1   **1. Title page**

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3   **Response of three krill species to hypoxia and warming: An experimental approach to**

4   **oxygen minimum zones expansion in coastal ecosystems**

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6   **Running head:** Response of krill to hypoxia and warming

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2. Abstract

To understand the adaptation of euphausiid (krill) species to oxygen minimum zones (OMZ), respiratory response and stress experiments combining hypoxia/reoxygenation exposure with warming were conducted. Experimental krill species were obtained from the Antarctic (South Georgia area), the Humboldt Current system (HCS, Chilean coast), and the Northern California Current system (NCCS, Oregon). *Euphausia mucronata* from the HCS shows oxyconforming pO_2-dependent respiration below 80% air saturation (18 kPa). Normoxic subsurface oxygenation in winter posed a "high oxygen stress" for this species. The NCCS krill, *Euphausia pacifica*, and the Antarctic krill, *Euphausia superba* maintain respiration rates constant down to low critical pO_2 values of 6 kPa (30% air saturation) and 11 kPa (55% air saturation), respectively. Antarctic krill had low antioxidant enzyme activities, but high concentrations of the molecular antioxidant glutathione (GSH) and was not lethally affected by 6 h exposure to moderate hypoxia. Temperate krill species had higher SOD (superoxide dismutase) values in winter than in summer, which relate to higher winter metabolic rate (*E. pacifica*). In all species, antioxidant enzyme activities remained constant during hypoxic exposure at habitat temperature. Warming by 7°C above habitat temperature in summer increased SOD activities and GSH levels in *E. mucronata* (HCS), but no oxidative damage occurred. In winter, when the NCCS is well mixed and the OMZ is deeper, +4°C of warming combined with hypoxia represents a lethal condition for *E. pacifica*. In summer, when the OMZ expands upwards (100 m subsurface), antioxidant defences counteracted hypoxia and reoxygenation effects in *E. pacifica*, but only at mildly elevated temperature (+2°C). In this season, experimental warming by +4°C reduced antioxidant activities and the combination of warming with hypoxia again caused mortality of exposed specimens. We conclude that a climate change scenario combining warming and hypoxia represents a serious threat to *E. pacifica* and, as a consequence, NCCS food webs.

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3. Introduction

One of the most important effects of climatic change at tropical and temperate latitudes is the expansion of oxygen minimum zones (OMZ), especially in coastal and shelf regions (Helly & Levin 2004). The expansion can be regional into areas previously not experiencing hypoxic conditions, or it can consist in vertical expansion of an existing OMZ. Notably the OMZ of the Eastern Tropical Pacific and the Eastern Atlantic off northwest Africa have expanded to higher latitudes during the past 50 years (Stramma *et al.* 2008), suggesting changes in zoogeographic distribution patterns and regionalization of biomass production (Stramma *et al.* 2011; Gilly *et al.* 2013). Global ocean warming is among the causes of OMZ expansion, and combined effects of warming, hypoxia and ocean acidification endanger many sensitive marine species (Rosa & Seibel 2008; Stramma *et al.* 2011). According to Cocco *et al.* (2013), circulation, pelagic production, remineralization processes, and temperature caused changes in the oxygen concentration of the upper mesopelagic layer (100-600 m). Five out of the seven predictive models for long-term changes of sea surface oxygenation agree with respect to a deoxygenation of the northern Pacific, the tropical and subtropical South Pacific, the Southern Ocean, the eastern part of the Indian Ocean, and the subpolar North Atlantic. The models were not unanimous regarding the expansion of the low-O_2 regions (<80 µmol L^{-1} or 5.9 kPa) with predictions ranging from 2 to 16% spatial increase.

Euphausiids (krill) are important marine biomass producers and link primary production and larger carnivorous secondary producers in marine food webs. They undertake daily vertical migrations (DVM), upwards at dusk to feed in the productive surface layers and downward at dawn to avoid visual predators and to digest their food. In so doing, they contribute strongly to the vertical biomass flux. During their daily migrations, krill cross important gradients of temperature, salinity, and oxygen, indicating that some species require

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72   a broad ecophysiological tolerance. Indeed, out of the total 86 krill species known worldwide,

73   54 occur in at least two different oceans (Brinton *et al.* 2003, updated 2008). Species

74   occupying a narrow range of distribution are mostly sub-tropical and temperate productive

75   species, relative specialists, and often constrained by physiological tolerance boundaries for

76   temperature and oxygen. For example, *Euphausia mucronata* in the Humboldt Current

77   System (HCS) has morpho-physiological adaptations to remain in the OMZ during daytime

78   vertical migration (Antezana 2010). In contrast, *Nyctiphanes simplex* and *Euphausia pacifica*,

79   a neritic subtropical and a temperate species from the North Pacific, reduce their DVM

80   maximal depth to stay above OMZ when hypoxic conditions worsen (Kunze *et al.* 2006;

81   Tremblay *et al.* 2010). Other productive species are associated with cold waters like the

82   Antarctic krill *Euphausia superba* and the north pacific neritic species *Thysanoessa spinifera*

83   and show greatly reduced abundance when warm anomalies occur (Brinton & Townsend

84   2003; Atkinson *et al.* 2004). Thus, upward migration of subtropical and temperate productive

85   species may be restricted by thermocline formation, whereas downward migration is limited

86   by an OMZ (Tremblay *et al.* 2010). Impairment of DVM can enhance visual predation

87   (Fernández-Álamo & Färber-Lorda 2006) or also cause mass mortality of krill under

88   physiological stress (Tyburczy *et al.* 2013; Oregon and Northern California).

89   One way to detect physiological disturbance is by measuring oxidative stress

90   parameters. The term *oxidative stress* refers to a state of respiratory imbalance in which

91   animals cannot maintain constant tissue oxygenation and instead experience rapid shifts

92   between over and under-oxygenation. In this case, especially when animals are re-oxygenated

93   after hypoxic exposure, reactive oxygen species (ROS: reactive molecules derived from

94   oxygen, such as the superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), and hydrogen peroxide

95   (H_2O_2)) are formed which, if not neutralized by the organism's antioxidant defence, cause

96   oxidative damage and eventually cellular disorder and death. Tremblay *et al.* (2010) showed

97 that OMZ/hypoxia adapted krill species in the Gulf of California (Mexico) possess

98 sufficiently high antioxidant protection, while less adapted species suffered severe oxidative

99 stress measurable as lipid peroxidation.

100   Aiming at a better understanding of the threat ocean warming and widening of the

101 OMZ presents to krill species on a global scale, we investigated metabolic and oxidative

102 stress indicators in three krill species known to differ in the level of adaptation to OMZ

103 conditions. Specifically we tested their response to hypoxia, reoxygenation, and warming, or a

104 combination of factors. We chose the north Pacific krill, *E. pacifica* (Adults: 11-25 mm

105 length; Brinton *et al.* 2003, updated 2008) which forms massive swarms in the northern

106 California Current system (NCCS) where mid-water hypoxia is common at the end of

107 summer since one decade (Chan *et al.* 2008; Connolly *et al.* 2010; Peterson *et al.* 2013). All

108 along the Pacific coast of the United States of America, juveniles and adults of this oceanic

109 species perform daily migrations between the surface and depths of at least 250 m (Brinton

110 1967). In fjords and bays their downward migration is often reduced to between 50 and 125 m

111 depth (Bollens *et al.* 1992), sometimes limited by seasonal hypoxic or anoxic conditions in

112 bottom water layers (Kunze *et al.* 2006). Off Newport (study area; Oregon, USA) the annual

113 range of variation of sea surface temperature (SST) is very constant, except for warm El Niño

114 events such as in August 1997 (SST reached 18°C along the coast; Feinberg & Peterson

115 2003), and under anomalous oceanographic conditions as in the year 2000 (SST drop to

116 8.3°C; Feinberg & Peterson 2003) when subarctic waters entered the region. Hypoxia

117 sensitivity of *E. pacifica* has already been investigated by Childress (1975) and Ikeda (1977).

118 Both authors recognized a critical limit of performance of this species at 20% air saturation

119 (4.2 kPa pO$_2$), visible in a dramatic reduction of swimming activity and also in high

120 mortalities below this critical pO$_2$ (pc). In the Strait of Georgia, these unprecedented year-to-

121 year fluctuations of krill biomass correlated positively with the North Pacific Gyre Oscillation

122 (NPGO) index, negatively with water column temperature anomalies, and positively to some

123 extent, with the survival of salmon and herring (Mackas *et al.* 2013). Thus, it was

124 hypothesized that climate change and enhanced predation may be responsible for the high

125 mortalities in low krill years.

126   A perfect counterpart to the hypoxia sensitive *E. pacifica* is its southern hemisphere

127 antipode, the hypoxia-tolerant *E. mucronata* (Adults: 17-22 mm length; Brinton *et al.* 2003,

128 updated 2008), endemic to the temperate HCS and, thus, pertaining to a similar climatic

129 background with respect to temperature and OMZ scenario. Here the OMZ is associated with

130 frequently upwelling of lowly oxygenated Equatorial Subsurface Water, producing a large

131 and stationary hypoxic area near the surface (Copin-Montegut & Raimbault 1994; Thiel *et al.*

132 2007). Off Concepción (study area; Chile), the annual SST varies normally between 12 and

133 18°C (Sobarzo *et al.* 2007). During the cold season of the El Niño event of 1997-1998, SST

134 increased to almost 16°C in this area (Contreras *et al.* 2007). Like *E. pacifica* in the NCCS, *E.*

135 *mucronata* plays a keystone role in the trophic dynamics of the HCS as principal prey of jack

136 mackerel and anchovy (Antezana 2010). The species performs extended DVM down to 250 m

137 into the OMZ in all seasons (Escribano *et al.* 2000; Antezana 2002a). Even if this migration

138 into OMZ is normal, the highest *E. mucronata* abundances occur in areas where the upper

139 boundary of the OMZ is deeper (Escribano *et al.* 2000), which suggests avoidance of extreme

140 hypoxia/anoxia. Larger gills/ cephalothorax surface ratio in *E. mucronata*, compared with

141 other Euphausiids, is another indicator of hypoxic adaptation (Antezana 2002b). The routine

142 metabolic rate (RMR) of *E. mucronata* is the same in well oxygenated surface waters as in the

143 hypoxic OMZ (Antezana 2002b), and Antezana (2009) observed *E. mucronata* to be one of

144 the lasts OMZ species to begin its ascent to the surface at dusk, thus stretching the deep

145 hypoxic residence time to a maximum. *E. mucronata* therefore qualifies as a highly hypoxia

146   adapted species in comparison to *E. pacifica*, and we hypothesized that its adaptive strategy

147   could involve better oxidative stress resistance.

148   Given the opportunity to participate in a summer ship cruise of the British Antarctic

149   Survey, we included the Antarctic krill *Euphausia superba* (Adults: 42-65 mm length;

150   Brinton *et al.* 2003, updated 2008), which so far rarely deals with hypoxia. *Euphausia*

151   *superba* forms large biomasses in the Southern Ocean and is central to Antarctic food webs

152   (Atkinson *et al.* 2004; Murphy *et al.* 2007). The southwest Atlantic sector of the Southern

153   Ocean around South Georgia where we sampled represents the northernmost limit of species

154   distribution where *E. superba* migration is restricted to the upper 100 m of water column

155   (Gaten *et al.* 2008). For this region and water depth (upper 100m), a strong and highly

156   seasonal warming trend has been detected since the beginning of the 20th century, amounting

157   to +0.9°C over 80 years in January (summer) and more than the double during winter in

158   August (Whitehouse *et al.* 2008). The authors report positive correlation between mean

159   annual summer SST at South Georgia and reduced densities of krill in this region. To

160   understand its response to oxygen deficiency at the high water temperature around South

161   Georgia, we measured pO_2 dependent metabolic rates (RMR) and oxidative stress parameters

162   of *E. superba* at control and under hypoxic conditions. Note that oxidative stress indicators

163   have not previously been reported for this Antarctic key species.

4. Materials and methods

Ethics statement

The present study is not involving any protected or endangered species. No specific permissions are required for sampling in the NCCS and HCS. For the species *Euphausia superba*, the British Antarctic Survey received a permit for its general operations in Antarctica from the Foreign and Commonwealth Office (United-Kingdom) as a requirement of the Antarctic Act.

Environmental data collection

Temperature, oxygen and salinity profiles were recorded with a Seabird SB09 "conductivity, temperature, depth" (CTD) system in all sampling areas. Each profile was plotted to detect the upper boundary of the OMZ and the depth of the thermocline, if present. As ecosystems with different salinity and temperature were compared, we defined the upper boundary of the OMZ at the depth where dissolved oxygen concentration was 20% of the maximal air saturation. SST (°C) and chlorophyll *a* concentration (mg m^{-3}) visualisations averaged monthly from the Moderate-resolution Imaging Spectroradiometer (MODIS) Aqua Global Level 3 (11 μm thermal infrared; 4 km spatial resolution) were produced with the Giovanni online data system (developed and maintained by the NASA GES DISC) for each sampling area and season.

Krill collection

Krill were collected during several day trips and some longer oceanographic cruises carried out in 2011 and 2012, details are given in Tables 1 and 2. Each area was visited during cold and warm seasons, except Antarctica, which was sampled only during the warm season. To reduce sampling stress, krill fishing was conducted at night when the krill are near the

189   surface. After heaving the sampling gear on deck, the collected zooplankton was immediately

190   transferred to 20 L buckets with seawater. Live adult euphausiids, in healthy condition

191   (showing a lot of movement and with no visible damage), were manually sorted into bins

192   (Colman boxes, or tanks of 100 L in Antarctica) filled with filtered seawater from the area

193   and transferred to a cold room (see Table 1 for holding temperature). Directly after sampling,

194   some specimens were snap frozen in liquid N_2 (HCS) or at -80°C (NCCS and Antarctica) for

195   biochemical analysis of *in situ* values (Table 2). Other animals were acclimated for at least 6

196   h in the cold room prior to starting respirometry and the experimental procedures.

197

198   *Respiration measurements*

199   As ROS formation can change as a function of animal O_2 consumption (although there

200   is no strict one to one relationship between both parameters, see also Buttemer *et al.* 2010),

201   we measured the routine metabolic rates (RMR) of all investigated species at *in situ*

202   temperature (see table 1 for temperature details) in the dark, using an OXY-4 channel PreSens

203   Oxygen Ingress Measurement system (Germany). The system was equipped with 4 chambers

204   for simultaneous measurement of three animals and a blank (for seawater bacterial oxygen

205   demand). All chambers were filled with filtered local seawater at 100% air saturation (21

206   kPa), and the oxygen concentration in each chamber was measured every 15 s in mBar (or

207   hPa). Cylindrical chambers of 20 mL volume were used, specially designed to reduce possible

208   differences in individual swimming activity, except for the Antarctic where chamber volume

209   was 250 mL to account for the larger size of *E. superba*. Upward and downward movements

210   were occurring during the first phase of the measurement, which was considered as RMR,

211   between 80 and 60% air saturation (16.8 to 12.6 kPa). Data recording was started at 80% air

212   saturation (16.8 kPa). This value was reached at different times between 30 to 60 min

213   depending on species and individual, which represents the chamber acclimation time before

214 data were considered. When substantial differences in activity or behaviour of a krill

215 happened during the measurement, the measurement was discarded. Chambers were equipped

216 with a magnetic stirrer (bottom) to achieve homogeneity of the oxygen concentration, and a

217 30-μm mesh gauze separated the stirrer from the euphausiids and served a substratum for

218 settling down. Below 60% air saturation (12.6 kPa), the specimens generally settled to the

219 substratum, moving only the pleopods. Movements of the pleopods of the animals were

220 visually monitored to make sure they were alive during the measurement. The duration of the

221 measurements varied between 4 and 12 h, lasting down to at least 20% air saturation (4.2 kPa)

222 in *E. superba* and *E. pacifica*, and to between 30 and 40% air saturation (6-8 kPa) in *E.*

223 *mucronata*. The measurement stopped when the oxygen concentration in two of the three

224 chambers was not decreasing, or when the krill died. The critical oxygen partial pressure (pc)

225 where respiration changes from oxyregulating to oxyconforming was visually determined by

226 the maximal change in the slope of the overall respiration curve, which represents the trend

227 line of the O_2 consumption data plotted against the oxygen concentration inside the chamber.

228 The contribution of anaerobic metabolism was confirmed by measuring lactate levels (in

229 mmol L^{-1}) in the hemolymph of each individual sacrificed after respirometry, using an

230 Accutrend R Lactate system (Roche Diagnostics, Germany). The surface of the krill was dried

231 with tissue paper and cut just below the cephalothorax. Drops of hemolymph from the

232 abdominal part were then directly applied to the testing strip, making sure it was completely

233 covered with hemolymph (approximately 15 μL).

234 Subsequently, both parts of the krill were frozen at -80°C, and dry mass (DM) of each

235 krill from the respiration experiments was measured after drying specimens 48 h at 50°C. The

236 bacterial O_2 demand in the blank chamber was subtracted from the O_2 consumption recorded

237 in the three chambers with krill in each run. RMR are expressed in μmol O_2 h^{-1} g DM^{-1} and

238 was calculated between 80 and 60% air saturation (16.8 to 12.6 kPa).

239

Experimental study of the synergic effect of hypoxia, reoxygenation, and warming exposure

At least one experiment was conducted for each krill species in the different areas (see Tab. 2). Krill were divided into eight replicates of 10 to 30 animals, according to the number of krill available and their size (see Tab. 2 for the number of krill used). Two experimental temperatures were simultaneously applied at 100% air saturation (21 kPa; 2 replicates for each temperature at normoxic conditions), and at 20% air saturation (4.2 kPa; 2 replicates for each temperature at hypoxic conditions) in which the animals were exposed to hypoxia over 6 h. The colder experimental temperature was always the one of the available cold room (Tab. 1), which was set closest possible to the *in situ* temperature at the sampling site. Higher temperature exposures were conducted by placing the aquaria in two boxes of water warmed with an aquarium heater (EHEIM, Germany). One control replicate (100% air saturation) and one hypoxic treatment replicate (20% air saturation) were incubated per box, which were covered with a lid to keep O_2 conditions and T°C homogenous. After transfer to the experimental aquaria, krill were allowed 1 h to acclimatize to the respective temperature. Then, water in the control aquarium was gently purged with air to achieve full saturation in the control group, while pure nitrogen (N_2) was purged in the treatment set-up to lower the oxygen level to 20% air saturation (4.2 kPa). Oxygen concentrations in hypoxic treatment aquaria were monitored using the OXY-4 channel PreSens Oxygen Ingress Measurement system at 30 min measuring intervals. Nitrogen was purged again when oxygen content started to rise in order to keep constant hypoxic levels of approx. 20% air saturation (4.2 kPa). After 6 h of hypoxia exposure, half of the surviving krill from the hypoxia treatments was sampled and immediately snap frozen in liquid N_2 (HCS) or at -80°C (NCCS and Antarctica). Half of control krill were sampled and preserved in the same way for each replicate. Krill left in the hypoxia treatment aquaria were reoxygenated for 1 h, by purging the aquaria with air,

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264 and then sampled along with a second control group maintained constantly oxygenated.

265 Frozen samples from all sampling locations were transported in dry ice to the Alfred Wegener

266 Institute, Helmholtz Centre for Polar and Marine Research for biochemical analysis.

267 *Deviations from this experimental set-up*: Since security legislations do not allow N_2

268 handling in ship laboratories in the Antarctic, we conducted hypoxia exposure experiments on

269 deck at ambient temperature. Here, we used a tank with a low certified O_2/N_2 mixture (4% O_2

270 which corresponded to 20% air saturation in seawater or 4.2 kPa) to constantly purge water in

271 the hypoxic treatment aquaria. The aquaria were placed in boxes with an entry and an exit to

272 allow a seawater flow from the research vessel water supply system to keep a constant and

273 cold temperature (between 3 and 3.5°C) during the experimental exposure, similar to SST at

274 South Georgia (Tab. 1). Working on the deck involved material limitations, and consequently

275 it was not possible to expose the Antarctic krill to warming and reoxygenation. In the HCS off

276 the Chilean coast, no hypoxic exposures were conducted in the cold season because N_2 was

277 not available. These data were therefore not generated.

278

279 *Biochemical analysis*

280 Citrate synthase (CS) is a mitochondrial matrix enzyme, pacemaker of the Krebs

281 cycle, which produces citrate from oxaloacetate, and acetyl-coenzyme A (acetyl-CoA). It is

282 frequently measured as indicator of mitochondrial capacity in a tissue. Here, CS activity was

283 measured in complete organisms immediately frozen after catch. Each organism was weighed

284 into a Precellys homogenization tube (Sartorius, LA230S, Germany) and diluted 1:20 (w/v)

285 with ice-cold Trizma® hydrochloride (Tris-HCl) buffer (20 mM Tris-HCL, 1 mM

286 ethylenediaminetetraacetic acid (EDTA), 0.1% (v/v) Tween† 20, pH 7.4). Subsequently, tubes

287 were placed in a homogenizer (Bertin Technologies Precellys 24 Dual, Germany) at 4°C with

288 the following cycle: 2 x 20 sec, 5000 rotations, 15 sec pause. After centrifugation at 7400 g

289 for 5 min, the supernatant of the homogenate was removed and used for the measurement.

290 The test by Sidell *et al.* (1987) optically records the catalytic turnover of acetyl-CoA-SH by

291 measuring the transfer of the sulfydryl groups to 5',5'-dithio-bis(2-nitro)benzoic acid (DTNB)

292 as absorbance increase at 412 nm. CS activity was measured at room temperature (20°C)

293 using a micro-plate reader (Berthold Technologies Multimode reader TriStar LB 941,

294 France). Soluble protein content was measured after Bradford (1976) in all supernatants. Data

295 were calculated as activity units (U) mg proteins^{-1}.

296   For the oxidative stress assays, each individual was cut into two pieces below the

297 cephalothorax. The front part (cephalothorax) was ground in liquid N_2 and homogenized on

298 ice with a micropistill after adding a 6-fold volume (w/v) of phosphate buffer solution (50

299 mmol L^{-1} potassium phosphate dibasic and monobasic mixture (K_2HPO_4/KH_2PO_4), 50 mmol

300 L^{-1} EDTA, 1 mmol L^{-1} phenylmethanesulfonyl fluoride, pH 7.5), and centrifuged at 23 897 g

301 velocity for 3 min at 4°C. The supernatant of this extraction was analysed in triplicates for the

302 antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione-S-

303 transferase (GST). If not enough supernatant was available, CAT and GST measurements

304 were prioritized over SOD, as less volume is requiring for these assays. SOD converts $O_2^{\bullet-}$ to

305 H_2O_2, and was measured using the xanthine-xanthine oxidase (XOD) as a superoxide radical

306 generating system and nitroblue tetrazolium as a detector (Suzuki 2000). CAT takes away

307 H_2O_2 preventing its increase in cells and tissues, which decreased was measured at 240 nm

308 (Aebi 1984). GST transforms xenobiotics into other conjugates using reduced glutathione

309 (GSH) as substrate, and was estimated by detecting the formation of the thioether product

310 from the reaction between GSH and 1-chloro, 2, 4-dinitrobenzene (CNDB; Habig & Jakoby

311 1981). The larger cephalothorax of *E. superba* allowed the additional analysis of glutathione

312 peroxidase (GPx) activity, which required a minimum of 150 μL per triplicate. Like CAT,

313 GPx removes H_2O_2 using nicotinamide adenine dinucleotide phosphate (NADPH) as

314 substrate, and was measured by monitoring the continuous decrease in the concentration of

315 NADPH upon addition of H_2O_2 to the assay mixture (Ahmad & Pardini 1988). All antioxidant

316 enzyme activities were measured at room temperature (20°C) using a spectrophotometer

317 (Beckman-Coulter DU 800 UV/Vis, USA). Soluble protein was also measured in all

318 supernatants to get enzyme activities expressed in activity units (U) mg proteins^{-1}.

319   A small selection of abdominal tissue from experimental and *in situ* samplings (*E.*

320 *mucronata* in cold season) was analysed for reduced and oxidized glutathione (GSH, GSSG)

321 concentration by high-performance liquid chromatography (HPLC) after de Almeida *et al.*

322 (2012) with some adjustments. For GSSH assay, 200 μL of 40 mmol N-ethylmaleimine was

323 added to 200 μL supernatant and incubated for 25 min at room temperature in the dark. Then,

324 700 μL of 0.1 mol NaOH was added and 50 μL of this mixture transferred to a fresh reaction

325 vial. After the addition of 1000 μL 0.1 mol NaOH, 20 μL aliquots of this mixture were

326 separated as replicates. To each replicate, 300 μL of 0.1 mol NaOH were added together with

327 20 μL of 0.1% *orto*-phthaldialdehyde (OPA) in methanol. The following steps were according

328 to the GSH assay (de Almeida *et al.* 2012). The replicates (three for GSH and five for GSSG)

329 were kept at -20°C and thawed four hours before analysis in the HPLC system (LaChrom

330 Elite®, Hitachi High Technologies America, USA). Five replicates were necessary for the

331 GSSG measurement because of the marginal amount of oxidized glutathione in the samples

332 (SD among replicates was >10% when only three were analysed). Separation was achieved on

333 a silica based C18 Hydro Reverse Phase column (250x4.6mm, 4μm particles, Phenomenex,

334 USA) at room temperature (20°C), using isocratic elution with a solvent composed of 15%

335 Methanol in 25 mmol NaH_2PO_4 (pH 6.0) at 100%. Flow rate was 0.7 mL min^{-1}, and the peak

336 area was recorded at a retention time of 9.8 min with excitation of 350 nm, and emission of

337 420 nm. This measurement of the actual redox state in the tissue is conducted to corroborate

338 the measurements of antioxidant enzymes for a better comparison of different species. The

339   total pool of glutathione, reduced and oxidized forms, was quantified as glutathione

340   equivalents (GSH-eq= GSH + 2 GSSG) and expressed as nmol g WM^{-1}. The ratio GSSG:

341   GSH was calculated from the determined GSSG and GSH concentrations.

342   More abdominal samples were further used for the detection of malondialdehyde

343   (MDA) formation, as indicator for lipid peroxidation, and protein carbonyl content, which

344   tells us about protein oxidative damages. MDA concentrations were assessed according to

345   Uchiyama & Mihara (1978) and expressed as nmol MDA g WM^{-1}. Protein carbonyl content

346   was measured using the OxiSelect Protein Carbonyl ELISA Kit (Cell Biolabs Inc., San Diego,

347   CA) according to the manufacturer's instructions. Because of the small size of *E. mucronata*

348   and *E. pacifica*, all experimental (control, hypoxia, and reoxygenation) abdominal samples

349   were used for HPLC and MDA analysis. For that reason, protein carbonyls were analysed in

350   abdominal samples of freshly caught *E. mucronata* and *E. pacifica*. As sufficient tissue was

351   available for the Antarctic species, *E. superba*, protein carbonyls were analysed in the

352   abdominal parts of experimental animals. Carbonyl results are expressed in nmol mg proteins^{-}

353   ^{1}.

354

355   *Data analysis*

356   Sea surface temperature and chlorophyll *a* concentration maps were elaborated with

357   Surfer (version 11, Golden Software Inc., USA). All statistic and figures were done with R (R

358   Core Team 2012). Interspecific differences were statistically tested among all species, and

359   between the two temperate species (as the polar thermal range of *E. superba* automatically

360   separates this species from the other two in many parameters). Differences between

361   experimental groups within each species (hypoxia/reoxygenation and warming) were tested

362   using ANOVA and Tukey post-hoc comparison. For all statistical comparisons, normality

363   (Shapiro test) and variance homogeneity (Bartlett test) tests were performed. Data were

364 (log(x), x^{-1}, $x^{1/2}$) transformed if criteria of normal distribution and homogeneity of variance

365 were not met. If no transformation of data allowed the use of analysis of variance (ANOVA),

366 the non-parametric Kruskal-Wallis test was applied. If a post-hoc comparison was necessary,

367 a Tukey test or, if non-parametric, a multiple comparison test after Kruskal-Wallis from the

368 package "pgirmess" (Giraudoux 2013) was applied. Significant level of all comparisons was

369 fixed at 95% (p=0.05).

5. Results

Environmental conditions

Figure 1 shows the vertical profiles of mean temperature, oxygen concentration, and salinity for each sampling site and season. At the sub Antarctic sampling site near South Georgia, surface temperatures were above 3°C and decreased steadily down to a thermal minimum in 120 m water depth. Between 120 and 300 m the temperature increased again to approximately 2°C (Fig. 1a). There was also a steep drop in surface salinity between 0 and 10 m water depth from 35.0 to 33.8 PSU (Fig. 1a). Similar to the temperature profile, salinity increased linearly with depth to near surface values in 400 m (Fig. 1a). Only the oxygen profile at the sub-Antarctic sampling site was homogenous and fully saturated between 0 and 400 m water depth (Fig. 1a). Antarctic krill was mainly fished between 40 and 200 m water depths in this study (Fig. 1a). Within the NCCS and HCS temperature profiles were similar, slightly stratified in the warm season and mixed in cold season, with SST ranging on average between 11 and 14°C in both locations (Fig. 1b-e). The upper boundary of the OMZ (at 20% air saturation or 4.2 kPa) was detected around 30 and 60 m in the HCS in the warm and cold season, respectively (Fig. 1b, c). Near anoxic conditions exist below 60 m at the HCS station in summer and winter (Fig. 1b, c). We caught the HCS krill species, *E. mucronata*, hauling the net from 40 m depth to the surface in both seasons (Fig. 1b, c). In the NCCS, hypoxia was less pronounced, never reaching as low as 20% air saturation (4.2 kPa; Fig. 1d, e). Between the surface and 60 m, a steep decline to approximately 30% air saturation occurred in the warm season (Fig. 1d), whereas in the cold season the decrease was less steep (Fig. 1e). Differences in the salinity gradient were more important during winter than during summer in the HCS and NCCS (Fig. 1c, e). Salinity was lower in the NCCS than in the HCS in both seasons, with a more pronounced lowering of surface salinity in the cold season (Apr 2012;

394 Fig. 1d, e). Krill collection in the NCCS occurred also in the first 40 m from the surface in

395 both seasons (Fig. 1d, e).

396   Sea surface temperature (SST; °C) and chlorophyll *a* (chl *a*) concentration (mg m^{-3})

397 averaged monthly means from MODIS-Aqua (4km) are presented in Figure 2 for each area

398 and sampling period. At South Georgia, krill were sampled in waters with relatively low chl *a*

399 concentrations compared to the temperate regions (Fig. 2a). Strong up-welling events can

400 easily be identified in the warm season in both temperate areas (Fig. 2b, d) with the SST

401 visualisations showing a cold-warm gradient of temperature from the coastline. Patches of

402 high chl *a* concentration (>20 mg m^{-3}) confirmed nutrient enrichment upwelling water masses

403 (Fig. 2b, d). In the cold season, SST was more homogenous in both regions (Fig. 2c, e).

404 Smaller patches of high chl *a* occurred in the HCS during the cold season (Fig. 2c), in contrast

405 to the NCCS (Fig. 2e).

406

407 *Respiration measurements*

408   Different patterns of O$_2$ consumption *vs* pO$_2$ were observed for each species (Fig. 3).

409 Not all individual respiratory tracks could be analysed over the full pO$_2$ range from 80% air

410 saturation (16.8 kPa) down to hypoxia, and the number of individuals graphed in both ends of

411 respiration curves in Fig. 3 corresponds to approximately one third of all measurements

412 conducted. The Antarctic krill, *E. superba*, had by far the largest body mass (Tab. 3) and O$_2$

413 consumption was constant (regulating) down to approx. 55% air saturation (11.5 kPa), below

414 which respiration was pO$_2$ dependent (oxyconforming) (Fig. 3a). Thus 11.5 kPa was

415 determined as pc for *E. superba*. A completely different respiration pattern was observed in

416 the HCS species *E. mucronata*: O$_2$ consumption first decreased linearly between 80 to 60%

417 air saturation as the animals ceased swimming and settled to the bottom (Fig. 3b). Below 46%

418 air saturation (9.7 kPa) down to 30% (6.3 kPa) oxygen uptake was constant. Thus, the pc of

419 6.3 kPa was interpreted as the point where the krill started additional anaerobic respiration.

420 No animal died below 6.3 kPa. The NCCS species *E. pacifica* maintained stable metabolic

421 rates down to 40% air saturation (8.4 kPa) in the warm season, with a transient increase of

422 respiration between 40% (8.4 kPa) and pc at 27% (5.7 kPa; Fig. 3c). In the cold season, the

423 same species was not able to maintain O_2 consumption constant as far down as in summer,

424 and pc was observed already at 34% (7.1 kPa; Fig. 3d), preceding immediate death. Animals

425 from the HCS and NCCS used in the respiration measurements in the winter season had

426 comparable body mass (Tab. 3).

427

428 *Interspecific basal metabolic and oxidative stress parameters comparison*
429
430 Basal metabolic and oxidative stress parameters analysed in the different krill species

431 from each ecosystem and season are presented in Table 3 and Fig. 4. The RMR of *E. pacifica*

432 during the warm season was significantly higher (λ^2=44.42; p<0.000, T_m: 10°C) than RMR of

433 the Antarctic krill *E. superba* (Tab. 3, T_m: 4°C). A seasonal comparison of the RMR is only

434 possible for the NCCS species *E. pacifica*, where RMR had was significantly higher SMR in

435 the cold season (F=7.84; p=0.008; Tab. 3, T_m: 10°C in both seasons). Lactate accumulation

436 measured at the end of the respiration experiment when the animals had reached their hypoxic

437 limit was significantly higher (λ^2=6.23; p=0.013) in the hypoxia-adapted species *E.*

438 *mucronata* than in *E. pacifica* during the cold season (Tab. 3). In *E. pacifica*, lactate

439 concentrations differed between seasons (λ^2=8.43; p=0.004), with higher values in September

440 2011 at the end of summer (Tab. 3). CS activity was significantly higher (λ^2=35.60; p<0.000)

441 in *E. superba* compared to both temperate species during the warm and the cold seasons (Fig.

442 4a).

443 Interspecific and seasonal comparisons (Fig. 4b-f) of oxidative stress parameters

444 correspond to the values obtained as control treatments for the hypoxia-reoxygenation

445   experiments at *in situ* temperature (*i.e.* cold room/respiration measurement temperatures, see

446   Tab. 2). The only exception was that protein carbonyls of *E. mucronata* and *E. pacifica* were

447   measured in samples frozen directly after catch. Superoxide dismutase (SOD) activity was

448   significantly lower in the Antarctic krill (λ^2=36.61; p<0.000) and the HCS krill *E. mucronata*

449   (λ^2=4.51; p<0.034; when the Antarctic krill is not considered) compared to the NCCS species

450   *E. pacifica* during the warm season (Fig. 4b). Both temperate species, *E. mucronata* (λ^2=4.02;

451   p=0.045) and *E. pacifica* (λ^2=31.46; p<0.000), had higher SOD activity in winter (Fig. 4b)

452   than in summer. In contrast, CAT activity was significantly higher in the warm than the cold

453   season (F=9.68; p=0.003) in *E. mucronata* (Fig. 4c). The hypoxia tolerant HCS species *E.*

454   *mucronata* also had the highest catalase (CAT) activity in the warm season (considering *E.*

455   *superba*: F=102.94; p<0.000; without *E. superba*: F=10.15; p<0.002; Fig. 4c). The activity of

456   the detoxifying antioxidant enzyme glutathione-S-transferase (GST) was significantly higher

457   in *E. mucronata* during both, the warm (considering *E. superba*: λ^2=104.65; p<0.000; without

458   *E. superba*: λ^2=33.11; p<0.000; Fig. 4d) and the cold season (F=61.85; p<0.000; Fig. 4d). The

459   Antarctic krill, *E. superba*, had the lowest GSSG:GSH ratio (least oxidized glutathione,

460   λ^2=31.91; p<0.000) corresponding to the highest concentration of reduced GSH (λ^2=43.04;

461   p<0.000) compared to both temperate species in the warm season (both; Fig. 4e, f). When

462   only comparing the temperate species, the GSSG:GSH redox ratio of *E. mucronata* was

463   significantly higher (F=8.95; p=0.010) than in *E. pacifica* (Fig. 4e) during the cold season.

464   Whereas *E. pacifica* had similar glutathione ratios in both seasons, *E. mucronata* had a higher

465   (more oxidized) GSSG:GSH ratio (F=5.84; p=0.023; Fig. 4e) and lower GSH concentration

466   (F=6.40; p=0.016; Fig. 4f) in the cold season.

467   The difference in malondialdehyde (MDA) concentrations were extremely subtle and

468   the only conspicuous difference was a very low MDA values in the hypoxia-adapted *E.*

469   *mucronata* during summer, which had significantly less MDA per g WM than Antarctic krill

470   *E. superba* (λ^2=7.02; p=0.030; Fig. 4g) and *E. mucronata* caught in winter (λ^2=8.04; p=0.005;

471   Fig. 4g). The Antarctic krill *E. superba* had negligible protein carbonyl levels (λ^2=22.23;

472   p<0.000; Fig. 4h) compared to the temperate species in both seasons. Both temperate species

473   had similar protein damage levels independently of the season (Fig. 4h).

474

475   *Intraspecific warming, hypoxia and reoxygenation responses*

476   No significant differences were detected in any of the three species between the

477   control groups run in parallel to the 6 h hypoxia exposure experiment and the control groups

478   maintained fully oxygenated during the additional 1 h reoxygenation phase. Results from both

479   control groups were therefore pooled in each species to form the control group for both

480   treatments. Table 4 summarizes the effect of both treatments on oxidative stress parameters in

481   all three species compared to their respective control group (the complete data set is available

482   in PANGAEA; Tremblay & Abele 2014). Oxidative stress parameters analysed in the

483   Antarctic krill *E. superba* at 4°C after 6 h of hypoxia exposure were similar to normoxic

484   control values (Tab. 4). Only MDA concentration (λ^2=7.02; p=0.030; Tab. 4) increased after 6

485   h of hypoxia. Minor mortality or loss of individuals occurred during exposure (3%; Tab. 4)

486   when heavy sea conditions started (movement of the water inside the aquaria allowed the krill

487   to jump out).

488   In the HCS during the cold season, no hypoxia-reoxygenation treatment was

489   conducted with *E. mucronata* due to N_2 unavailability. Warming by +7°C (to 15°C) reduced

490   the activity of all antioxidant enzymes analysed, but the effect was only significant for CAT

491   (F=7.80; p=0.013; Tab. 4). All 15°C abdomen samples were used for GSH and GSSG HPLC

492   measurements; which is why we could not compare MDA concentrations during experimental

493   warming in the cold season. During the warm season, a hypoxia-reoxygenation experiment

494   was conducted with *E. mucronata* which revealed increased SOD activity (λ^2=11.20;

495 p=0.001; Tab. 4) and a reduction of the GSSG: GSH ratio (F=7.53; p=0.009; Tab. 4) under

496 hypoxic and reoxygenation exposure at 15°C. No other parameters were affected by the

497 treatments, and no mortalities occurred in the experiments with this species in either season

498 (Tab. 4).

499   The NCCS species *E. pacifica* was the only one that we could expose to warming and

500 hypoxia-reoxygenation in both seasons. Mortality in the 6 h hypoxia experiments increased

501 with increasing exposure temperature from 24% of exposed krill dying at 10°C, 30% at 12°C,

502 and 95% at 14°C in the cold season. Better survival was recorded in the warm season with 8%

503 mortality at 10°C, 17% at 12°C, and 48% at 14°C (Tab. 4). No significant change in any of

504 the oxidative stress indicators as effect of hypoxia and reoxygenation treatments and/or

505 warming was observed in individuals from the treatment groups in the cold season (Tab. 4).

506 Similar to *E. mucronata*, there was no combined effect of hypoxia/reoxygenation treatments

507 and warming (from 10 to 14°C) on the oxidative stress parameters in *E. pacifica* in the warm

508 season. At 14°C, CAT activity was significantly lower in all hypoxia/reoxygenation

509 treatments compared to both 10 and 12°C (λ^2=19.08; p<0.000; Tab. 4). GSH-eq from all

510 treatments was significantly depleted at 12°C compared to 10°C (λ^2=5.75; p=0.017; Tab. 4).

511 Because of the high mortality in these experiments, no samples were available for the GSH

512 and GSSG analysis at 14°C. Oxidative damage of lipids (MDA) was less intensive at 12°C

513 than at colder (10°C) or warmer (14°C) temperature (λ^2=20.46; p<0.000; Tab. 4). At 10°C,

514 SOD activity (λ^2=6.05; p=0.049; Tab. 4) and GSSG:GSH ratios (λ^2=6.36; p=0.042; Tab. 4)

515 decreased in both hypoxia and reoxygenation treatments, while MDA values were elevated in

516 the reoxygenation group over normoxic (control) and hypoxic samples (F=4.67; p=0.018;

517 Tab. 4). Further, two-way ANOVA also revealed increased GST activity under reoxygenation

518 treatment at all tested temperatures (F=5.57; p=0.005; Tab. 4) compared to normoxic (control)

519 and hypoxic incubations.

6. Discussion

Effects of climatic adaptation on metabolism and oxidative stress parameters: polar vs. temperate regions

Antarctic marine ectotherms are often characterized as especially sensitive to oxidative stress and damage (*e.g.* Lesser 2006; Abele & Puntarulo 2004). Their high sensitivity is associated with less saturated membrane lipids, which are more susceptible to ROS damage. In our comparison, the polar species *E. superba* clearly sticks out among the three investigated species in terms of *in situ* metabolic parameters RMR, CS activity, antioxidant enzyme activities of SOD and CAT, and the cellular oxidative stress and damage parameters (GSSG:GSH ratio, GSH-eq, and carbonyl concentrations). Although strongly hypoxic OMZ conditions are not expected in Antarctic shelf regions in the near future, Antarctic krill at South Georgia is able to regulate RMR down to 55% air saturation (*p*c) and moreover accumulate lactate from anaerobic metabolism below *p*c, as seen in our respiration measurements. *E. superba* from our study had a *p*c above the one reported by Torres *et al.* (1994; *p*c between 30 and 52 mm Hg corresponding to 19-33% air saturation and 4-7 kPa at 0.5°C) for the Scotia Sea. Upward shift of *p*c is caused by a higher RMR of Antarctic krill at South Georgia water temperatures, as was observed in other krill species when respiration was measured at maximal habitat temperature (*Meganyctiphanes norvegica*: Strömberg & Spicer 2000; *Euphausia hanseni*: Werner 2012). Note that moderate hypoxia (50% air saturation or 10.5 kPa) is already observed in the Indian sector of the Southern Ocean at depths below 500 m (Dehairs *et al.* 1990). This might become problematic for the krill and perhaps explains why biomass in this Antarctic sector is 10-fold lower than in the well-oxygenated regions of the Southern Ocean (Nicol *et al.* 2000).

The lower RMR recorded for the Antarctic compared to the temperate krill species relates to its larger body mass and the lower habitat and experimental temperatures (Ikeda

545 2012). Even when normalizing mean RMR of *E. superba* to 10°C, with Q10=2.68 calculated

546 from McWhinnie (1964) which gives a $RMR_n=58$ µmol O_2 h^{-1} g DM^{-1}, RMR remains

547 significantly lower compared with both temperate species.

548   Lactate accumulation in *E. superba* was in the same range as in hypoxia-adapted *E.*

549 *mucronata* from HCS. Lactate accumulation has previously been shown for one OMZ

550 species, *Euphausia eximia* (summarized in Seibel 2011), and the polar-temperate species

551 *Meganyctiphanes norvegica* (Spicer *et al*. 1999). Euphausiids are active swimmers and lactate

552 accumulation may be problematic and supports only transient survival under extremely

553 hypoxic conditions. In our case, animals respiring in a closed system were measured and had

554 no other choice than to enhance anaerobic energy production in their confined water volume.

555 Thus, our data show experimentally re-enforced glycolytic capacities that have presumably no

556 relevance in nature. For example, lactate concentration in *E. superba* measured directly after

557 capture (data not shown; mean and S.E. with *n*=51: 2.3 ± 0.3 mmol L^{-1}) was lower than the

558 concentrations after respiration measurements (reported in Tab. 3).

559   The slow routine activity of the Antarctic krill goes hand in hand with low

560 hemocyanin O_2 affinity (Bridges *et al.* 1983) and high citrate synthase (CS) activity (our

561 data), indicating enhanced mitochondrial densities to compensate for the permanently low

562 temperatures in Antarctic waters (Johnston *et al.* 1998; Guderley 2004; Morley *et al.* 2009).

563 Metabolic cold adaptation of mitochondrial densities at South Georgia indicates this trait to be

564 either genetically based, or that the residence time of the krill in warmer sub-Antarctic waters

565 is not sufficiently long to allow for an adaptive change.

566   SOD and CAT activities align with RMR for the three krill species. In fact, activities

567 of both enzymes were almost not detectable in polar krill, which speaks for lower

568 mitochondrial ROS generation. High mitochondrial densities are characteristic for many

569 Antarctic marine invertebrates and often associated with higher mitochondrial cristae

570 densities and higher proton leak than related temperate species (Philipp *et al.* 2005b:

571 comparing marine clams). A higher proton leak, not yet investigated in Antarctic or other krill

572 species, can almost certainly mitigate metabolic ROS formation (Brand 2000) and could be

573 instrumental in further restraining oxidative stress in *E. superba*. The unchanged levels of

574 antioxidants, the limited increase of lipid peroxidation, and a negligible mortality rate of *E.*

575 *superba* after 6 h of hypoxia treatment further support a robust hypoxia tolerance in Antarctic

576 krill at habitat temperature.

577 To compensate the low SOD and CAT activities, polar krill has considerable amounts

578 of low molecular antioxidants such as vitamin E and GSH. In fact, vitamin E concentration in

579 *E. superba* was in the range of Antarctic fish (Dunlap *et al.* 2002), and glutathione

580 concentration was twice as high as in temperate euphausiids (our data). The high GSH

581 concentration, and the very low GSSG: GSH ratio, match the idea of overall low basal

582 oxidative stress in *E. superba* and indicate that glutathione may be involved in buffering ROS

583 induced by environmental insult as UV radiation or warming. Low GSSG: GSH ratios (below

584 0.3) were earlier detected in the Antarctic clam *Laternula elliptica* (Philipp *et al.* 2005a)

585 compared to temperate species, and in the Antarctic limpet *Nacella concinna* (Weihe *et al.*

586 2010). Indeed it may be characteristic of permanently cold adapted marine ectotherms to rely

587 on chemical rather than enzymatic mechanisms for ROS quenching. Note that *E. superba*

588 further had the lowest levels of protein damage, supporting our hypothesis of low ROS

589 formation and sufficient protection. The comparison of the three krill species clearly argues

590 against elevated oxidative stress as an inherent attribute to life in cold environments such as

591 Arctic and Antarctic, and instead may support the concept that low metabolic ROS promote

592 long lifespan in many polar species (Philipp & Abele 2010; Clark *et al.* 2013).

593

594 *OMZ adaptation and "normoxic stress" in the hypoxia-adapted species Euphausia*

595 *mucronata*

596   The outstanding GST activity in the hypoxia tolerant *E. mucronata*, which was at least

597 4-fold higher than GST in *E. pacifica* in both seasons, may be pertinent to its success in the

598 OMZ. A high GST activity means extra removal of GSH for detoxification purposes, which

599 also explains the conspicuously higher GSSG: GSH ratio in this species. Such a high GSSG:

600 GSH ratio in tissues (as a rule: GSSG: GSH should be around 0.1) often indicates oxidative

601 stress, as it reflects oxidation of reduced GSH to the oxidized (GSSG) form. However, neither

602 lipid nor protein oxidative damage indicators were higher in *E. mucronata* than in *E. pacifica*,

603 which argues against enhanced oxidative stress in the hypoxia tolerant *E. mucronata*. The

604 power of glutathione has earlier been noticed in two OMZ species from the Gulf of California

605 (Mexico), *Nematoscelis difficilis* and *Euphausia eximia* (Tremblay *et al*. 2010). As the

606 thermocline forms in this region and the OMZ extends upwards during the warm summer

607 season, higher superoxide radical ($O_2^{\cdot -}$) production was counteracted by substantial use of

608 GSH by GST and GPx activities, which prevented lipid oxidative damage.

609   The hypoxia-adapted *E. mucronata* does not regulate the O_2 consumption above 60%

610 air saturation (12.6 kPa) and, instead, respires in a pO_2-dependent manner in hypoxic and

611 even in normoxia (> 17 kPa or 80% air saturation). The linear decrease could be due to i)

612 decreasing movements in the respiration chambers at declining pO_2, earlier found by Teal and

613 Carey (1967) or ii) an oxyconforming behavior over broad ranges of pO_2 as the animal adjust

614 metabolism (ATP demand and ATP production) to lowering outside oxygenation. This was

615 shown mainly for benthic species such as the polychaete worm *Heteromastus filiformis*

616 (Abele et al 1998), the flatworm *Macrostomum lignano* (Rivera Ingraham *et al*. 2013), and

617 the sipunculid worm described in Pörtner and Grieshaber (1993). In hypoxic OMZ conditions,

618 aerobic energy production can be partly covered by less energy efficient anaerobic glycolysis,

619   as seen in the relatively high lactate accumulation rate in *E. mucronata* compared to *E.*

620   *pacifica* when letting them respire to severely hypoxic conditions at the limits of survival

621   (below *pc* in *E. pacifica*).

622   A seasonal difference in SOD activity with lower values in summer and higher in

623   winter was observed in *E. mucronata*. Whether or not this reflects higher winter RMR (as in

624   *E. pacifica*) remains unclear, as summer RMR were not measured. However, higher GSSG:

625   GSH ratio and lipid oxidation levels, together with lower CAT activity and GSH-eq in the

626   cold season, corroborate a state of comparatively higher oxidative stress during winter.

627   Hypoxia is a permanent condition in the Chilean stretch of the HCS and, as we stated before,

628   the temperature and food conditions did not vary much between seasons in this up-welling

629   ecosystems. As *E. mucronata* is physiologically and morphologically adapted to extremely

630   hypoxic OMZ conditions, oxygenation conditions above the hypoxic range down to 60 m

631   water depth in the cold season may already represent a scenario of "stress due to over-

632   oxygenation". Examples of fish and scallops in which environmental hyper-oxygenation

633   induces oxidative stress were recently compiled by Lushchak (2011) and observed in the

634   infaunal polychaete *Heteromastus filiformis* (Abele *et al.* 1998), the freshwater clam

635   *Sphaerium* sp. (Joyner-Matos *et al.* 2007), and in marine sedimentary meiofauna (Rivera-

636   Ingraham *et al.* 2013).

637   Hypoxia-reoxygenation treatments alone were not *per se* stressful to *E. mucronata.*

638   However the cross effect of warming was crucial, and even damaging in the cold season (Aug

639   2011), much more than in the warm season (Feb 2012). Experimental warming of the habitat

640   especially during winter is bound to reduce antioxidant defences and support oxidative stress

641   and damage in *E. mucronata*, compromising survival. Paradoxically, the same temperature

642   increment applied in summer conditions (Feb 2012) mobilized SOD activity, especially in

643   control and reoxygenation treatments. This combined with a more reduced GSSG: GSH ratio,

644   indicates better control of oxidative stress in summer, possibly preventing additional lipid

645   peroxidation. Indeed, no mortality was recorded in the oxygen treatments with or without

646   warming, although +7°C (15ºC) represents extreme warming stress not currently predicted for

647   this part of the HCS region. Thus, in spite of being extremely well adapted to life in the

648   OMZ, *E. mucronata* can suffer oxidative stress when moving upwards to normoxic

649   environmental conditions in the cold season. This also partly explains their reluctance to

650   surface and instead to remain longer in their hypoxic OMZ niche than more oxygen tolerant

651   species.

652

653   *Hypoxia-reoxygenation stress is accentuated by warming in the north Pacific species*

654   *Euphausia pacifica*

655   In a warming and deoxygenated scenario, the future is not so rosy for the NCCS

656   species *E. pacifica*. As for the Antarctic krill, *E. pacifica* switch from oxyregulation to

657   oxyconformity at 5.7 and 7.1 kPa, during the warm and the cold season, respectively. In both

658   seasons, the pc we measured was higher than the pc reported in the southern part of the

659   California current system for the same temperature (Childress 1975; pc of 18 mm Hg which

660   corresponds approximately to 2.3 kPa at 10°C). Nevertheless, our findings are similar to

661   values obtained by Ikeda (1977; pc of near 40% air saturation corresponds approximately to

662   8.4 kPa at 13°C) at Saanich Inlet (Canada), a fjord located north of the NCCS, where deep

663   water presents anoxic conditions during most of the year (Herlinveaux 1962). Higher RMR in

664   April 2012 reflects slower growth of krill in spring seasons with weak upwelling, because the

665   adult individuals from the spring cohort in our catches were comparatively small (Shaw *et al*.

666   2010). As mitochondrial capacities (CS activities) remained unchanged between September

667   2011 (warm) and April 2012 (cold), higher spring RMR effectively means more oxygen

668   reduction and faster electron transport in each mitochondrion. This was kept in balance by

669 enhanced SOD activity in April 2012, whereas none of the other oxidative stress parameters

670 and damage indicators changed, matching the view that a non-stressful increase in metabolic

671 rate rarely causes oxidative stress. However, absence of significant lactate concentration in

672 hypoxia, the higher pc recorded during the cold season, and the mortality of the animals at the

673 end of the respiration measurements speak for a lower capacity to deal with hypoxia in cold

674 adapted winter animals (or better hypoxia tolerance in late summer-collected animals).

675 Lactate concentration in *E. pacifica* measured directly after capture in April 2012 was under

676 the level of detection (<0.8 mmol L^{-1}, data not shown; n=10), consistent with the lack of

677 lactate accumulation below pc (Tab. 3). Thus, a seasonal adaptation to the shallower OMZ

678 conditions in summer take place in this species. The same seasonal pattern of adjustments was

679 observed in the hypoxia reoxygenation plus warming experiments, with no visible effect on

680 either oxidative stress or metabolic indicators in the cold season. At first sight, this seems

681 encouraging, but note that biochemical analyses were only performed on survivors

682 (practically no krill survived exposure temperature of 14°C). Yet, due to the small number of

683 animals surviving warming treatments, our capacity to interpret what happened at the cellular

684 level is quite limited.

685 In the warm season, when mild OMZ conditions occur already at 100 m depth,

686 fighting low oxygen and warming conditions was also a challenge for *E. pacifica*. At *in situ*

687 temperature of 10°C the species dealt well with hypoxia. Still, reoxygenation seems to be a

688 challenge since MDA levels increased and SOD activity had not recovered after 1 h of

689 reoxygenation. The oxidative damage could be the result of the increased metabolic rates

690 during reoxygenation, as the organisms make up for the hypoxic oxygen deficit (Welker *et al.*

691 2013). De Oliveira *et al.* (2005) observed a decrease in SOD activity during anoxia in the gills

692 of the crab *Chasmagnathus granulata* from Rio Grande do Sul (Brazil). Further, as in the

693 krill, GST activities in *C. granulata* also increased at all times of reoxygenation, indicating

694 strong detoxification requirements (de Oliveira *et al.* 2005).

695   The antioxidant system of *E. pacifica* buffered the oxidative stress arising in the +2°C

696 exposure to control lipid peroxidation. Depletion of GSH-eq at 12°C backed-up the activation

697 of non-enzymatic antioxidants relative to the 10°C experiments. Another 2°C of warming

698 (14°C) was already lethal for almost half of the specimens and was accompanied by a

699 reduction in antioxidant enzyme activities. Clearly, *E. pacifica* has a narrow thermal windows

700 (see Pörtner, 2010), and short term warming to 14°C brings the species to its upper lethal

701 temperature (14°C is the temperature at which 50% of the specimens die during the warmer

702 season according to Richard *et al*. 2012) where stress is exacerbated by hypoxia-

703 reoxygenation exposure during DVM.

704

705 *The consequences of OMZ expansion in a warming world*

706   The order Euphausiacea encompasses many different ecotypes, and krill tolerance to

707 warming and OMZ expansion is species specific with metabolic and antioxidant strategies

708 shaped strongly by species evolutionary background. This is bound to cause habitat shifts of

709 krill species, mass stranding and mortality of sensitive species as climate change progresses.

710 In spite of their pelagic swarm swimming and thus energy consuming lifestyle, all krill

711 species compared could tolerate hypoxia to some extent, even the Antarctic species *E.*

712 *superba*. This underlines the general tolerance of krill to survive at least moderate hypoxia.

713 Lactate accumulation is not a true benefit for an active swimmer as it causes exhaustion of

714 energy reserves and causes tissue acidification which impinges on swimming and, therewith,

715 the capacities to escape predation. Anaerobic capacities are not always helpful to bridge an

716 energetic gap as seen in our lactate measurement in *E. pacifica* in winter, indicating increased

717 sensitivity of NCCS krill to hypoxia. Both, the latitudinal and seasonal comparisons indicate

718 that enzymatic antioxidants are not the prime mechanisms of ROS protection and at least need

719 to be complemented by high GSH levels in the cold. Major complications arise also in cold

720 temperate species (*E. pacifica*) when warming and OMZ stress during DVM come together,

721 as they rely more strongly on the antioxidant enzymes, which lose activity as water

722 temperatures peak at 14°C. Oxidative stress normally occurs when more ROS are produced

723 than the antioxidant system can handle. In this case, ROS can act as signalling molecules for

724 the activation of protective responses (e.g. stress gene transcription, membrane pore opening

725 and metabolic down regulation). At mildly stressful conditions (12°C) the stress signal may

726 be helpful, but extreme temperature stress breaks down this fragile balance.

727   In the hypoxia adapted krill from the HCS, oxidative stress is compromising the

728 wellbeing of the species when it eventually has to move up to the surface in the cold season,

729 entering a well-mixed upper water layer with >70% (15 kPa) oxygenation. Here oxidative

730 stress manifested in depletion and oxidation of the redox buffer glutathione, oxidation of

731 membrane lipids, while catalase activity was suppressed. To this end, evolutionary adaptation

732 to hypoxia, which has shaped species morphology in *E. mucronata* (greater gill surface area;

733 Antezana 2002b), can act as a double-edged sword under full oxygenation. In addition, under

734 warm-hypoxic conditions, the enzymatic antioxidant system of the Chilean species was more

735 versatile and inducible. Prospectively, although present habitat temperature ranges are very

736 similar for both temperate species, *E. mucronata* is dealing much better with OMZ and

737 warming than its northern relative. Better understanding of the physiological mechanisms

738 underlying the response of krill to climate driven changes of the seasonal OMZ and

739 thermocline formation by the analysis of biomarkers for sublethal effects may help us to

740 explain why and predict when years of poor survival may occur. This will be of major

741 importance in future risk assessment, life stocks management, and ecosystem modelling in

742 these regions.

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Table 1. Sampling areas (Latitud/Longitud), periods, sampling gears, R/V, off board localities, and temperature conditions in Antarctica (South Georgia), in the Humboldt current system (HCS) and in the northern California current system (NCCS).

Area	Lat/Lon	Period (season)	Water depth (m)	Sampling gear	R/V; off board facilities (if applied)	Sea surface* T°C	Water column¥ T°C	Cold room T°C
Antarctica (South Georgia)	53-55°S 37-41°W	3 - 10th Jan 2012 (summer)	>400	pelagic net, rectangular midwater trawl (RMT), 8 m² mouth area	James Clark Ross	3.2	1.5	4.0
HCS (Chile)	36.5°S 73.1°W	23th Aug - 13th Sep 2011 (winter)	80	zooplankton net, 1 m diameter, 5 m long, 300 μm black mesh with nonfiltering cod end (0.22 m diameter and 0.70 m long)	Kay-Kay II; Universidad de Concepción, Marine biology laboratory (Dichato, Región del Biobío)	11.9	11.7	8.0
		24th Jan - 3rd Feb 2012 (summer)				12.9	11.0	8.0
NCCS (United-States of America)	44.7°N 124.7°W	14 - 30th Sep 2011 (summer)	275	bongo net, 0.6 m diameter, 333 μm black mesh with nonfiltering cod end	Elakha; Oregon State University, Hatfield Marine Science Center (Newport, Oregon)	11.9	8.1	10.0
		7 - 14th Apr 2012 (winter)				9.9	7.7	10.0

*between 0 and 20 m; ¥from 20 m until maximum depth

Table 2. Number of catches, number of samples frozen just after catch, experimental temperatures (Exp T°C), experiments (Exp), number of experiments conducted (n exp), replicates (n rep), individuals at the beginning (n_i ind), and at the end (n_f ind) of experiments in South Georgia (SG), the Humboldt current system (HCS) and the northern California current system (NCCS).

Area	Period	n catch	n frozen after catch	Exp. T°C	Exp[*]	n exp	n rep	n_i ind	n_f ind
SG	Jan 2012	9	290	-	-	-	-	-	-
				4.0	Resp.	7	21	21	20
				3.5	C_6	4	8	60	56
					H	4	8	80	78
HCS	Aug 2011	4	81	-	-	-	-	-	-
				8.0	Resp.	4	12	12	12
					C_6	1	20	20	20
				15.0	C_6	1	6	6	6
	Feb 2012	2	71	-	-	-	-	-	-
				8.0	C_6	1	2	20	20
					H	1	4	40	40
					C_7	1	2	20	20
					R	1	4	40	40
				15.0	C_6	1	2	20	20
					H	1	2	20	20
					C_7	1	2	20	20
					R	1	2	20	20
NCCS	Sep 2011	3	60	-	-	-	-	-	-
				10.0	Resp.	11	33	33	31
					C_6	2	4	30	30
					H	2	4	37	34
					C_7	2	4	30	30
					R	2	4	17	17
				12.0	C_6	1	2	20	20
					H	1	2	30	25
					C_7	1	2	20	20
					R	1	2	12	12
				14.0	C_6	1	2	20	20
					H	1	2	40	19
					C_7	1	2	20	20
					R	1	2	5	5
	Apr 2012	2	90	-	-	-	-	-	-
				10.0	Resp.	4	12	12	11
					C_6	2	4	50	49
					H	2	6	140	106
					C_7	2	4	50	50
					R	2	6	50	50
				12.0	C_6	1	2	20	20
					H	1	2	30	21
					C_7	1	2	20	19
					R	1	2	10	10
				14.0	C_6	1	2	20	20
					H	1	2	20	1
					C_7	0	0	0	0
					R	0	0	0	0

[*]Resp.=respiration measurements; C_6=control (100% O_2 saturation for 6 h); H=hypoxia (20% O_2 saturation for 6h); C_7=control (100% O_2 saturation for 7 h); R=reoxygenation (100% O_2 saturation after for 1 h after H treatment)

Table 3. Mean oxygen (O₂) consumption rate per mg dry mass (DM), lactate concentration at the end of the respiration measurement and critical oxygen partial pressure (*pc*) of *Euphausia superba* (South Georgia; Jan 2012), *Euphausia mucronata* (Humboldt current system; Aug 2011), and *Euphausia pacifica* (Northern California current system; Sep 2011 and Apr 2012); data are mean ± SE; (n).

Species	Period	O₂ consumption (μmol O$_2$ h^{-1} g DM^{-1})	DM (mg)	[Lactate] (mmol L^{-1})	*pc* (kPa)
E. superba	Jan 2012	31 ± 1$^{¥}$ (30)	260 ± 114 (30)	3.5 ± 0.7 (24)	11.5
E. mucronata	Aug 2011	78 ± 19 (12)	5 ± 3 (12)	3.0 ± 1.1$^{¥}$ (6)	-
E. pacifica	Sep 2011	77 ± 5*$^{¥}$ (31)	14 ± 3 (31)	1.8 ± 0.3* (31)	5.7
	Apr 2012	115 ± 6* (11)	4 ± 2 (11)	0*$^{¥}$ (5)	7.1

*Intraspecific and ¥Interspecific significant differences.

Table 4. Effect of hypoxia and/or reoxygenation and/or warmer temperature on the oxidative stress parameters analysed in Antarctic krill *Euphausia superba* (*Es*; Jan 2012), *Euphausia mucronata* (*Em*; Aug 2011, Feb 2012), and *Euphausia pacifica* (*Ep*; Sep 2011, Apr 2012) compared to control (6 h 100% air saturation or 21 kPa at cold room temperature). Sp= species, T°C= temperature, Treat= Treatments (H= hypoxia, 6 h, 20% air saturation or 4.2 kPa; R= reoxygenation, 1 h, 100% air saturation after H treatment; HT= higher temperature, 6h), oxidative stress parameters (SOD= superoxide dismutase, CAT= catalase; GST= glutathione S-transferase; GSSG: GSH= oxidized/reduced glutathione; GSH-eq= glutathione equivalents; MDA= malondialdehyde concentration), %m= percentage of mortality; Arrows indicate significant changes compared to control value; nd= no data; The complete data set is available in PANGAEA (Tremblay & Abele 2014).

Sp	Period	T°C	Treat	SOD	CAT	GST	GSSG: GSH	GSH-eq	MDA	%m
Es	Jan12	4	H	=	=	=	=	=	↑	3
Em	Aug11	15	HT	=	↓	=	=	=	nd	0
	Feb12	8	H	=	=	=	=	=	=	0
		8	R	=	=	=	=	=	=	0
		15	HT	↑	=	=	↓	=	=	0
		15	HT+H	↑	=	=	↓	=	=	0
		15	HT+R	↑	=	=	↓	=	=	0
Ep	Sep11	10	H	↓	=	=	↓	=	=	8
		10	R	↓	=	↑	↓	=	↑	0
		12	HT	=	=	=	=	↓	↓	0
		12	HT+H	=	=	=	=	↓	↓	17
		12	HT+R	=	=	↑	=	↓	↓	0
		14	HT	=	↓	=	nd	nd	=	0
		14	HT+H	=	↓	=	nd	nd	=	48
		14	HT+R	=	↓	↑	nd	nd	nd	0
	Apr12	10	H	=	=	=	=	=	=	24
		10	R	=	=	=	=	=	=	0
		12	HT	=	=	=	=	=	=	5
		12	HT+H	=	=	=	=	=	=	30
		12	HT+R	=	=	=	=	=	=	0

1063 **Figure captions**
1064

1065 Figure 1: Vertical profiles of abiotic parameters and krill sampling depth in each area during

1066 sampling periods. Mean temperature (°C), oxygen concentration (% air saturation), and

1067 salinity (PSU) in South Georgia (SG; a), the Humboldt current system (HCS; b, c), and the

1068 northern California current system (NCCS; d, e).

1069

1070 Figure 2: Sea surface temperature (°C; 11 μm day) and chlorophyll a concentration (mg m^{-3})

1071 MODIS-Aqua (4 km) in each area. Contour maps produced with the Giovanni online data

1072 system (developed and maintained by the NASA GES DISC), during sampling periods in

1073 South Georgia (SG; a), the Humboldt current system (HCS; b, c), and the northern California

1074 current system (NCCS; d, e). Euphausiid sampling stations are marked with a black cross (+).

1075

1076 Figure 3: Oxygen consumption (μmol O$_2$ h^{-1} g^{-1} DM) associated to chamber oxygen

1077 concentration (% air saturation) and chamber oxygen partial pressure (pO$_2$; kPa) of $Euphausia$

1078 $superba$ (South Georgia; a), $Euphausia$ $mucronata$ (HCS; b), and $Euphausia$ $pacifica$ (NCCS,

1079 c: warm:, d: cold); mean ± SE.

1080

1081 Figure 4: Basal metabolic and oxidative stress parameters in $E.$ $superba$ (Es), $E.$ $mucronata$

1082 (Em), and $E.$ $pacifica$ (Ep) in warm (dark grey) and cold seasons (white). (a) CS in U g^{-1} WM,

1083 (b) SOD in U mg^{-1} protein, (c) CAT in U mg^{-1} protein, (d) GST in U mg^{-1} protein, (e)

1084 oxidized/reduced glutathione (GSSG: GSH), (f) glutathione equivalents (GSH-eq) in nmol g^{-1}

1085 WM, (g) malondialdehyde (MDA) in nmol g^{-1} WM, (h) carbonyl concentrations in nmol mg^{-1}

1086 protein. AB: interspecific differences in warm season among all species; "+" and "*":

1087 interspecific differences in warm and cold seasons among temperate species, respectively; ab:

1088 intra-specific seasonal differences; (n): number of samples analyzed; Dash lines separate the

1089   species; ND: no data; Horizontal bars in the box plots indicate median. Upper and lower

1090   edges of the rectangle show the 1st and 3rd quartiles, respectively. Vertical error bars extend

1091   to the lowest and highest value in a 1.5-fold inter-quartile range (R Core Team 2012).

Fig. 1

(a) SG – Jan12 (warm)
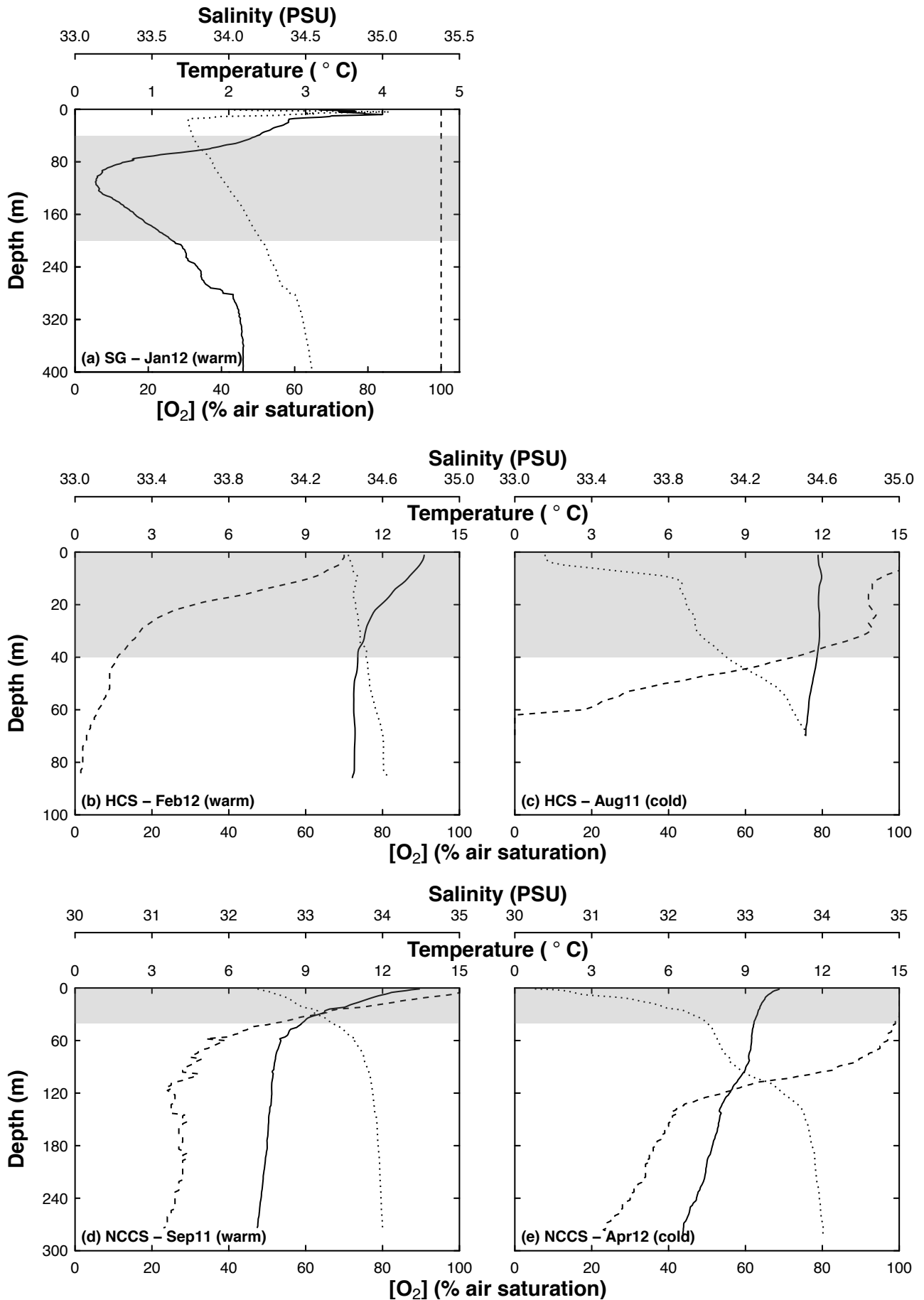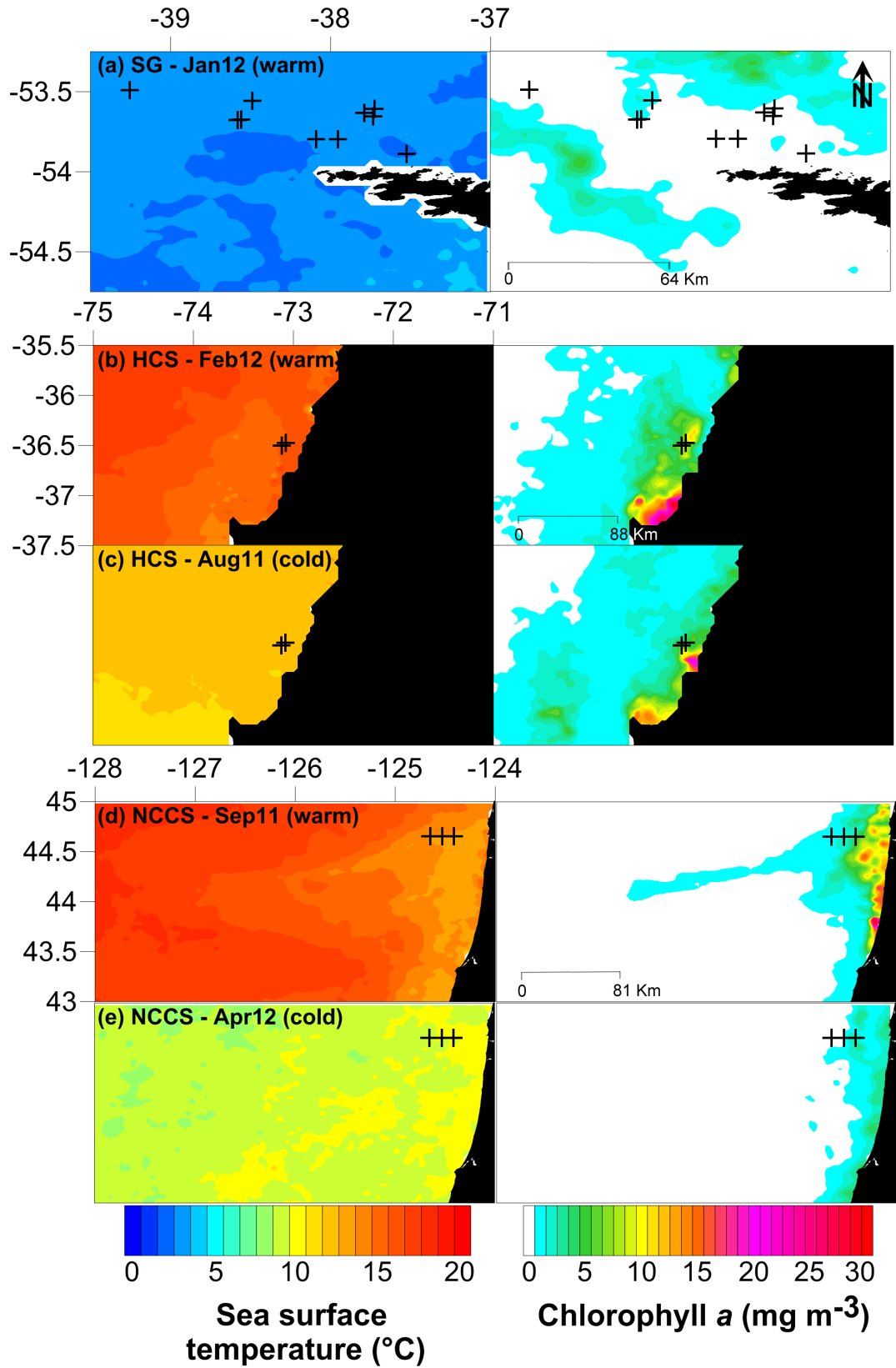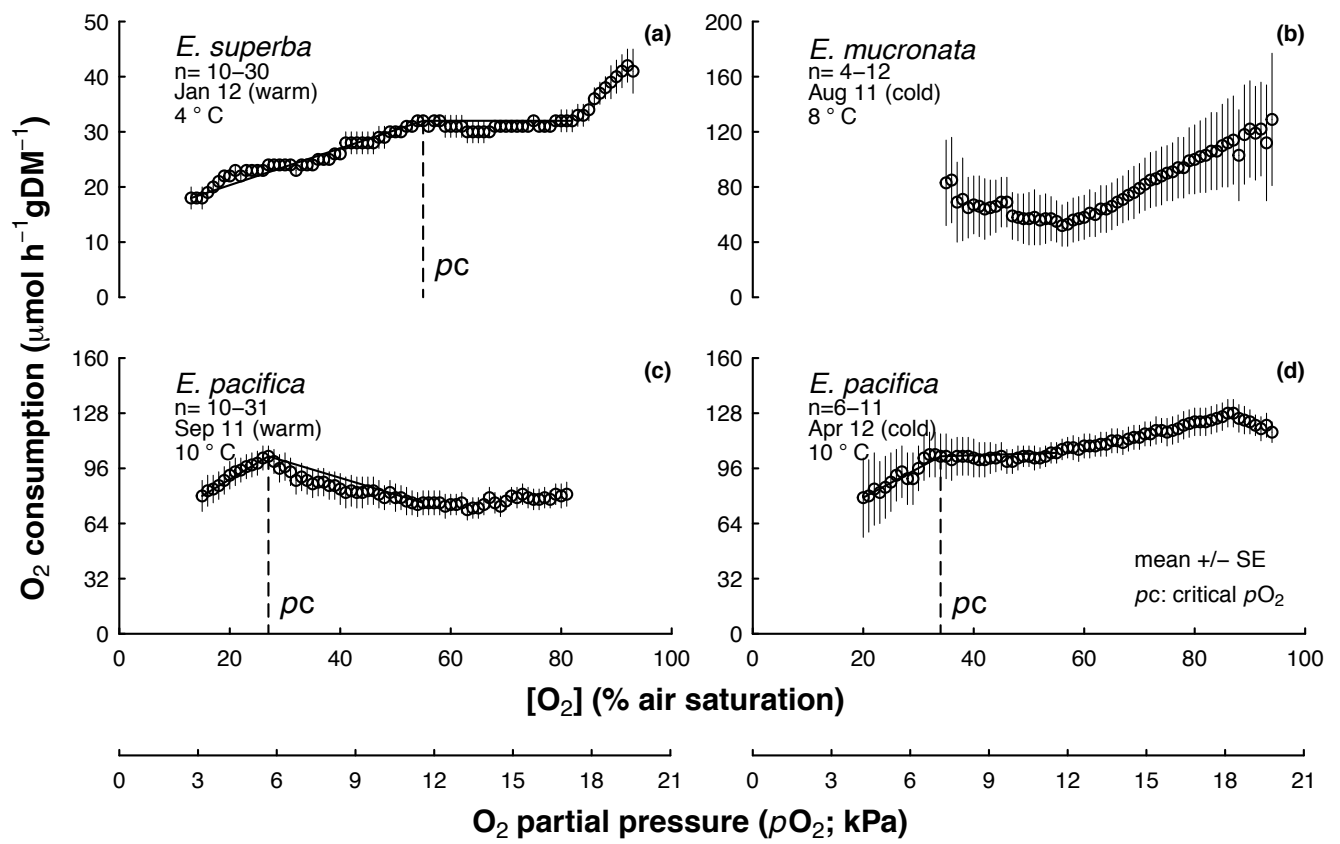
(b) HCS – Feb12 (warm)

(c) HCS – Aug11 (cold)

(d) NCCS – Sep11 (warm)

(e) NCCS – Apr12 (cold)

Fig. 2

Fig. 3

Fig. 4