Moderate reductions in dissolved oxygen may compromise performance in an ecologically-important estuarine invertebrate

Michael Collins*[1], Oliver Tills[1], Lucy M Turner[1], Melody S. Clark[2], John I. Spicer[1], Manuela Truebano[1]

1 Marine Biology and Ecology Research Centre, School of Biological and Marine Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, UK

2 British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge CB3 OET, UK

*Corresponding author: michael.collins@plymouth.ac.uk

ABSTRACT

Coastal ecosystems, including estuaries, are increasingly pressured by expanding hypoxic regions as a result of human activities such as increased release of nutrients and global warming. Hypoxia is often defined as oxygen concentrations below 2 mL O_2 L^{-1}. However, taxa vary markedly in their sensitivity to hypoxia and can be affected by a broad spectrum of low oxygen levels. To better understand how reduced oxygen availability impacts physiological and molecular processes in invertebrates, we investigated responses of an estuarine amphipod to an ecologically-relevant level of moderate hypoxia (~ 2.6 mL O_2 L^{-1}) or severe hypoxia (~ 1.3 mL O_2 L^{-1}). Moderate hypoxia elicited a reduction in aerobic scope, and widespread changes to gene expression, including upregulation of metabolic genes and stress proteins. Under severe hypoxia, a marked hyperventilatory response associated with maintenance of aerobic performance was accompanied by a muted transcriptional response. This included a return of metabolic genes to baseline levels of expression and downregulation of transcripts involved in protein synthesis, most of which indicate recourse to hypometabolism and/or physiological impairment. We conclude that adverse ecological effects may occur under moderate hypoxia through compromised individual performance and, therefore, even modest declines in future oxygen levels may pose a significant challenge to coastal ecosystems.

Keywords

hypoxia, estuary, integrative, ecophysiology, Crustacea

1 Introduction

Shallow coastal ecosystems, including estuaries, are pressured by increasing severity and duration of hypoxia driven by increased nutrient pollution and climate change (Breitburg et al., 2018). Hypoxia was originally defined by ecologists as a threshold oxygen concentration of < 2 mL O_2 L^{-1} based upon avoidance behaviour and mass mortality of benthic organisms (Diaz and Rosenberg, 2008). However, the use of a singular 'limit' to define hypoxia has been the subject of considerable discussion given that taxa vary markedly in their sensitivity to reduced oxygen (Galic et al., 2019; Vaquer-Sunyer and Duarte, 2008). The incorporation of physiological evaluations in hypoxia studies has identified dissolved oxygen thresholds detrimental to a range of taxonomic groups with fish and crustaceans thought to be most sensitive (Galic et al., 2019; Vaquer-Sunyer and Duarte, 2008). As a result, more conservative thresholds for dissolved oxygen have been proposed to support fisheries and the conservation of coastal biodiversity (Steckbauer et al., 2011).

While it is now widely recognised that biota can be affected by a broad spectrum of dissolved oxygen levels (Galic et al., 2019; Vaquer-Sunyer and Duarte, 2008), the underpinning integrated mechanisms are largely unknown, particularly for invertebrate species (Spicer, 2014). In-depth analyses of these mechanisms will aid prediction of how individuals, species, communities and ecosystem function will be affected by the chronically reduced oxygen levels predicted to occur under climate change (Breitburg et al., 2018; Galic et al., 2019; Spicer, 2014). Integrative analyses have largely been restricted to understanding mechanisms elicited by severe hypoxia (Boutilier and St-Pierre, 2000) which may be associated with mass mortality in nature (Diaz and Rosenberg, 1995). Mortality is thought to be driven by disruption

of aerobic metabolism at a critical oxygen tension (P_c), which compromises essential cellular energy stores (ATP) resulting in time-limited survival, dependent on the ability of organisms to suppress metabolic ATP demand (Boutilier and St-Pierre, 2000). Molecular evidence from fish and a small number of crustacean species appears to support this paradigm but requires assessment for a wider variety of species (Rathburn et al., 2013; Richards, 2009). In fish and crustaceans, metabolic suppression may be achieved through reduced activity and a reduction of ATP-demanding cellular processes such as protein synthesis (Gracey et al., 2001; Seibel et al., 2018). This may be accompanied by up-regulation of a suite of genes, despite being energetically-compromised, to enhance mitochondrial activity and oxygen carriage by respiratory pigments, increase anaerobic (glycolytic) ATP production and prevent cellular damage (Larade and Storey, 2009; Nikinmaa and Rees, 2005; Richards, 2009).

While the effects of severe hypoxia are relatively well characterised, our understanding of responses to moderate hypoxia is more disjointed, despite it being prevalent in nature with consequences for estuarine assemblage composition (Farrell and Richards, 2009; Froehlich et al., 2015; Spicer, 2016). Under moderate hypoxia, fish and invertebrates can experience altered activity, ecological interactions and fitness traits such as growth and reproduction (Galic et al., 2019; Vaquer-Sunyer and Duarte, 2008). The mechanisms supporting function under moderate hypoxia have received some attention, albeit indirectly, as part of studies where acutely declining oxygen tensions are employed. Transitioning through moderate hypoxia does not typically disrupt resting aerobic metabolism, which is maintained by alterations to ventilation and circulation (Grieshaber et al., 1994). In

fish, the increased challenge of sustaining resting rates of aerobic metabolism may impact aerobic scope (Farrell and Richards, 2009), which underpins many facets of fitness and ecological performance (Pörtner, 2010). However, changes to aerobic scope under hypoxia are not well characterised for most ecologically-important coastal invertebrates. In the longer term, resting rates of aerobic metabolism may continue to be sustained through enhanced rates of ventilation or gill plasticity (McMahon et al., 1974; Sollid et al., 2003). However, at lower levels of organisation, arguably, the only response which has been well characterised is adjustments to oxygen carriage by respiratory pigments (Pan et al., 2017). The limited evidence available at the molecular level for aquatic invertebrates points to longer term moderate hypoxia eliciting a minimal response in terms of global gene expression (Brouwer et al., 2007).

Given the increasing prevalence of hypoxia in estuarine ecosystems and the predicted increase in both its intensity and duration (Breitburg et al., 2018), this multidisciplinary study investigated the physiological and molecular mechanisms elicited by more 'moderate' hypoxia compared to those elicited by severe hypoxia. The brackishwater amphipod, *Gammarus chevreuxi* was used as a model as it is an ecologically-important decomposer in brackishwater habitats (Lincoln, 1979) and its transcriptome has recently been sequenced (Collins et al., 2017; Truebano et al., 2013). Its life history and physiological responses to environmental stress have also received attention (Girisch et al., 1974; Lowenstein, 1934; Subida et al., 2005) including hypoxia, where the P_c for the species lies at ~ 12% air saturation (% a.s.) (~ 2.4 kPa) but long term fitness effects have been documented at 40% a.s (~ 8 kPa) (Truebano et al., 2018). A number of key physiological, biochemical and

transcriptomic responses to hypoxia were investigated after 7 d exposure to moderate (40 % a.s., ~ 2.6 mL O_2 L^{-1}, ~ 8 kPa) and severe hypoxia (20 % a.s., ~ 1.3 mL O_2 L^{-1}, ~ 4 kPa). Organismal responses were characterised by measurement of resting and active rates of oxygen uptake (a proxy for metabolism) and calculation of aerobic scope. Oxygen uptake and transport systems (ventilation and circulation), and biochemical indicators of anaerobic metabolism (end-product L-lactate) were investigated alongside transcriptome profiling, *via* RNA-Seq. This discovery-led NGS (next-generation sequencing) approach provides the first insight into the molecular response to hypoxia for this species and pinpoints which mechanisms are regulated by moderate and severe hypoxia, and may contribute to altered performance.

2 Methods

2.1 Sampling site and pre-exposure conditions in the laboratory

Gammarus chevreuxi were collected using a hand-held net from the Plym estuary, Devon (−50 ° 39 ′ 03 ″ N, 4 ° 08 ′ 56 ″ W). The site is subject to tidal influence experiencing variable salinities (S = 0 – 30) on a daily basis (Houston, 2013). Spot measurements of dissolved oxygen were made on one day at low tide using a hand-held dissolved oxygen probe (ProDO 2030, YSI Inc., Ohio, USA). The site experiences considerable variation in oxygen tensions including normoxia within the main river channel (102 – 106 % a.s.) (Fig. 1A) to hyperoxia (up to 134 % a.s) in areas of high algal density (Fig. 1B). Different intensities of hypoxia are present in small pools isolated from the river channel at low tide (18 – 35 % a.s.) (Fig. 1C) and regions of the main channel of low flow (13 – 55 % a.s.) (Fig.1D). Within an hour of collection, amphipods were returned to the laboratory and kept in stock aquaria (Vol. = 10 L), where they were acclimated to controlled conditions (T = 15 °C, S = 15, 12

h:12 h L:D regime) for at least one week before use in any experiment. During this time, they were fed carrot *ad libitium*. Full water changes were performed weekly. Only adult males (wet mass = 7.79 ± 1.67 mg) were used in the experiments described below.

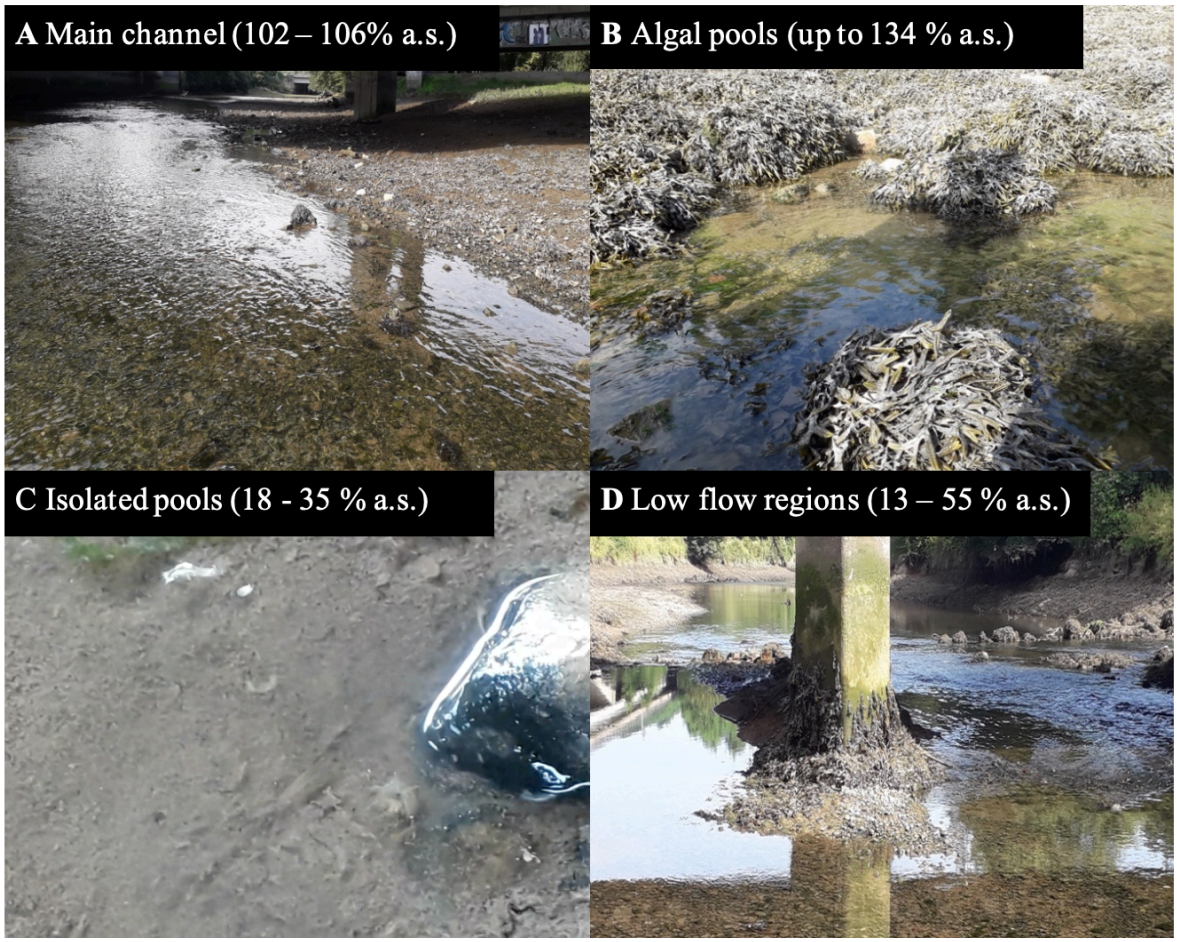


Fig. 1. *G. chevreuxi* inhabiting the River Plym experience considerable variation in oxygen tensions such as in (A) the main channel (normoxic), (B) algal pools (hyperoxic), (C) shallow pools isolated from the main channel at low tide (moderately to severely hypoxic) and (D) regions of low flow (moderately to severely hypoxic). Images illustrate the range of environments in which amphipods are found, and the variation in dissolved oxygen that characterise them.

164

165 2.2 Exposure to different intensities of hypoxia

166 Exposure of amphipods to different intensities of hypoxia was achieved using a
167 mesocosm system consisting of 24 sealed aquaria (Vol. = 1.4 L, eight aquaria per
168 treatment, eight individuals in each) maintained in a temperature-controlled facility (T
169 = 15 °C). After the pre-exposure period, individuals were exposed to one of three
170 oxygen regimes: normoxia (100 % a.s.: 90.6 ± 0.2 % a.s), moderate hypoxia (40 %
171 a.s.: 39.1 ± 0.7 % a.s) consistent with seasonal hypoxia in local estuaries (Morris et
172 al., 1982; Uncles et al., 2002), or severe hypoxia (20 % a.s.: 22.9 ± 0.9 % a.s). Other
173 environmental factors were kept constant (T = 14.2 ± 0.1 °C, S = 14.7 ± 0.1, 12 h L:
174 12 h D).

175

176 Different intensities of hypoxia were produced by aspirating a gas mixture,
177 constructed from nitrogen and “carbon dioxide-scrubbed” air (air previously aspirated
178 through 1 M NaOH solution) directly into the water through an airline, with the flow
179 controlled using adjustable flow valves (100 % a.s.: 5 L min^{-1} air; 40 % a.s.: 0.6 L
180 min^{-1} N_2 gas to 0.4 L min^{-1} air; 20 % a.s.: 1.2 L min^{-1} N_2 gas to 0.4 L min^{-1} air)
181 (FR2000 Flowmeter, Key Instruments, Pennsylvania, USA). Temperature and
182 oxygen tension in aquaria waters were recorded daily using an oxygen microsensor
183 (Pm-Pst7, Presens, Regensburg, Germany) and temperature probe (Pst 100,
184 Presens, Regensburg, Germany) coupled to a dissolved oxygen meter (Microx 4,
185 Presens, Regensburg, Germany). Salinity was measured every 1 - 2 d using a
186 refractometer (HI96822 Digital Refractometer, Hanna Instruments Ltd., Leighton
187 Buzzard, UK). Amphipods were fed carrot *ad libitium* during the experiment and
188 water was fully changed every 3 - 4 d to ensure good water quality. All amphipods

were kept under these conditions for 7 d, which is a sufficient time period to allow acclimation of individuals (Truebano et al., 2018), before their responses to hypoxia were characterised as outlined below.

2.3 Physiological responses to different intensities of hypoxia

Individuals were starved *in situ* for 12 h prior to any measurements of oxygen uptake taking place. To measure rates of oxygen uptake individuals were carefully placed in plastic mesh envelopes (mesh size = 1 mm) which mimicked the tight spaces between rocks where these animals are found *in situ* and to try to minimise activity. Each envelope was then transferred to a holding aquarium (vol. = 5 L), containing sea water at the appropriate oxygen tension and allowed to settle for 30 min. Keeping them submerged, individuals were carefully transferred to a 5 mL glass chamber containing filtered (25 µm), autoclaved, diluted sea water (S = 15). The initial oxygen tension (% a.s.) within the chamber was recorded using a needle-type oxygen micro-sensor (NTH-PSt7, Presens, Regensburg, Germany) connected to an oxygen meter (Microx 4, Presens, Regensburg, Germany). The chamber was then sealed, gently transferred to a water bath (T = 15 °C) and the individuals were kept for 2 h to consume ~10 % a.s. (100 % a.s.: ~ 96 - 81 % a.s., moderate hypoxia: ~ 39 – 27 % a.s., severe hypoxia: ~ 22 – 10 % a.s.), after which period chambers were mixed by inversion and the oxygen tensions within the chamber were measured again as described. The rate of oxygen uptake under resting conditions was calculated from the difference between oxygen tension in the water at the beginning and at the end of the experiment. Data are expressed as μL O_2 mg wet mass^{-1} h^{-1} STP.

214 To estimate the rate of oxygen uptake under active conditions, individuals were
215 chased for 1 min with a plastic pipette before being returned to their mesh envelope
216 and re-inserted into their respirometry chamber. The chamber was immediately
217 resealed and the individuals were left for 1 h. The oxygen tension within the chamber
218 was then remeasured as previously described and the aerobic scope was calculated
219 by subtracting resting metabolic rate from active metabolic rate. This end-point
220 metabolic rate assay was utilised in order to minimise disturbance to the amphipods
221 within the respirometry chamber. Active metabolic rate following chasing of the
222 amphipod did not return to resting conditions during the respirometry period, a notion
223 supported by higher ventilation rates observed at the end of the metabolic rate
224 measurements (Fig. 2).
225
226 Upon removal from the respirometers individuals were gently blotted dry and their
227 wet mass determined using a microbalance (MSA225P-000-DA, Göttingen Sartorius
228 AG, Germany, ± 0.01 mg). After weighing, these active individuals were quickly
229 frozen in liquid N_2 and stored separately at T = - 80 °C for subsequent determination
230 of whole body L-lactate concentration.
231
232 To measure the effect of different oxygen regimes on ventilation and perfusion, in
233 resting and active animals, individuals were observed visually during their time in the
234 respirometers. The resting and active pleopod beat frequency and heart rate were
235 observed and quantified in the respirometers (measured twice for 15 s for each
236 individual) under low power magnification (x 10) using a light microscope (MZ15,
237 Leica Microsystems Ltd, Cambridge, UK). Ventilation, *via* the beating of pleopods, is
238 a key mechanism of oxyregulation under hypoxia in gammarid amphipods (Sutcliffe,

1984). Therefore, we also characterised scope for ventilation by subtracting resting pleopod rate from active pleopod rate, due to its importance as a potential mechanism in changing aerobic scope.

2.4 Biochemical responses

Frozen individuals (wet mass = 7.72 ± 1.76 mg) were sonicated (60 % amplification for 60 s) in 50 µL of 10 % TCA (Fisher Scientific Ltd., Loughborough, UK). The concentration of L-lactate was quantified using a commercially-available lactate assay kit (Lactate Kit 735-10, Trinity Biotech, Bray, Ireland, limit of detection = 2 mg/dL). Lactate reagent (100 µL) was added to a 10 µL subsample of sonicated supernatant and incubated at room temperature for 10 min. Absorbance (λ = 540 nm) of this mixture was measured using a microplate reader (Versamax Microplate Reader, Molecular Devices LLC, California, USA) and calibrated against standards (Lactate Standard Solution 826-10, Trinity Biotech, Bray, Ireland).

2.5 Statistical analyses of physiological and biochemical data

All statistical analyses were performed in R v. 3.3.1. For physiological responses, data showed equal variance when tested using Levene's Test ($P > 0.05$). Nine one-way ANOVA were utilised to test for the effect of oxygen regime (100, 40 and 20 % a.s.) on (1) resting metabolic rate, (2) resting pleopod rate, (3) resting heart rate, (4) active metabolic rate, (5) active pleopod rate, (6) active heart rate, (7) aerobic scope, (8) scope for ventilation and (9) L-lactate concentration of active individuals. Significant differences between treatments were identified using *post-hoc* Tukey tests. Statistical significance was assigned at $P < 0.05$. Data are expressed as means ± SEM.

264

265 2.6 Transcriptomic responses

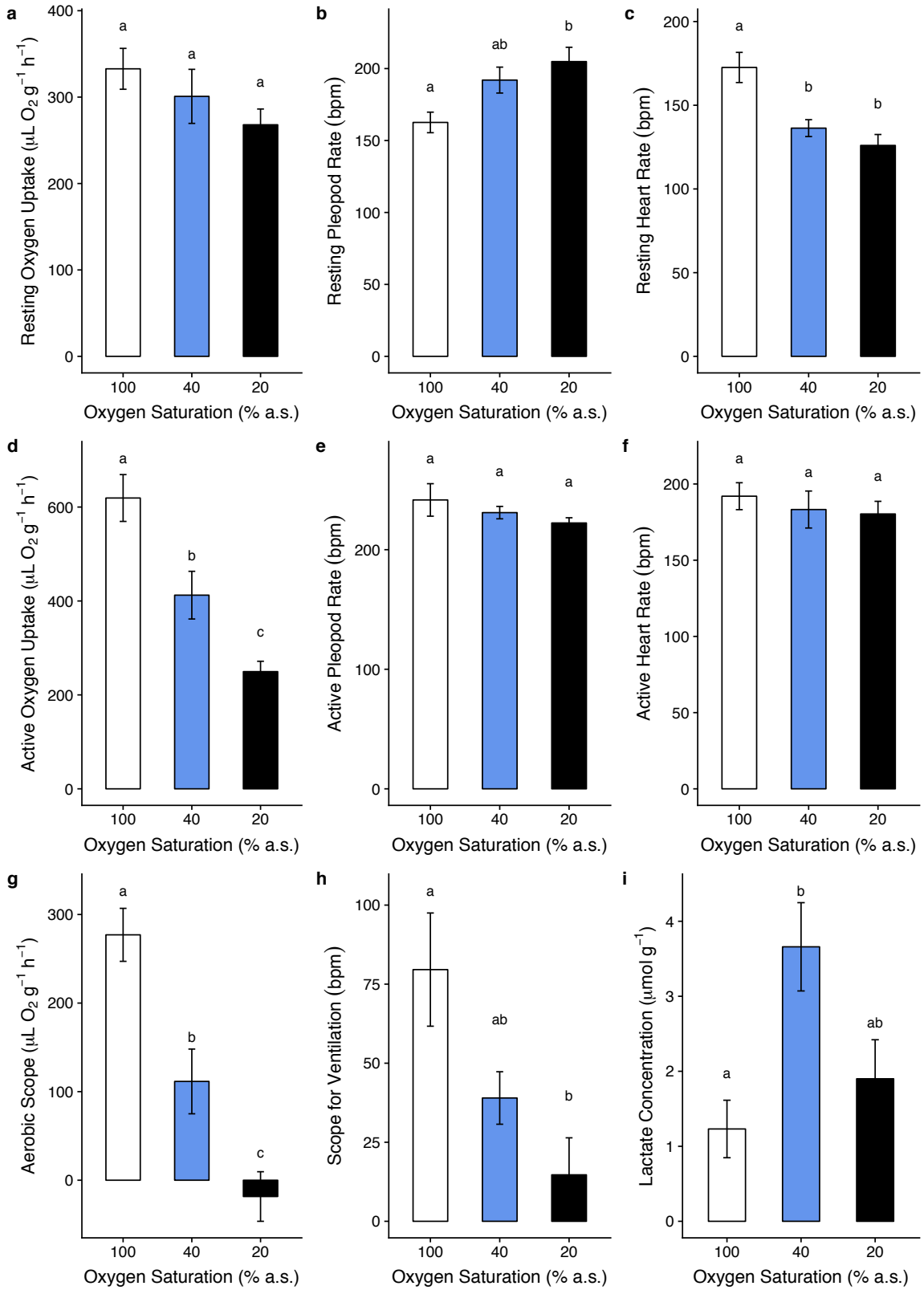
266 An RNA-Seq experiment to determine responses to different intensities of hypoxia
267 were performed according to Collins et al., (2017). Briefly, individuals exposed to
268 100, 40 or 20 % a.s. for 7 d were snap frozen in liquid N_2 and stored at T = - 80 °C
269 for subsequent transcriptomic analysis. Total RNA was extracted from three pools of
270 10 individuals (one amphipod from each aquarium and then two from random
271 aquaria) per treatment using the PureLink RNA Mini Kit (Ambion Inc., California,
272 USA) and used to construct TruSeq RNA libraries (Illumina, San Diego, USA).
273 Sequencing was performed on a single lane of an Illumina HiSeq 2000 using 100
274 base paired-end sequencing (HiSeq 2000, Illumina, San Diego, USA) at The
275 Genome Analysis Centre, Norwich, UK. Transcriptome assembly was performed
276 using Trinity v. 2.2.0 (Haas et al., 2013) using default parameters.

277

278 Differentially expressed genes (DEGs) between treatments were identified by
279 aligning the sequenced reads to the assembled transcriptome using Bowtie v. 1.1.1
280 (Langmead et al., 2009). Gene counts were then generated using RSEM v. 1.2.29
281 (Li and Dewey, 2011). Counts data were imported into R v. 3.3.1 using tximport v.
282 1.0.3 (Soneson et al., 2015). Differential gene expression analysis was performed
283 using DESeq2 v. 1.12.4 (Love et al., 2014) to identify significantly differentially
284 expressed genes ($P_{adj} < 0.05$) in pairwise comparisons of 40 % a.s. and 20 % a.s.
285 against the normoxic control (100 % a.s.). Gene ontology (GO) enrichment analysis
286 of DEGs ($P_{adj} < 0.01$, and $\log_2$ fold change < -1 or > 1) was performed using TopGO
287 v. 2.24.0 (Alexa and Rahnenfuhrer, 2016) and KEGG enrichment analysis using
288 clusterProfiler v. 3.0.5 (Yu et al., 2012) to identify biological pathways regulated

289 under exposure to 40 % a.s. and 20 % a.s. compared with the control. Differentially
290 expressed genes (P_{adj} < 0.05) putatively associated with physiological responses to
291 different severities of hypoxia were further explored. This included genes encoding
292 for oxygen transporters (hemocyanin) previously identified in Truebano et al., (2018),
293 aerobic metabolic enzymes (tricarboxylic acid (TCA) cycle enzymes and
294 mitochondrial electron transport chain (ETC) complexes), anaerobic metabolic
295 enzymes (glycolytic enzymes), and cellular defences (antioxidant enzymes and heat
296 shock proteins (HSPs)).
297
298 3 Results
299 3.1 Physiological and biochemical responses to different severities of low oxygen
300 For resting individuals, there was no significant effect of exposure to either moderate
301 (40 % a.s) or severe (20 % a.s.) hypoxia on mean mass specific oxygen uptake
302 compared to normoxia (Fig. 2a, ANOVA $F_{2,19}$ = 1.51, P = 0.246). Ventilation rate only
303 increased during exposure to severe hypoxia (Fig. 2b, ANOVA $F_{2,19}$ = 5.79, P =
304 0.011) but heart rate decreased significantly upon exposure to both hypoxia
305 treatments for 7 d (Fig. 2c, ANOVA $F_{2,16}$ = 11.60, P < 0.001). For active individuals,
306 mass-specific rate of oxygen uptake was significantly lower in individuals exposed to
307 both moderate (Tukey P = 0.013) and severe hypoxia (Tukey P < 0.001) compared
308 to those under normoxic conditions (Fig. 2d, ANOVA $F_{2,17}$ = 15.82, P < 0.001). For
309 active individuals, there was no effect of hypoxia exposure on either ventilation rate
310 (Fig. 2e, ANOVA $F_{2,16}$ = 1.40, P = 0.275) or heart rate (Fig. 2f, ANOVA $F_{2,16}$ = 0.04, P
311 = 0.966). Significant reductions in aerobic scope (Fig. 2g, ANOVA $F_{2,17}$ = 17.25, P <
312 0.001) occurred under both moderate (Tukey P = 0.009) and severe (Tukey P <
313 0.001) hypoxia. The slight negative value for aerobic scope observed under 20 %

314 a.s. may reflect zero aerobic scope as it did not differ significantly from zero (One
315 sample T-test, T_6 = -0.65, P = 0.268). Declining aerobic scope may be associated
316 with a significant decline in the ability to increase ventilation above resting rates
317 under hypoxia, measured as scope for ventilation (Fig. 2h, ANOVA $F_{2,16}$ = 6.46, P =
318 0.009). Declining aerobic scope was also associated with an increase in L-lactate
319 concentration (Fig. 2i, ANOVA $F_{2,13}$ = 5.28, P = 0.021) in individuals exposed to
320 moderate (Tukey P = 0.026), but not severe hypoxia which displayed a response
321 intermediate of 100 % a.s. (Tukey P = 0.726) and 40 % a.s. (Tukey P = 0.09).



322

323 Fig. 2. The physiological effects of 7 d exposure to normoxia (100 % a.s.), moderate

324 hypoxia (40 % a.s.) or severe hypoxia (20 % a.s.). (a) resting oxygen uptake (100 %:

325 n = 7, 40 %: n = 8, 20 %: n = 7) (b) resting pleopod rate (100 %: n = 7, 40 %: n = 8,
326 20 %: n = 7) (c) resting heart rate (100 %: n = 6, 40 %: n = 8, 20 %: n = 6) (d) active
327 oxygen uptake (100 %: n = 5, 40 %: n = 8, 20 %: n = 7) (e) active pleopod rate (100
328 %: n = 5, 40 %: n = 8, 20 %: n = 6) (f) active heart rate (100 %: n = 5, 40 %: n = 8,
329 20 %: n = 6) (g) aerobic scope (100 %: n = 5, 40 %: n = 8, 20 %: n = 7) (h) scope for
330 ventilation (100 %: n = 5, 40 %: n = 8, 20 %: n = 6) (i) L-lactate concentration of
331 active individuals (100 %: n = 4, 40 %: n = 7, 20 %: n = 5) (mean values ± s.e.m).
332 Letters indicate significant differences between treatments identified by one-way
333 ANOVA and *post-hoc* Tukey test (P < 0.05). For supporting data see Table S1.
334
335 3.2 Transcriptomic features subject to regulation by moderate and severe hypoxia
336 Principal Component Analysis (PCA) of all genes revealed that samples were
337 predominately separated along the first principal component (PC1), which accounted
338 for 81 % of the variance. Along PC1, amphipods exposed to normoxia and moderate
339 hypoxia differed the most based on their global expression profiles; whereas there
340 was little separation between normoxia and severe hypoxia exposed amphipods
341 along this axis (Fig. 3a). Differential expression analysis identified a total of 11,686
342 unique significantly differentially expressed transcripts (P_{adj} < 0.05) between
343 amphipods exposed to 40 % and 100 %, of which approximately 67 % were up-
344 regulated. In comparison, a more limited transcriptional response was observed in
345 animals exposed to 20 % a.s. compared to the normoxic controls, with 1,721
346 significantly differentially expressed unique genes, 52 % of which were up-regulated.
347 An additional 1,466 significantly differentially expressed genes overlapped between
348 40 % and 20 % a.s. giving an overall total of 13,152 significantly differentially

expressed transcripts between 40 % and 100 % a.s. and 3,187 between 20 % and 100 % a.s. (Fig. 3b).

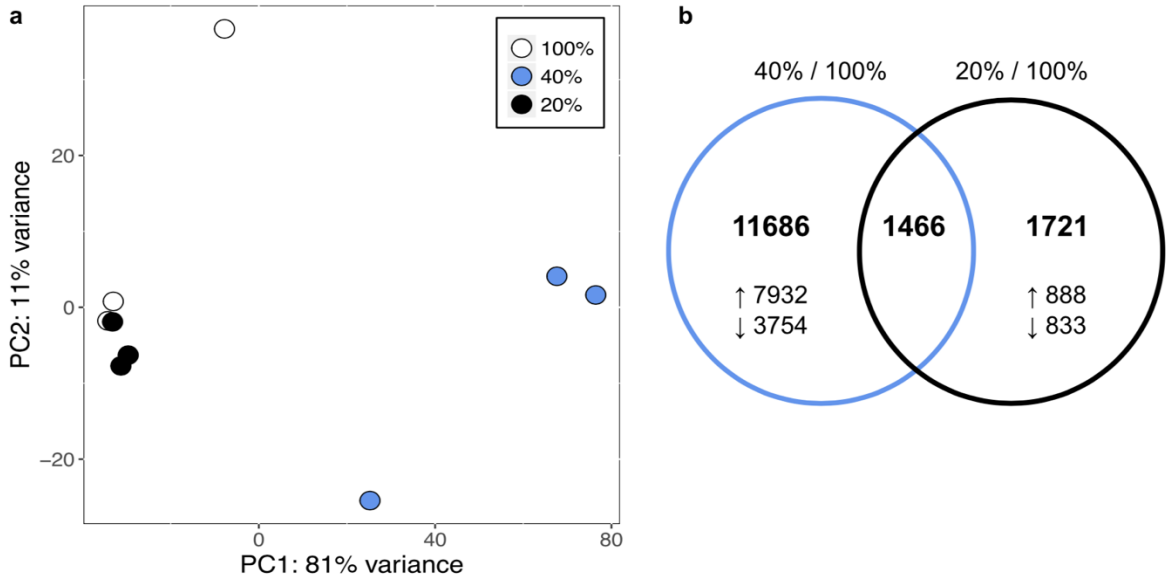


Fig. 3. Transcriptomic responses to moderate (40 % a.s.) and severe hypoxia (20 % a.s.). (a) Principal components 1 and 2 from principal component analysis performed using variance stabilised counts of all tested genes (n = 198,862) across all tested samples (n = 3 pools per treatment) (b) number of DEGs (P_{adj} < 0.05) in comparison to control for 40 % a.s. and 20 % a.s. Upward and downward arrows indicate up and down-regulation respectively in each treatment compared to the normoxic control.

Functional enrichment analysis of significantly up-regulated genes following exposure to moderate hypoxia (40 % a.s.) compared to normoxia identified 23 significantly affected KEGG pathways (P_{adj} < 0.05) (Fig. S1). These were predominantly linked to protein synthesis and cellular repair/defence. GO term analysis revealed significant enrichment of processes involved in protein synthesis

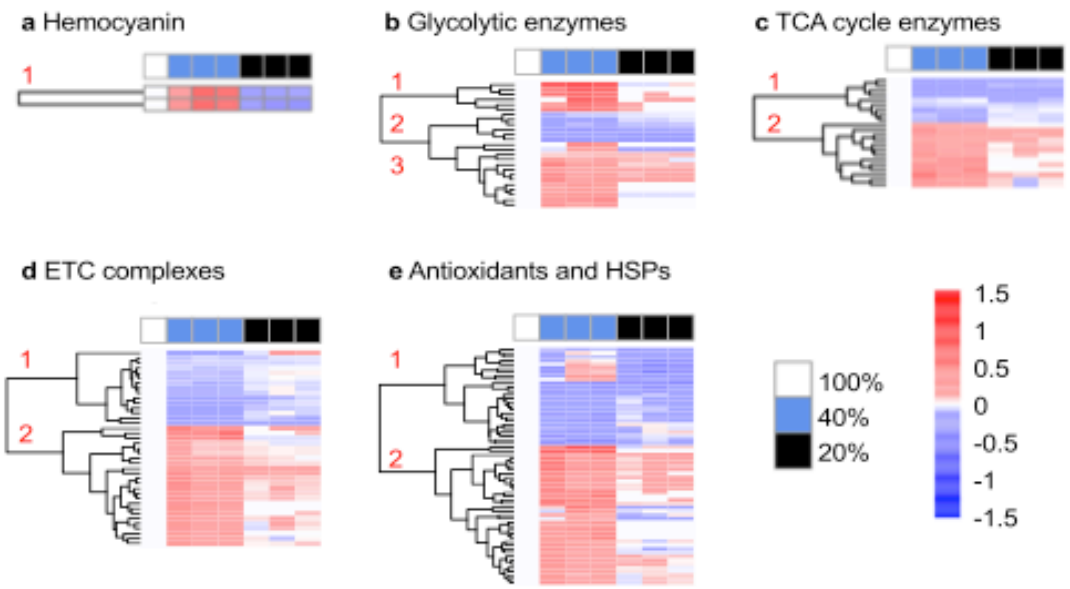
and oxygen carriage by respiratory pigments, amongst others (Fig. S2). Down-regulated genes under moderate hypoxia compared to normoxia were significantly enriched for GO terms involved in muscle structure (Fig. S2).

In response to severe hypoxia, up-regulated DEGs were significantly enriched for multiple GO terms involved in chitin metabolism and cuticle structure (cuticle proteins/resilins) (Fig. S3). Coagulation was the only KEGG pathway significantly enriched for upregulated DEGs under severe hypoxia. Down-regulated DEGs under severe hypoxia were also significantly enriched for chitin metabolism. Thus, there was mixed regulation of chitin metabolic pathways consisting primarily of chitin catabolic pathways (Fig. S3). Also, protein degradation and glucose metabolism GO terms were significantly enriched (Fig. S3). Ribosomal pathways were the only significantly affected KEGG pathway for down-regulated genes under 20 % a.s (P_{adj} < 0.05).

3.3 Transcripts putatively associated with the physiological responses to moderate and severe hypoxia

Hemocyanin (Fig. 4a) and metabolic enzyme genes including multiple glycolytic enzymes (Fig. 4b), TCA cycle enzymes (Fig. 4c), and mitochondrial subunits (Fig. 4d) exhibited increased levels of expression under 40 % a.s. compared to normoxia. Two hemocyanin transcripts corresponding to two different hemocyanin subunits were putatively identified, both of which were up-regulated under moderate hypoxia. Multiple glycolytic enzyme contigs (e.g. phosphofructokinase (*PFK*), fructose bisphosphate aldolase (*FBP*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)) were significantly up-regulated which may be associated with the

391 significant higher L-lactate concentration found in active individuals. Several TCA
392 cycle enzymes including five transcripts annotated as isocitrate dehydrogenase
393 (*IDH*) and mitochondrial ETC complexes were up-regulated, such as the 11
394 transcripts annotated as ATP synthase subunits (*ATPα* and *ATPß*) and two
395 cytochrome c oxidase 1 (*COX1*) contigs. Putative antioxidant enzymes were mostly
396 up-regulated under 40 % a.s. including two contigs annotated as catalase and seven
397 contigs annotated as superoxide dismutase isoforms (Fig. 4e). Under severe
398 hypoxia, a significant reduction in the expression of one hemocyanin contig occurred
399 (Fig. 4a). Glycolytic genes largely returned to baseline levels of expression in
400 individuals exposed to severe hypoxia (*PFK, GAPDH*) or were down-regulated (*FBP*)
401 (Fig. 4b) and may be associated with the less pronounced accumulation of L-lactate
402 under 20% a.s. compared to moderate hypoxia. TCA cycle (*IDH*), and mitochondrial
403 ETC complexes (*ATPα* and ATP*ß*) also returned to baseline levels of expression in
404 amphipods exposed to 20 % a.s. (Fig. 4c-d). Cellular antioxidants also mostly
405 returned to a baseline level of expression but six glutathione-S-transferases were
406 significantly down-regulated in amphipods exposed to severe hypoxia (Fig. 4e).
407 Within different heat shock protein families (*HSP70, HSP90*), contigs which may
408 represent different isoforms showed different patterns of regulation under both
409 moderate and severe hypoxia (Fig. 4e).
410



411

412 Fig. 4. Heat map of $\log_2$ fold changes of DEGs ($P_{adj} < 0.05$) for moderate and severe
413 hypoxia (n = 3 pools per treatment) in comparison to the mean of the normoxic
414 control (100 % a.s.). Counts were subjected to variance stabilising transformation
415 using the DESeq2 library prior to calculation of $\log_2$ fold changes. DEGs belonging to
416 selected functional categories thought to underlie the responses to hypoxia are
417 shown including (a) Hemocyanin (b) Glycolytic enzymes (c) TCA cycle enzymes, (d)
418 ETC complexes and (e) Antioxidants and HSPs. Different clusters are indicated by
419 numbers on the dendrogram. The full list of contigs contained within (a-e) and cluster
420 information is presented in Table S2.

421

422 4 Discussion

423 We investigated the physiological and molecular responses of the estuarine
424 invertebrate *Gammarus chevreuxi* to moderate and severe hypoxia. Previous studies
425 have highlighted a range of reduced oxygen levels can impact aquatic invertebrates
426 at the organismal level (Galic et al., 2019) and therefore taking cognizance of the
427 type of hypoxia experienced *in situ* is required to accurately predict responses
428 (Spicer, 2014). We have demonstrated that aquatic invertebrates rely on markedly

429 different strategies upon encountering different intensities of hypoxia. The integrated
430 mechanisms utilised to deal with less extreme, and often ecologically-relevant, levels
431 of moderate hypoxia may have been overlooked across species from a range of
432 coastal environments (Spicer, 2016). For *G. chevreuxi* exposed to moderate
433 hypoxia, there was a widespread transcriptional response and a significant reduction
434 in aerobic scope. Under severe hypoxia, however, individuals appeared to adopt a
435 hypometabolic strategy, characterised by limited recourse to anaerobic metabolism
436 and a significant downregulation of genes involved in protein synthesis. Given these
437 differences in the mechanisms affected, hypoxic intensity must be carefully
438 considered when assessing the ecological effects of low oxygen.
439
440 4.1 Moderate hypoxia has significant implications for estuarine animals
441 The ability to sustain the metabolic demand for oxygen from the environment is
442 thought to be important in determining species ecological distributions and habitat
443 use (Deutsch et al., 2015). Moderate hypoxia did not disrupt the ability to regulate
444 resting metabolism without recourse to a significant hyperventilatory response and
445 despite a significant bradycardia as previously observed by Truebano et al., (2018).
446 However, the ability to remain metabolically viable under moderate hypoxia may
447 come at a significant cost which was only revealed through the use of a discovery-
448 led NGS approach. The molecular response of *G. chevreuxi* to moderate hypoxia
449 was far more complex than previous studies on crustaceans seemed to suggest
450 (Brouwer et al., 2007) with significant changes in the expression of over 13,000
451 genes compared to normoxia. It is not always clear how transcriptomic responses of
452 marine invertebrates to hypoxia integrate with those observed at the protein level
453 (Spicer, 2014) due to potential modifications to translational efficiency under hypoxia

454 (Hardy et al., 2013). However, differences in gene expression profiles may reflect the
455 metabolic needs of different tissues and be reasonably accurate in its representation
456 of phenotypic changes (Whitehead and Crawford, 2005).
457
458 For *G. chevreuxi*, molecular changes which included up-regulation of genes
459 significantly enriched for transcription and translation pathways, may suggest that
460 amphipods have to actively expend energy to produce novel gene products and
461 rearrange cellular metabolism (Larade and Storey, 2009). The ability to regulate
462 whole-organism rates of resting metabolism under moderate hypoxia may be
463 associated with the up-regulation of multiple genes involved in aerobic metabolism
464 (TCA cycle enzymes and mitochondrial subunits). This may compensate for reduced
465 environmental oxygen availability and maintain aerobic ATP production in the
466 mitochondria (Brouwer et al., 2007) despite bradycardia and absence of a significant
467 hyperventilatory response. Furthermore, the up-regulation of two hemocyanin genes
468 may potentially enhance oxygen transport by the respiratory pigment (Johnson et al.,
469 2016; Truebano et al., 2018).
470
471 Despite an apparent attempt to meet energetic demands aerobically at the molecular
472 level, these amphipods may be compromised by even fairly moderate levels of
473 hypoxia. For *G. chevreuxi*, an up-regulation of glycolytic enzyme genes was
474 observed, including the enzyme *PFK* suggesting that amphipods may be primed for
475 a transition to less energetically-efficient anaerobic metabolism (Cota-Ruiz et al.,
476 2015), a notion that is supported by a significant accumulation of L-lactate when
477 individuals were forced to be active.
478

The accumulation of L-lactate in active individuals may be associated with a significant decline in aerobic scope which theoretical models suggest may also be compromised as a result of oxidative stress (Sokolova, 2013). This conclusion is supported by the enhanced expression of several key antioxidant enzymes. Although antioxidant gene expression may not always correlate with antioxidant enzyme activity in hypoxia-exposed crustaceans as hypoxia may also affect mRNA stability (Trasviña-Arenas et al., 2013). However, an upregulation of antioxidant genes has been used to indicate enhanced levels of oxidative stress in several marine invertebrates exposed to prolonged hypoxia (Clark et al., 2013; Sussarellu et al., 2010). The reduction in aerobic scope and the increased levels of transcripts associated with cellular stress may provide an early warning of the longer-term fitness consequences (Pörtner, 2010; Sokolova, 2013) of moderate hypoxia on coastal invertebrates. For example, we have directly observed the reduced fitness of *G. chevreuxi* under moderate hypoxia where the F_1 generation of hypoxia-treated parents displayed reduced size at hatching and impaired hypoxic performance (Truebano et al., 2018).

4.2 Severe hypoxia elicits markedly different responses

Studies describing how aquatic animals respond to severe hypoxia at the physiological level predict limitation of resting aerobic metabolism and recourse to anaerobic or hypometabolism (Grieshaber et al., 1994). Under the tested level of severe hypoxia (20 % a.s., ~ 1.3 mL O_2 L^{-1}), *G. chevreuxi* maintained the ability to regulate aerobic metabolism under resting conditions. A bradycardic response was also observed but, in this instance, was accompanied by pronounced hyperventilation, which is thought to improve the extraction of oxygen from the

environment at the gills (Sutcliffe, 1984). In isolation, the strong ability to regulate resting metabolism could indicate that *G. chevreuxi* is fairly hypoxia tolerant and may be resilient to future increases in the intensity of hypoxia. However, unlike the situation in moderate hypoxia, regulation of metabolism under severe hypoxia did not appear to be supported by changes at the molecular level. A surprisingly limited transcriptomic response was observed under severe hypoxia. As gene expression was only measured at a singular time point, it is possible that changes to gene expression could have been induced earlier during exposure to severe hypoxia, which may have contributed to the reduced magnitude of response compared to moderate hypoxia. The temporal dynamics of global gene expression under different intensities of hypoxia remains understudied. For crustaceans, the time course of global gene expression under different severities of hypoxia has only been investigated for a singular species (Brouwer et al., 2007). In *Palaemon* (as *Palaemonetes*) *pugio*, marked changes to gene expression were only observed under severe hypoxia but not moderate hypoxia (Brouwer et al., 2007), in contrast to *G. chevreuxi*. However, the magnitude of change elicited by different intensities of hypoxia seemed consistent across the time course. Severe hypoxia elicited marked changes to gene expression across all time points whilst moderate hypoxia elicited limited effects (Brouwer et al., 2007).

The extremely limited transcriptomic response of *G. chevreuxi,* including baseline levels of expression of metabolic enzymes and downregulation of one hemocyanin gene, may suggest the beginning of an alternate hypometabolic strategy under severe hypoxia particularly as 20 % a.s is approaching the critical oxygen tension (P_c) for the species (approximately 12 % a.s.) (Truebano et al., 2018). A recent study

529 suggests that signals of hypometabolism can occur above P_c as increasing rates of
530 ventilation elicited by hypoxia, such as the hyperventilatory response of pleopods
531 observed for *G. chevreuxi* at 20 % a.s., may utilise an increasing proportion of
532 consumed oxygen leaving less available to support cellular energy demands
533 (McMahon, 1988; Wood, 2018). This may lead to metabolic suppression despite
534 resting rates of oxygen uptake continuing to be regulated at the organismal level
535 (Wood, 2018).
536
537 Hypometabolism has long been recognised as a key strategy for survival of
538 organisms under severely low oxygen levels (Larade and Storey, 2002), but the
539 underlying cellular and molecular pathways are still being characterised for many
540 non-model marine invertebrate species (Seibel et al., 2018; Spicer, 2014). The
541 described changes in transcription profiles may indicate that amphipods at 20 % a.s.
542 were poised for metabolic depression. This only became apparent at the whole
543 organismal level when the amphipods were forced to be active. Despite an increase
544 in heart rate, active metabolism could not be sustained resulting in zero aerobic
545 scope which may be more attributable to there being no scope for increased
546 ventilation. A similar response has been observed in fish where aerobic scope also
547 declined to zero under severe hypoxia (Claireaux and Chabot, 2016). A transition to
548 anaerobic metabolism could have been predicted on the basis of previous studies
549 (Pörtner, 2010) and, while some accumulation of L-lactate did occur in active
550 individuals under 20 % a.s. it was, perhaps surprisingly, not as pronounced as
551 observed under moderate hypoxia. However, this may reflect the limited changes to
552 gene expression of glycolytic enzymes in individuals exposed to severe hypoxia
553 compared to the widespread changes to regulation under moderate hypoxia. Limited

554 changes to anaerobic glycolysis genes have also been observed under severe
555 hypoxia in the prawn *Litopenaeus vannamei* and are thought to be indicative of
556 metabolic suppression (Rathburn et al., 2013). A hypometabolic strategy could
557 reduce the need for anaerobic metabolism and slow the accumulation of toxic
558 anaerobic end products such as L-lactate (Boutilier and St-Pierre, 2000). Costly
559 cellular processes may be down-regulated to reduce ATP demand and avoid cellular
560 death through ATP imbalance (Boutilier and St-Pierre, 2000). The limited
561 transcriptional response of *G. chevreuxi* may therefore reflect the need to reduce the
562 energetically-demanding production of mRNA and protein (Storey and Storey, 2004)
563 as previously observed in fish exposed to severe hypoxia (Mandic et al., 2014).
564 Hypometabolic states are thought to be characterised by enhanced cellular defences
565 to prolong cellular longevity (Storey and Storey, 2011) but we observed a muted
566 antioxidant response. However, minimal changes to antioxidants have been
567 observed under severe hypoxia in deep-sea crabs (Seibel et al., 2018) and baseline
568 levels of stress proteins could still be sufficient to prevent cellular stress under
569 severe hypoxia given the general reduction in cellular metabolism (Seibel et al.,
570 2014).
571
572 Alternatively, the limited antioxidant response in combination with zero aerobic scope
573 and reduced capacity for anaerobic metabolism could indicate a severely impaired
574 state at multiple levels of organisation rather than adaptive hypometabolism. In such
575 a state, there may be no excess aerobic energy available to support physiological
576 functions essential for fitness, such as growth (Pörtner, 2012). Reduced moulting
577 frequency rates have been observed in crustaceans exposed to hypoxia (Das and
578 Stickle, 1993). Whilst not directly addressed in this study, the significant enrichment

of genes involved in chitin metabolism may indicate altered aspects of moulting and growth (Peruzza et al., 2018). These changes included mixed regulation of chitin catabolic pathways but upregulation of genes related to cuticle structure such as cuticle proteins and resilin. Upregulation of cuticle structure genes have been observed in other hypoxia-exposed crustaceans but the consequences for cuticle structure remains to be determined (Graham and Barreto, 2019). Models suggest that zero aerobic scope may ultimately be lethal (Sokolova, 2013) and so amphipods exhibiting this response may even be close to death. Future increases in prolonged episodes of severe hypoxia (Diaz and Rosenberg, 2008) may therefore be detrimental to the persistence of this species.

4.3 Conclusions

We clearly demonstrate, through the adoption of a multilevel approach, that even moderate levels of hypoxia have implications for aquatic organisms through reductions in performance. The intensity of environmental oxygen reduction experienced *in situ* should be considered in any attempt to both understand and predict the effects of hypoxia on coastal invertebrates. Future increases in the frequency of fairly moderate hypoxia may threaten the future growth, reproduction and resilience of coastal species with significant ecological consequences.

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605

606 Data availability

607 Availability for the assembled transcriptome (TSA:GFCV01000000) and raw reads
608 (SRA: SRR5109797-SRR5109805) (Bioproject number: “PRJNA357029”) are
609 detailed in Collins *et al.*, (2017). Datasets generated and analysed during the
610 current study are available on request.
611

612 Competing Interests

613 The authors declare no competing interests.

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