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

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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.

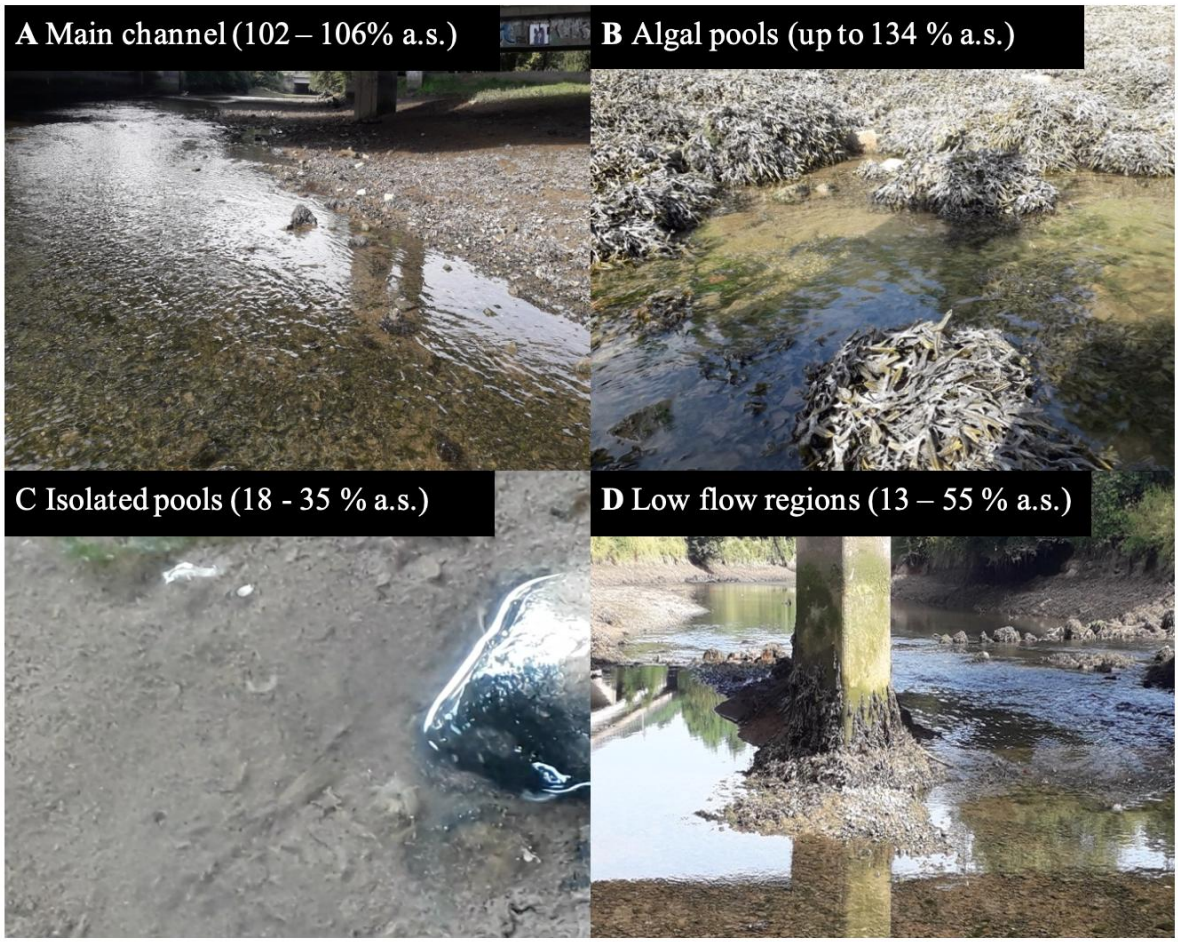

Fig. 1. *G. chevreuxi* inhabiting the River Plym experience considerable variation in oxygen tensions such as in (A) the main channel (normoxic), (B) algal pools (hyperoxic), (C) shallow pools isolated from the main channel at low tide (moderately to severely hypoxic) and (D) regions of low flow (moderately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

2.2 Exposure to different intensities of hypoxia

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

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

To estimate the rate of oxygen uptake under active conditions, individuals were chased for 1 min with a plastic pipette before being returned to their mesh envelope and re-inserted into their respirometry chamber. The chamber was immediately resealed and the individuals were left for 1 h. The oxygen tension within the chamber was then remeasured as previously described and the aerobic scope was calculated by subtracting resting metabolic rate from active metabolic rate. This end-point metabolic rate assay was utilised in order to minimise disturbance to the amphipods within the respirometry chamber. Active metabolic rate following chasing of the amphipod did not return to resting conditions during the respirometry period, a notion supported by higher ventilation rates observed at the end of the metabolic rate measurements (Fig. 2).

Upon removal from the respirometers individuals were gently blotted dry and their wet mass determined using a microbalance (MSA225P-000-DA, Göttingen Sartorius AG, Germany, ± 0.01 mg). After weighing, these active individuals were quickly frozen in liquid N_2 and stored separately at T = - 80 °C for subsequent determination of whole body L-lactate concentration.

To measure the effect of different oxygen regimes on ventilation and perfusion, in resting and active animals, individuals were observed visually during their time in the respirometers. The resting and active pleopod beat frequency and heart rate were observed and quantified in the respirometers (measured twice for 15 s for each individual) under low power magnification (x 10) using a light microscope (MZ15, Leica Microsystems Ltd, Cambridge, UK). Ventilation, *via* the beating of pleopods, is 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.

2.6 Transcriptomic responses

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

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

under exposure to 40 % a.s. and 20 % a.s. compared with the control. Differentially expressed genes (P_{adj} < 0.05) putatively associated with physiological responses to different severities of hypoxia were further explored. This included genes encoding for oxygen transporters (hemocyanin) previously identified in Truebano et al., (2018), aerobic metabolic enzymes (tricarboxylic acid (TCA) cycle enzymes and mitochondrial electron transport chain (ETC) complexes), anaerobic metabolic enzymes (glycolytic enzymes), and cellular defences (antioxidant enzymes and heat shock proteins (HSPs)).

3 Results

3.1 Physiological and biochemical responses to different severities of low oxygen

For resting individuals, there was no significant effect of exposure to either moderate (40 % a.s) or severe (20 % a.s.) hypoxia on mean mass specific oxygen uptake compared to normoxia (Fig. 2a, ANOVA $F_{2,19}$ = 1.51, P = 0.246). Ventilation rate only increased during exposure to severe hypoxia (Fig. 2b, ANOVA $F_{2,19}$ = 5.79, P = 0.011) but heart rate decreased significantly upon exposure to both hypoxia treatments for 7 d (Fig. 2c, ANOVA $F_{2,16}$ = 11.60, P < 0.001). For active individuals, mass-specific rate of oxygen uptake was significantly lower in individuals exposed to both moderate (Tukey P = 0.013) and severe hypoxia (Tukey P < 0.001) compared to those under normoxic conditions (Fig. 2d, ANOVA $F_{2,17}$ = 15.82, P < 0.001). For active individuals, there was no effect of hypoxia exposure on either ventilation rate (Fig. 2e, ANOVA $F_{2,16}$ = 1.40, P = 0.275) or heart rate (Fig. 2f, ANOVA $F_{2,16}$ = 0.04, P = 0.966). Significant reductions in aerobic scope (Fig. 2g, ANOVA $F_{2,17}$ = 17.25, P < 0.001) occurred under both moderate (Tukey P = 0.009) and severe (Tukey P < 0.001) hypoxia. The slight negative value for aerobic scope observed under 20 %

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

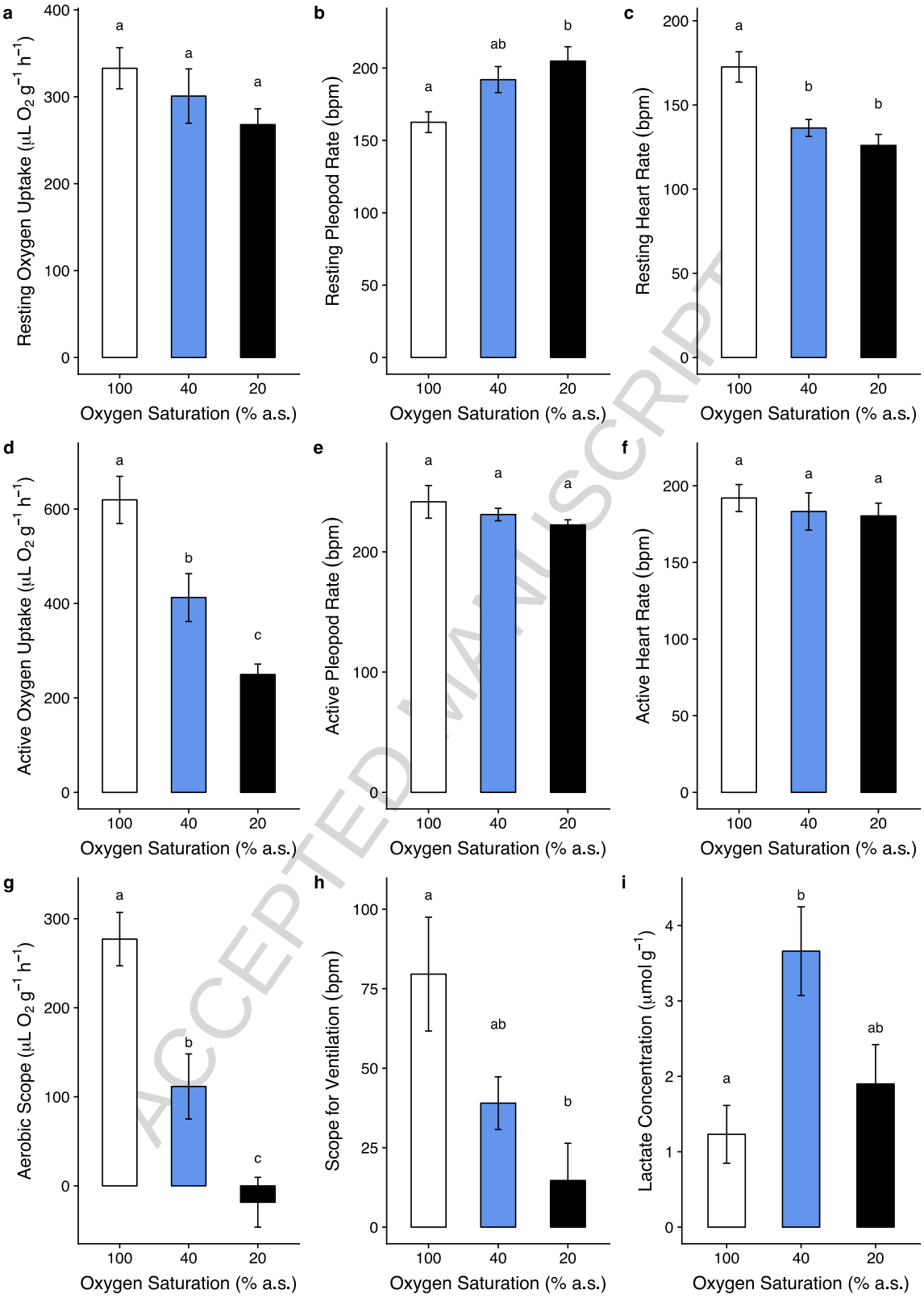

Fig. 2. The physiological effects of 7 d exposure to normoxia (100 % a.s.), moderate hypoxia (40 % a.s.) or severe hypoxia (20 % a.s.). (a) resting oxygen uptake (100 %:

n = 7, 40 %: *n* = 8, 20 %: *n* = 7) (b) resting pleopod rate (100 %: *n* = 7, 40 %: *n* = 8, 20 %: *n* = 7) (c) resting heart rate (100 %: *n* = 6, 40 %: *n* = 8, 20 %: *n* = 6) (d) active oxygen uptake (100 %: *n* = 5, 40 %: *n* = 8, 20 %: *n* = 7) (e) active pleopod rate (100 %: *n* = 5, 40 %: *n* = 8, 20 %: *n* = 6) (f) active heart rate (100 %: *n* = 5, 40 %: *n* = 8, 20 %: *n* = 6) (g) aerobic scope (100 %: *n* = 5, 40 %: *n* = 8, 20 %: *n* = 7) (h) scope for ventilation (100 %: *n* = 5, 40 %: *n* = 8, 20 %: *n* = 6) (i) L-lactate concentration of active individuals (100 %: *n* = 4, 40 %: *n* = 7, 20 %: *n* = 5) (mean values ± s.e.m). Letters indicate significant differences between treatments identified by one-way ANOVA and *post-hoc* Tukey test ($P < 0.05$). For supporting data see Table S1.

3.2 Transcriptomic features subject to regulation by moderate and severe hypoxia

Principal Component Analysis (PCA) of all genes revealed that samples were predominately separated along the first principal component (PC1), which accounted for 81 % of the variance. Along PC1, amphipods exposed to normoxia and moderate hypoxia differed the most based on their global expression profiles; whereas there was little separation between normoxia and severe hypoxia exposed amphipods along this axis (Fig. 3a). Differential expression analysis identified a total of 11,686 unique significantly differentially expressed transcripts ($P_{adj} < 0.05$) between amphipods exposed to 40 % and 100 %, of which approximately 67 % were up-regulated. In comparison, a more limited transcriptional response was observed in animals exposed to 20 % a.s. compared to the normoxic controls, with 1,721 significantly differentially expressed unique genes, 52 % of which were up-regulated. An additional 1,466 significantly differentially expressed genes overlapped between 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).

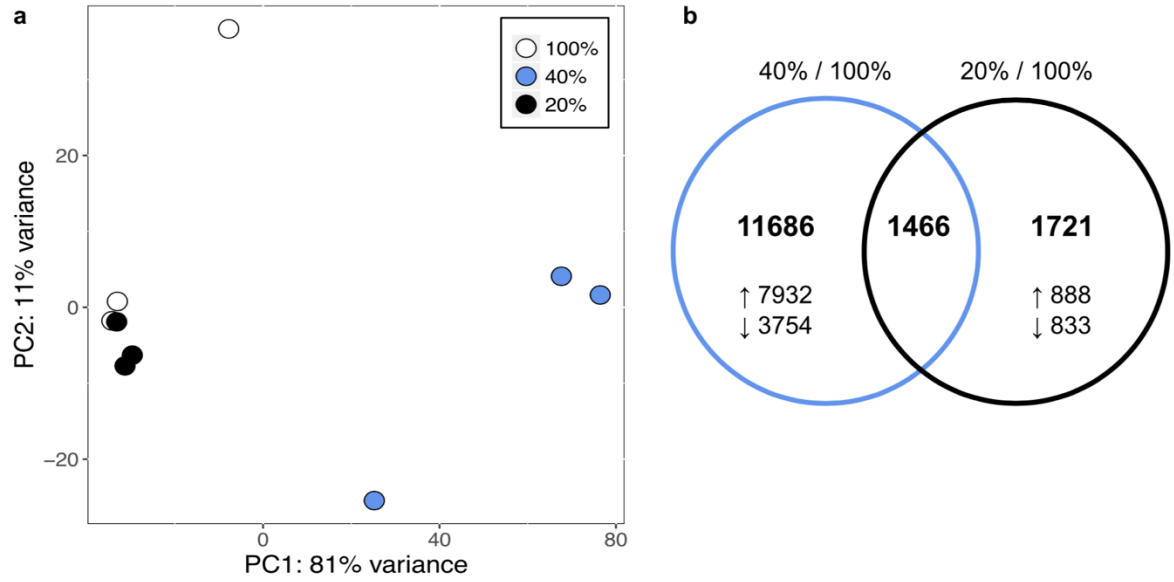

Fig. 3. Transcriptomic responses to moderate (40 % a.s.) and severe hypoxia (20 % a.s.). (a) Principal components 1 and 2 from principal component analysis performed using variance stabilised counts of all tested genes (*n* = 198,862) across all tested samples (*n* = 3 pools per treatment) (b) number 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

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

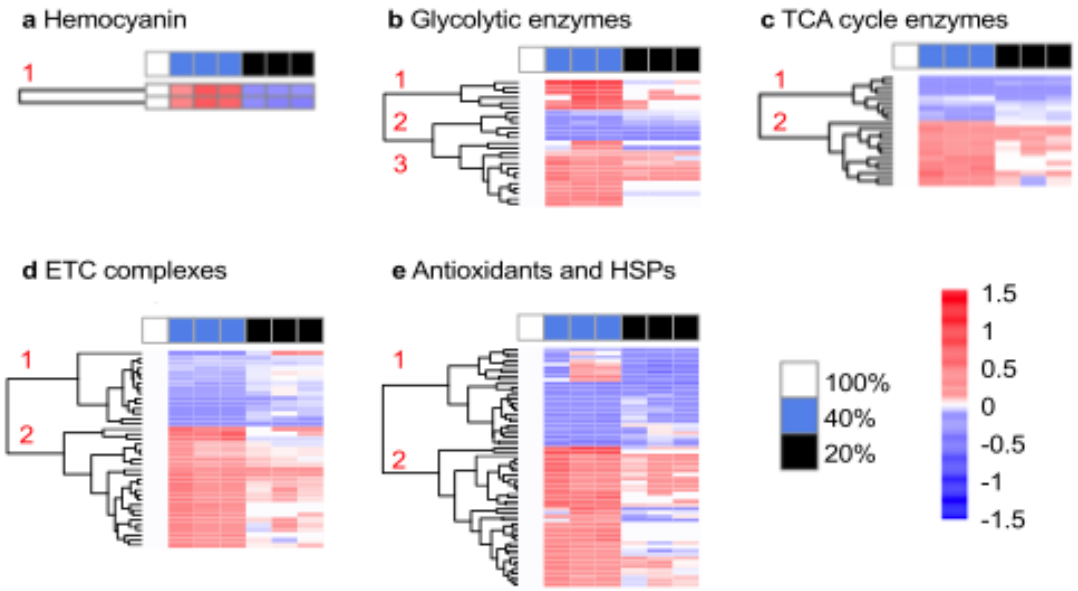

Fig. 4. Heat map of $\log_2$ fold changes of DEGs ($P_{adj} < 0.05$) for moderate and severe hypoxia (n = 3 pools per treatment) in comparison to the mean of the normoxic control (100 % a.s.). Counts were subjected to variance stabilising transformation using the DESeq2 library prior to calculation of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

4 Discussion

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

different strategies upon encountering different intensities of hypoxia. The integrated mechanisms utilised to deal with less extreme, and often ecologically-relevant, levels of moderate hypoxia may have been overlooked across species from a range of coastal environments (Spicer, 2016). For *G. chevreuxi* exposed to moderate hypoxia, there was a widespread transcriptional response and a significant reduction in aerobic scope. Under severe hypoxia, however, individuals appeared to adopt a hypometabolic strategy, characterised by limited recourse to anaerobic metabolism and a significant downregulation of genes involved in protein synthesis. Given these differences in the mechanisms affected, hypoxic intensity must be carefully considered when assessing the ecological effects of low oxygen.

4.1 Moderate hypoxia has significant implications for estuarine animals

The ability to sustain the metabolic demand for oxygen from the environment is thought to be important in determining species ecological distributions and habitat use (Deutsch et al., 2015). Moderate hypoxia did not disrupt the ability to regulate resting metabolism without recourse to a significant hyperventilatory response and despite a significant bradycardia as previously observed by Truebano et al., (2018). However, the ability to remain metabolically viable under moderate hypoxia may come at a significant cost which was only revealed through the use of a discovery-led NGS approach. The molecular response of *G. chevreuxi* to moderate hypoxia was far more complex than previous studies on crustaceans seemed to suggest (Brouwer et al., 2007) with significant changes in the expression of over 13,000 genes compared to normoxia. It is not always clear how transcriptomic responses of marine invertebrates to hypoxia integrate with those observed at the protein level (Spicer, 2014) due to potential modifications to translational efficiency under hypoxia

(Hardy et al., 2013). However, differences in gene expression profiles may reflect the metabolic needs of different tissues and be reasonably accurate in its representation of phenotypic changes (Whitehead and Crawford, 2005).

For *G. chevreuxi*, molecular changes which included up-regulation of genes significantly enriched for transcription and translation pathways, may suggest that amphipods have to actively expend energy to produce novel gene products and rearrange cellular metabolism (Larade and Storey, 2009). The ability to regulate whole-organism rates of resting metabolism under moderate hypoxia may be associated with the up-regulation of multiple genes involved in aerobic metabolism (TCA cycle enzymes and mitochondrial subunits). This may compensate for reduced environmental oxygen availability and maintain aerobic ATP production in the mitochondria (Brouwer et al., 2007) despite bradycardia and absence of a significant hyperventilatory response. Furthermore, the up-regulation of two hemocyanin genes may potentially enhance oxygen transport by the respiratory pigment (Johnson et al., 2016; Truebano et al., 2018).

Despite an apparent attempt to meet energetic demands aerobically at the molecular level, these amphipods may be compromised by even fairly moderate levels of hypoxia. For *G. chevreuxi*, an up-regulation of glycolytic enzyme genes was observed, including the enzyme *PFK* suggesting that amphipods may be primed for a transition to less energetically-efficient anaerobic metabolism (Cota-Ruiz et al., 2015), a notion that is supported by a significant accumulation of L-lactate when individuals were forced to be active.

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

suggests that signals of hypometabolism can occur above P_c as increasing rates of ventilation elicited by hypoxia, such as the hyperventilatory response of pleopods observed for *G. chevreuxi* at 20 % a.s., may utilise an increasing proportion of consumed oxygen leaving less available to support cellular energy demands (McMahon, 1988; Wood, 2018). This may lead to metabolic suppression despite resting rates of oxygen uptake continuing to be regulated at the organismal level (Wood, 2018).

Hypometabolism has long been recognised as a key strategy for survival of organisms under severely low oxygen levels (Larade and Storey, 2002), but the underlying cellular and molecular pathways are still being characterised for many non-model marine invertebrate species (Seibel et al., 2018; Spicer, 2014). The described changes in transcription profiles may indicate that amphipods at 20 % a.s. were poised for metabolic depression. This only became apparent at the whole organismal level when the amphipods were forced to be active. Despite an increase in heart rate, active metabolism could not be sustained resulting in zero aerobic scope which may be more attributable to there being no scope for increased ventilation. A similar response has been observed in fish where aerobic scope also declined to zero under severe hypoxia (Claireaux and Chabot, 2016). A transition to anaerobic metabolism could have been predicted on the basis of previous studies (Pörtner, 2010) and, while some accumulation of L-lactate did occur in active individuals under 20 % a.s. it was, perhaps surprisingly, not as pronounced as observed under moderate hypoxia. However, this may reflect the limited changes to gene expression of glycolytic enzymes in individuals exposed to severe hypoxia compared to the widespread changes to regulation under moderate hypoxia. Limited

changes to anaerobic glycolysis genes have also been observed under severe hypoxia in the prawn *Litopenaeus vannamei* and are thought to be indicative of metabolic suppression (Rathburn et al., 2013). A hypometabolic strategy could reduce the need for anaerobic metabolism and slow the accumulation of toxic anaerobic end products such as L-lactate (Boutilier and St-Pierre, 2000). Costly cellular processes may be down-regulated to reduce ATP demand and avoid cellular death through ATP imbalance (Boutilier and St-Pierre, 2000). The limited transcriptional response of *G. chevreuxi* may therefore reflect the need to reduce the energetically-demanding production of mRNA and protein (Storey and Storey, 2004) as previously observed in fish exposed to severe hypoxia (Mandic et al., 2014). Hypometabolic states are thought to be characterised by enhanced cellular defences to prolong cellular longevity (Storey and Storey, 2011) but we observed a muted antioxidant response. However, minimal changes to antioxidants have been observed under severe hypoxia in deep-sea crabs (Seibel et al., 2018) and baseline levels of stress proteins could still be sufficient to prevent cellular stress under severe hypoxia given the general reduction in cellular metabolism (Seibel et al., 2014).

Alternatively, the limited antioxidant response in combination with zero aerobic scope and reduced capacity for anaerobic metabolism could indicate a severely impaired state at multiple levels of organisation rather than adaptive hypometabolism. In such a state, there may be no excess aerobic energy available to support physiological functions essential for fitness, such as growth (Pörtner, 2012). Reduced moulting frequency rates have been observed in crustaceans exposed to hypoxia (Das and 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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Data availability

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

Competing Interests

The authors declare no competing interests.

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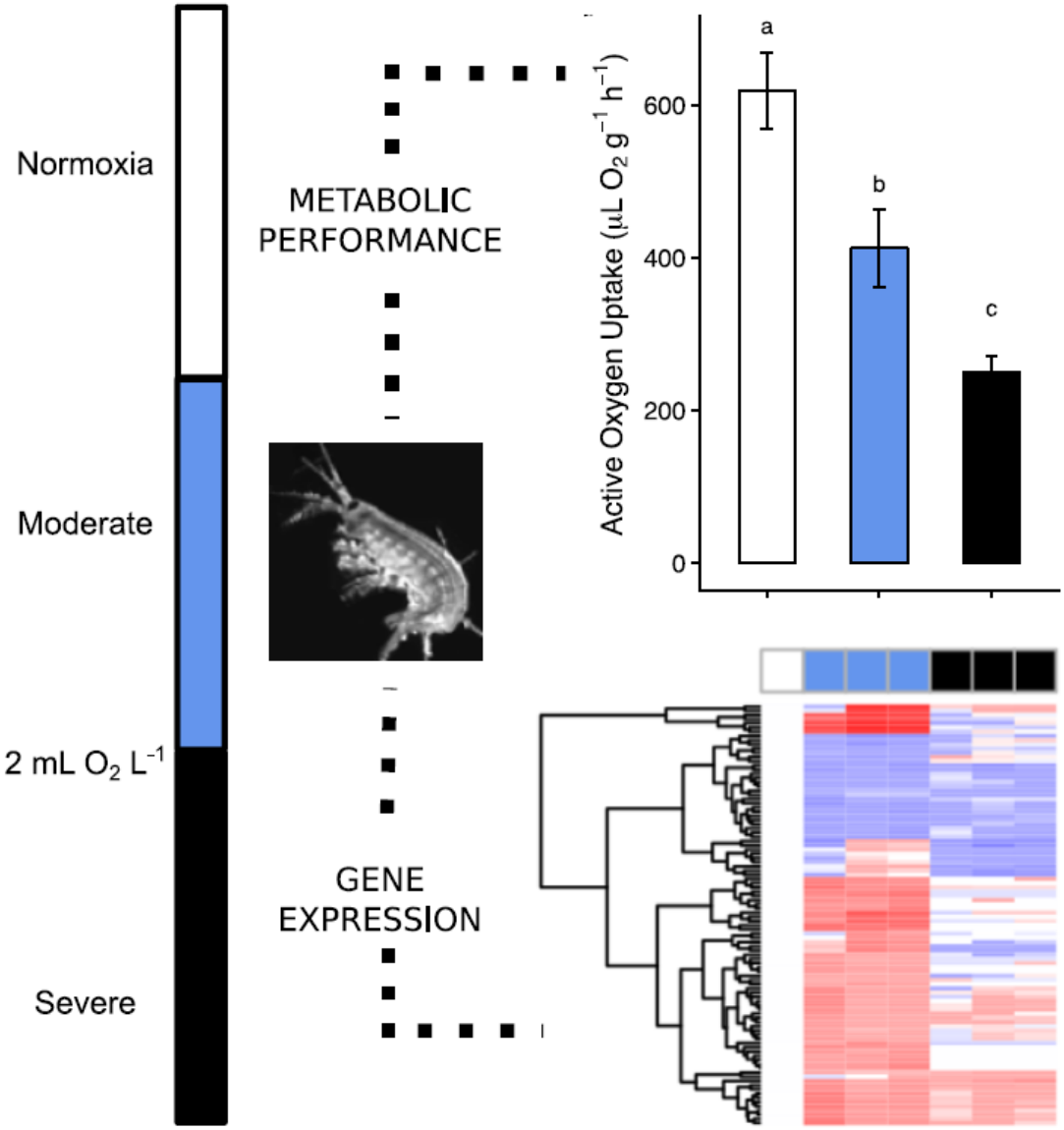

Graphical abstract

Highlights

- Coastal regions pressured by hypoxia, often defined as < 2 mL O_2 L^{-1}
- Investigation of ecologically-relevant levels of hypoxia experienced in estuaries.
- Evaluation of physiological and transcriptomic responses in coastal invertebrate
- Compromised performance and marked molecular response seen under moderate hypoxia
- Modest declines in future oxygen levels will have implications for coastal systems