GR. GPx, which is responsible for reducing both
319 hydrogen and lipid peroxides, protecting the cells against the damaging effects of lipid
320 peroxidation (Winston and Di Giulio, 1991), was in general more affected by temperature,
321 especially at the extremes (5 and 18 °C), than by elevated CO₂. The activities of two additional
322 antioxidant enzymes, SOD and CAT, correlate significantly with one another in control fish but
323 not in elevated CO₂-treated fish. A mismatch in SOD and CAT activities can result in an
324 ineffective metabolism of ROS, allowing ROS to interact with other molecules, causing oxidative
325 damage (Halliwell and Gutteridge, 1999).

CYP1A activity (EROD) was higher in the elevated CO₂-treated fish in all studied temperatures, thus indicating that there is a CO₂ effect on EROD activity, which is independent of xenobiotic exposure. The effects of pH on EROD activity are not clear, yet most studies to date have exposed fish to pH levels much lower than in the current study ((Whyte et al., 2000) and references therein). Research on the mechanisms of toxicity behind CYP1A induction show that EROD activity not only indicates chemical exposure but may also reflect effects of other abiotic factors (Whyte et al., 2000). Induction of CYP1A in fish requires the activation of cytosolic aryl hydrocarbon receptors (AhR), followed by the transcription of the Ah-gene battery and subsequent synthesis of proteins, including CYP1A and many phase II enzymes (Whyte et al., 2000). Hence, the observed CO₂-dependent increase of EROD activity in the current study cannot be due to a xenobiotic induction, but rather a post-translational or kinetic regulation of the enzyme. In addition, EROD had a strong negative correlation with temperature, in contrast to antioxidant enzyme activities measured here, indicating that these proteins may have different stability or regulatory pathways linked to temperature (Regoli and Giuliani, 2013; Solé et al., 2015). Temperature impacts the composition and hence the fluidity of membranes ((Nikinmaa, 2013) and references therein), so this may have a greater impact on EROD, a membrane bound protein, than on the cytosolic antioxidant enzymes. In addition, it is possible that cell membrane properties are influenced by oxidative stress-dependent lipid peroxidation, changing membrane fluidity and the integrity of biomolecules associated with membranes (Carney Almroth et al., 2005).

In this study, we also measured effects in activity of two cholinesterases, AChE which is known for its role in neurotransmission via hydrolysis of acetylcholine, and BChE, suggested to play a role in ghrelin regulation and aggression (Brimijoin et al., 2016; Chen et al., 2015). AChE occurs mostly in brain, neurons and muscle but is also present in many other tissues, while BChE predominates in liver, lungs, plasma, and neuroglia (Chen et al., 2015; Pohanka, 2014). Both enzymes have been used as indicators for xenobiotic exposure (Sanchez et al., 2011; Sturm et al.,

1999). AChE has been shown to be sensitive to stress and inflammation (Bresolin de Souza et al., 2014b; Ming et al., 2015). In addition, the activity of serum BChE is an indicator of systemic inflammation in humans, and the more severe the inflammation, the smaller the concentration of serum BChE (Zivkovic et al., 2015). In the present study, both AChE and BChE show a positive correlation with temperature, but this tendency was less evident in the elevated [CO₂]-treated fish. However, current understanding of the effects of temperature on AChE and BChE activities are conflicting. Some studies show positive effects and others negative effects, while still others find no significant differences ((Solé et al., 2015) and references therein). Interestingly, both AChE and BChE were decreased at elevated [CO₂] treatment at 14 °C, which is within the optimal growth temperature range for this species (Gräns et al., 2014b) but outside normal water pH levels. We would also like to propose that further investigation into BChE and its role in physiological responses to environmental stressors could prove interesting since: BChE has been indicated as playing a role in ghrelin regulation and aggression in knockdown mice (Chen et al., 2015); ocean acidification can result in decreased BChE activity as shown here; and previous studies have shown behavioural changes in stickleback when exposed to increased [CO₂], including effects on boldness and exploratory behaviour (Jutfelt et al., 2013; Näslund et al., 2015).

Metabolic acclimations to deal with changes in environmental temperature have been previously described (Pörtner et al., 2005; Somero, 2012). Arrhenius' law dictates that acclimation to lower temperatures includes increased protein synthesis to compensate for lower reaction rates (Solé et al., 2015; Whyte et al., 2000). This mechanism could explain the negative relationship of EROD activity and temperature in our study, which may be due to increased amounts of CYP1A at lower temperatures, and vice-versa. There are previous studies with fish showing the same negative correlation between EROD activity and temperature acclimation (Solé et al., 2015; Whyte et al., 2000).

375 Ocean acidification is thought to raise metabolic rates in aquatic organisms in order to supply the
 376 cells with additional energy to cope with the physiological changes caused by such environmental
 377 variations (Nikinmaa, 2013). In addition, when animals are close to their thermal limits, highest or
 378 lowest preferred temperature tolerated, even a small disturbance (small rise in temperature) may
 379 reduce their activity scope and consequently reduce ecological success (Nikinmaa, 2013). The
 380 extra energetic cost associated with these processes is expected to result in an increase oxidative
 381 stress (Tomanek, 2014a). Evidences of metabolic changes resulting from exposure to elevated
 382 CO₂ are found in these experimental fish in a previous study (Bresolin de Souza et al., 2014b),
 383 seen in the modulation of proteins such as glyceraldehyde 3-phosphate dehydrogenase, fructose-
 384 1,6-phosphate aldolase, and malate dehydrogenase. Similar changes regarding higher expression
 385 of metabolic enzymes and increased oxidative stress have been shown in the Pacific oyster
 386 (*Crassostrea gigas*) exposed to elevated CO₂ (Timmins-Schiffman et al., 2014). Oxidative stress is
 387 a co-stress of temperature and elevated CO₂ low pH, and stress-mediated ROS can lead to shifts in
 388 energy metabolism. This can be accompanied by the activation of pathways of ATP production;
 389 excess ROS and shifts in energy metabolism might impact protein homeostasis through e.g.
 390 protein denaturation or lack energy for protein synthesis and normal function (Tomanek, 2011b;
 391 Tomanek, 2014b).

392

393 **Conclusion**

394 This study provides insights regarding how fish are affected by the combined stress of elevated
 395 CO₂ and temperature. We show physiological changes, possibly related to detrimental effects
 396 and/or acclimation mechanisms, that indicate impacts of future climate change, as modelled in the
 397 conditions in our experiments. Here we show that elevated CO₂ exposure can induce oxidative
 398 stress, evident in accumulation of protein carbonyls. In addition activities of esterases and

detoxification enzymes were shown to be affected by temperature and CO₂ levels. The results presented here support the hypothesis that CO₂ levels estimated to occur at the end of this century could pose physiological challenges to marine fish.

Acknowledgements

We thank the GRIP project members for their collaboration and support, and Niklas Dahr for technical assistance. The study was supported by the University of Gothenburg Platform for Integrative Physiology (GRIP) and the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS).

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