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The effects of thermal acclimation on hypoxia thresholds: an integrative approach

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THE EFFECTS OF THERMAL ACCLIMATION ON HYPOXIA THRESHOLDS: AN INTEGRATIVE APPROACH

by

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At no time during the registration for the degree of *Doctor of Philosophy* has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

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Abstract

The effects of thermal acclimation on hypoxia thresholds: an integrative approach

Michael Hinton Collins

Marine animals are increasingly threatened by a combination of reduced environmental oxygen (hypoxia) and chronically rising sea temperatures. Our current understanding of the effects of temperature on hypoxic performance is based predominantly upon studies of acute warming demonstrating reductions in hypoxic performance in numerous species. However, these studies do not take account of the possibility for altered performance via thermal acclimation. They are also largely focused at the organismal level with integration of different levels of biological organisation just beginning to appear. This thesis takes an integrative approach to investigate the effects of thermal acclimation on hypoxia thresholds using gammarid amphipods as models. The metabolic, biochemical and transcriptomic responses to hypoxia in isolation were first characterised in the brackishwater amphipod *Gammarus chevreuxi*. The consequences of warm acclimation for hypoxic performance were then identified and compared across closely-related gammarid species. Finally, the underpinning molecular mechanisms driving altered metabolic responses to hypoxia were investigated in the intertidal amphipod *Echinogammarus marinus*. This thesis demonstrates that, firstly, the integrated responses to hypoxia in isolation are complex and dependent upon the severity of hypoxia organisms experience. Secondly, thermal acclimation may enhance oxyregulatory capacity under hypoxia in some species, contrary to predictions from studies of acute warming, which may be associated with warm acclimated changes to metabolic rate. Thus, some

species with a greater capacity for thermal acclimation may cope better with increasingly hypoxic environments. Intraspecific alterations to metabolic performance under declining oxygen tensions may be associated with widespread change at the molecular level. Warm acclimated *E. marinus* displayed a greater degree of hypometabolism under hypoxia compared to cold acclimated individuals, which may be associated with transcriptional changes occurring during the acclimation period associated with reductions in cellular energy demand. Thermal acclimation therefore induces changes at multiple levels of organisation which may prepare for hypoxia. Acclimation should be considered in any attempt to predict the consequences of future climate change driven hypoxia on marine species.

TABLE OF CONTENTS

List of figures

List of Tables

No tables contained within main text, please see Appendices for supporting tables.

Abbreviations

MO_2 – metabolic rate(s), all MO_2 are actually $\dot{M}O_2$

PO_2 – partial pressure(s) of oxygen

P_c – critical partial pressure of oxygen/critical oxygen tension(s)

T_a – acclimation temperature(s)

T_t – test temperature(s)

Chapter 1 General Introduction: Does increased temperature always impair hypoxic performance in marine ectotherms?: the role of thermal acclimation

1.1 Introduction

Oxygen is an essential molecule, with very few exceptions, for all animal life as it enables metabolism to proceed aerobically and supply sufficient energy to meet the energetic costs of living (Semenza, 2007). Oxygen plays a pivotal role as the final electron acceptor in the mitochondrial respiratory chain allowing the generation of fifteen times more energy (ATP) than anaerobic metabolism alone (Grieshaber *et al.*, 1994). However, through its use within the mitochondria, oxygen can become highly toxic as approximately 1-4 % is converted to reactive oxygen species (ROS) causing damage to cells and tissues (oxidative stress) if not detoxified by cellular antioxidants (Storey, 1996). ROS production can be exacerbated by environmental stress, including oxygen levels that are too low (hypoxia) or too high (hyperoxia) (Storey, 1996). That said, many marine animals are physiologically capable of inhabiting regions where pronounced variations in oxygen tension is a natural feature (Spicer, 2014). Shallow coastal habitats can experience hypoxia over diurnal to seasonal scales (Diaz and Rosenberg, 1995). Intertidal rockpools, experience daily cycles of hypoxia and reoxygenation (cyclic hypoxia) which may even become hyperoxic (< 1.5 % a.s. to 300 % a.s.) (Truchot and Duhamel-Jouve, 1980). This can result from photosynthetic oxygen production by algae during the day and oxygen consumption via respiration at night time (Truchot and Duhamel-

Jouve, 1980). Estuarine systems are also subject to these cycles of hypoxia but also episodic (days to weeks) or seasonal hypoxia (weeks to months) (< 28 % a.s.) (Diaz and Rosenberg, 1995; Tyler *et al.*, 2009). Seasonal hypoxia usually occurs in summer months when raised summer temperatures drive stratification and disrupt the circulation of oxygen to deeper waters below the thermocline (Tyler *et al.*, 2009). Seasonal hypoxia also typifies fjord systems driven by poor circulation of oxygen by current systems to deeper waters (Spicer, 2014). Certain species even inhabit, or make frequent excursions into almost permanent severe hypoxia such as the inhabitants of the extensive deep sea oxygen minimum zones (OMZs) (< 1.5 % a.s.) (Childress and Seibel, 1998; Seibel, 2011).

Application of a singular threshold to define hypoxia for marine animals inhabiting environments naturally exposed to reductions in dissolved oxygen of different severities and duration is extremely challenging (Nikinmaa, 2013). Hypoxia has historically been defined by ecologists as dissolved oxygen levels less than 2 mL O_2 L^{-1} (~ 28 % a.s.) (Diaz and Rosenberg, 2008). However, there has been considerable discussion about the use of a single oxygen level to define hypoxia (Vaquer-Sunyer and Duarte, 2008), and other definitions have been suggested. Physiological definitions of hypoxia include those of Farrell and Richards (2009), who consider hypoxia to be any reduction in the partial pressure of oxygen that elicits a physiological effect, and Seibel (2011), who focusses specifically upon how low oxygen affects resting rates of aerobic metabolism. Adopting such physiological approaches may be required to avoid the difficulties of applying a single threshold oxygen concentration to species which differ in hypoxia tolerance (Seibel, 2011).

Although hypoxia can occur naturally, organisms inhabiting both coastal and pelagic ecosystems are potentially at risk from anthropogenically-driven reductions in oxygen tension (Breitburg *et al.*, 2018). The spread of coastal hypoxia is being driven by excessive nutrient pollution (eutrophication), which triggers algal bloom formation followed by enhanced rates of oxygen consumption by microorganisms (Diaz and Rosenberg, 1995). Such prolonged bouts of severe hypoxia result in reduced levels of biodiversity and ecosystem function (Diaz and Rosenberg, 2008). Global warming, via temperature-related decreases in the solubility of oxygen in sea water, is further exacerbating hypoxia in coastal regions and reducing the oxygen content of the ocean more generally (Breitburg *et al.*, 2018). Rising sea temperatures have been driving a global decline in dissolved oxygen levels (~ 2 %) over the past fifty years (Schmidtko *et al.*, 2017). Future declines in ocean oxygen levels of up to 7 % are predicted by 2100 (Keeling *et al.*, 2010) which may be driven, in part, by the predicted 2.7 °C increase in average sea surface temperature by 2100 (Pörtner *et al.*, 2015). Increasing temperatures will intensify hypoxia by reducing oxygen solubility, but also possibly by enhancing stratification, disrupting oxygen circulation by current systems and enhancing rates of biological oxygen consumption (Rabalais *et al.*, 2009; Altieri and Gedan, 2015). Based on these predictions, marine animals will increasingly experience hypoxia in combination with chronically raised temperatures and therefore an understanding of their interactive effects is urgently required (Pörtner, 2010; McBryan *et al.*, 2013). While hypoxic performance in isolation has been well characterised, knowledge of the potentially modifying effects of increased temperature is somewhat disjointed. Most published studies have investigated the effect of acute

warming, typically demonstrating impaired tolerance and performance (Herreid, 1980; Vaquer-Sunyer and Duarte, 2011). However, less attention has been given to the effects of longer term thermal acclimation, a consideration which is essential if we are to extrapolate to, and predict, the effects of climate change-driven hypoxia (McBryan *et al.*, 2016).

1.2 Physiological and molecular responses to hypoxia in isolation

The physiological and biochemical responses of marine animals to environmental hypoxia have been the subject of numerous reviews (Mangum and Van Winkle, 1973; Herreid, 1980; Grieshaber *et al.*, 1994; Burnett and Stickle, 2001; McMahon, 2001; Wu, 2002; Farrell and Richards, 2009; Spicer, 2016), and the underlying molecular responses have also started to receive attention (Brouwer *et al.*, 2004; Nikinmaa and Rees, 2005; Richards, 2009; Johnson *et al.*, 2016). Under acutely declining oxygen tensions (PO_2), aerobic metabolic rate (measured as oxygen consumption, MO_2) can display a spectrum of responses ranging from oxyconformity, where MO_2 declines directly with decreasing environmental PO_2, to oxyregulation, where MO_2 remains independent of environmental PO_2 down to a critical PO_2 value (P_c). Below P_c, organisms begin to conform and recourse to anaerobic metabolism and/or hypometabolism (Herreid, 1980; Grieshaber *et al.*, 1994) (Fig. 1.1A.). However, for some species, a P_c estimated based upon a decline in oxygen consumption, may not necessarily mark a clear transition from aerobic metabolism to the onset of anaerobic or hypometabolism as, in some species, anaerobic metabolites may accumulate before animals become oxygen-limited (Pörtner *et al.*, 1985). Therefore it has been suggested that P_c could be defined based

upon the PO_2 at which recourse to anaerobic metabolism begins (Pörtner *et al.*, 1985). Additionally, MO_2 may not necessarily be regulated at 100 % of the normoxic rate leading to partial, but regulated, reductions in metabolism before P_c is reached (Verberk *et al.*, 2018).

Notwithstanding the caveats, P_c are the most widely used threshold of hypoxic performance and have been used to define hypoxia for individual species (Seibel, 2011). They represent the capacity of organisms to extract oxygen from their environment and therefore, compensate for environmental hypoxia (Mandic *et al.*, 2009). A plethora of statistical methods have been generated to identify P_c such as “broken-stick regression” (Yeager and Ultsch, 1989), “segmented regression” (Muggeo, 2008) or non-linear regression (NLR) methods (Marshall *et al.*, 2013).

For species which do not display a clear P_c, alternate methods to determine hypoxic performance have been developed such as identification of the PO_2 at which resting MO_2 declines to 50 % of the normoxic rate (Sutcliffe, 1984; Lagos *et al.*, 2017). Alternatively, the degree of oxyregulation can be determined using “regulation values (*R*-values)” (Alexander and McMahon, 2004) or the “regulatory index (RI)” (Mueller and Seymour, 2011). While a reduction in RI or R-values could indicate a reduction in hypoxic performance, it is changes to P_c that have traditionally received the greatest attention. It has long been documented that P_c are not fixed at a single PO_2 but vary depending upon the physiological state of an organism (Herreid, 1980). This makes P_c useful thresholds to identify the modifying effects of other environmental factors, as

changes to P_c can reflect either improved or impaired hypoxic performance (Herreid, 1980; Rogers *et al.*, 2016).

The mechanisms underpinning the metabolic response to hypoxia at all levels of organisation are a function of severity and duration (Burnett and Stickle, 2001). Acutely, MO_2 can be sustained above P_c, under what could be considered 'moderate' hypoxia, by increased ventilation of gas exchange surfaces, altered circulation and allosteric modulation of the O_2 affinity of the respiratory pigment (Burnett and Stickle, 2001; McMahon, 2001; Spicer, 2016). Oxygen affinity of respiratory pigments is an important component of hypoxic performance and species which possess high affinity pigments may be more hypoxia tolerant (Mandic *et al.*, 2009). The possession of respiratory pigments allows the delivery of more oxygen to the tissues than would be possible from oxygen just being transported in solution (Bridges, 2001). Increasing the oxygen affinity of respiratory pigments, measured as a reduction in the half-saturation or P_{50}, favours the loading of oxygen at the respiratory surface (Bridges, 2001). Changes which increase oxygen affinity may therefore become particularly important under environmental hypoxia to maintain oxygen uptake in the face of low PO_2 at the gill surface (McMahon, 2001).

Attempts to elucidate the molecular bases of responses to acute hypoxia have largely been limited to 'severe' hypoxia below P_c. Models have long existed to explain the integrated mechanisms which drive mortality under severe hypoxia (Hochachka *et al.*, 1996; Boutilier and St-Pierre, 2000). These models suggest that severe hypoxia disrupts MO_2 and compromises essential energy stores

(ATP) at the cellular level, leading to apoptosis and cellular death. In order to promote survival, ATP demand requires metabolic suppression to a level that can be met by anaerobic ATP supply (Boutilier and St-Pierre, 2000). In marine animals, acute changes in gene expression are induced, which may contribute towards an energetically-balanced hypometabolic state. Genes may be downregulated to reduce ATP demand from cellular processes such as protein synthesis, upregulation of anaerobic (glycolytic) ATP supply and enhanced expression of cellular stress proteins to minimise mitochondrial damage caused by low oxygen (Gracey *et al.*, 2001; Brouwer *et al.*, 2007; Richards, 2009). Despite being below P_c, genes responsible for increasing oxygen transport in the hemolymph, such as genes encoding for fish hemoglobins or crustacean hemocyanins, surprisingly display rapidly increased expression (Nikinmaa and Rees, 2005; Johnson *et al.*, 2015). In combination with changes to mitochondrial gene expression, such changes may allow the organism to sustain at least some aerobic ATP production (Richards, 2009).

For studies investigating the effects of chronically reduced oxygen levels, moderate hypoxia has often been utilised to acclimate animals to promote survival of test organisms. Under longer term exposure to moderate hypoxia, MO_2 may be sustained by remodelling of gas exchange surfaces (Sollid *et al.*, 2003), and/or increasing the concentration or oxygen affinity of the respiratory pigment through increased synthesis of protein subunits with a higher oxygen affinity, continued allosteric modulation or changes to ionic composition (Defur *et al.*, 1990; Mangum, 1997) (Fig. 1.1B). The regulation of respiratory pigments is, arguably, the only transcriptional response which has been reasonably well characterised following exposure to chronic moderate hypoxia (Johnson *et al.*,

2016; Pan *et al.*, 2017). The limited studies focussing on elucidating other cellular effects of moderate hypoxia have identified minimal changes in global gene expression (Brouwer *et al.*, 2007, 2008). The effects of moderate hypoxia should not be overlooked as it is often more prevalent in nature than severe hypoxia (Farrell and Richards, 2009). Moderate hypoxia can reduce active MO_2 and aerobic scope, representing the energy available to support all functions important to fitness above resting metabolism (Pörtner, 2010; Claireaux and Chabot, 2016). A recent meta-analysis also highlights that many invertebrate species undergo reductions in fitness traits such as growth, feeding and reproduction under modest declines in dissolved oxygen (Galic *et al.*, 2019).

The longer term global transcriptional responses to severe hypoxia have received attention in selected fish and crustaceans using dissolved oxygen levels just below P_c to allow survival of organisms for several days to weeks (Li and Brouwer, 2009; Mandic *et al.*, 2014) (Fig. 1.1C). Acutely, the transcriptional responses to severe hypoxia appear to conform to the disruption of MO_2 observed at the physiological level. Time-course data of gene expression for fish and crustaceans exposed to severe hypoxia reveals complex transcriptional regulation where genes involved in glycolysis, protein synthesis and mitochondrial activity can be dynamically upregulated and downregulated over hours to days in a species-specific manner (Li and Brouwer, 2009; Mandic *et al.*, 2014). However, it is not clear what the functional consequences of these dynamic changes to gene expression are or how they scale to the protein level as hypometabolic states are thought to be subject to considerable regulation by post-translational modifications (Storey and Storey, 2004). Linking transcriptomic responses to changes at even the protein level, let alone the

whole organism, remains a key challenge for non-model marine animals exposed to hypoxia (Spicer, 2014). A combined proteomic-transcriptomic approach may aid interpretation of global molecular and cellular responses to hypoxia, but studies using such approaches are limited for marine species (Tiedke *et al.*, 2015).

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Fig. 1.1 Integrated responses of marine animals to hypoxia. (A) Acute hypoxia: MO_2 can display a range of responses with declining PO_2 from oxyconformity (dashed line) to oxyregulation (solid line) where MO_2 is regulated down to the P_c before resorting to anaerobic metabolism. Ventilation can increase towards P_c alongside maintenance of heart rate, both of which decline below P_c. The molecular responses to acute, moderate hypoxia (above P_c) have largely been disregarded in favour of severe hypoxia (below P_c) where

increases to gene expression of respiratory pigments, cellular defences and anaerobic enzymes have been observed. Downregulation of costly cellular processes such as ribosomal protein synthesis may reduce ATP demand. (B) Chronic moderate hypoxia can induce longer term changes to sustain aerobic metabolism including a reduction in increased rates of ventilation and perfusion accompanied by increased respiratory pigment concentration (hemoglobin Hb, or hemocyanin Hc), increased gill (respiratory surface) area and increased oxygen affinity of respiratory pigments via molecular changes to protein subunit composition coordinated at the transcriptional level or biochemical changes through allosteric modulator substances or ion composition (C) Chronic severe hypoxia typically limits survival time. Metabolic depression at the physiological level is accompanied by a depression of ATP demand at the cellular level to achieve a lower, but energetically balanced steady state (simplified from Hochachka *et al.*, 1996). This may be supported by changes in global gene expression but regulation under chronic severe hypoxia is complex in fish and crustaceans. The heatmap shows the time course of global gene expression under severe hypoxia in the grass shrimp *Palaemon* (as *Palaemonetes*) *pugio* indicating complex cycles of up and down regulation between 6 and 240 h (adapted from Brouwer *et al.*, 2007). Responses depicted in this figure were collated from references contained in Section 1.2

The metabolic and respiratory consequences of hypoxia have been well characterised, particularly through the identification of P_c points under acutely declining PO_2 in numerous fish and invertebrate species (Grieshaber *et al.*, 1994; Rogers *et al.*, 2016). While our understanding of the molecular mechanisms underpinning these responses is in its infancy for the majority of

marine species, this is rapidly changing with the incorporation of -omic technologies. An integrative approach has been successful in characterising the effects of acute, severe hypoxia, but the responses become complex when severe hypoxia persists over several days to weeks (Li and Brouwer, 2009; Mandic *et al.*, 2014). The integrated responses to more 'moderate' hypoxia, both acutely and chronically, require further assessment as it is currently, and predicted to become, more common in nature under climate change (Spicer, 2014; Breitburg *et al.*, 2018) and may have significant consequences for the life history of marine organisms (Vaquer-Sunyer and Duarte, 2008).

As the integrated responses to hypoxia in isolation are clearly still being explored for marine species, it is perhaps not surprising that the responses measured under hypoxia in combination with other abiotic factors are more limited in scope. Studies investigating the modifying effects of temperature are largely restricted to key hypoxia thresholds such as P_c and survival and I therefore focus on these responses for the remainder of the review part of this chapter.

1.3 The interaction of temperature and hypoxic performance: evidence from acute warming

The interaction of temperature and hypoxia has long received attention in both fish and invertebrates (Fry, 1971; Herreid, 1980). For ectotherms, warming increases physiological rates through thermodynamic effects on biochemical reactions (Schulte, 2015). The thermal sensitivity of a given physiological rate

can be modelled using Q10 values, which represents the fold change in a physiological rate standardised to a 10 ºC temperature increase (Cossins and Bowler, 1987). The physiological rate which has received a large amount of attention is MO_2 (Fry, 1971; Schulte, 2015; Seebacher *et al.*, 2015). Fry (1971) noted both temperature and hypoxia can interact to influence MO_2. Fry described temperature as a "controlling factor" as it controls the rate at which metabolism proceeds whilst oxygen was described as a "limiting factor" as it can potentially restrict metabolism. Models of the interaction of temperature and hypoxia on MO_2 have therefore existed for several decades. An acute temperature increase (typically over several hours) tends to reduce the survival time of a number of marine species exposed to different levels of low oxygen (Vaquer-Sunyer and Duarte, 2011) and increase P_c (Wiens and Armitage, 1961; Varo *et al.*, 1991, 1998; Dupont-Prinet *et al.*, 2013; González-Ortegón *et al.*, 2013; Lapointe *et al.*, 2014). This has commonly been attributed to a rise in MO_2 at higher temperatures which increases the difficulty of sustaining MO_2 when external PO_2 are low (Herreid, 1980) (Fig. 1.2). There may also be detrimental effects of increased temperature on the oxygen affinity of respiratory pigments under hypoxia where a rise in P_{50} may reduce the loading of oxygen at the respiratory surface (Mangum, 1980; Bridges, 2001).

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Fig. 1.2 The effect of an acute increase in temperature on MO_2 under declining PO_2. Resting MO_2 is regulated under declining PO_2 down to P_c at an initial colder/control temperature (blue line). P_c is typically raised as a result of increased temperature (red line) due to a temperature-induced increase in MO_2 (adapted from Herreid, 1980).

More recently, the Oxygen and Capacity Limitation of Thermal Tolerance (OCLTT) hypothesis has provided a mechanistic framework for the interaction of temperature and hypoxia. Initially, the hypothesis aimed to explain the role of oxygen limitation in setting thermal tolerance (Pörtner, 2002), but was subsequently modified to explicitly incorporate the reverse role of temperature on hypoxic performance (Pörtner, 2010, 2012). One of the predictions from the hypothesis is that marine animals have an optimal temperature for metabolic performance but at sub-optimally high “pejus” temperatures, the increased MO_2 cannot be met via hyperventilation and increased heart rate. This results in hemolymph deoxygenation and a decline in aerobic scope, even under

normoxic conditions. At a critical temperature, an individual becomes entirely reliant on anaerobic metabolism and survival time becomes limited. The hypothesis has also been used to predict hypoxia thresholds where critical oxygen tensions are at their lowest, i.e. best hypoxic performance, at optimal temperatures. Raised critical oxygen tensions are predicted at “pejus” temperatures in response to increasing internal hypoxia (Fig. 1.3).

The applicability of the OCLTT as an overarching hypothesis for all species is contentious, particularly concerning whether sub-optimal temperatures drive an internal hypoxia and loss of aerobic scope under normoxic conditions (Ern *et al.*, 2016; Lefevre, 2016; Verberk *et al.*, 2016; Jutfelt *et al.*, 2018). The effects of sub-optimally low temperatures on P_c are not well characterised but evidence is limited for oxygen limitation in the cold (Verberk *et al.*, 2016). However, it is plausible that an increased internal hypoxia could occur under the combination of warm temperature and environmental hypoxia, contributing to increased P_c. Irrespective of the underpinning mechanisms by which increased P_c occurs, its consistent increase in a large number of marine species under acute warming predicts marine ectotherms will perform worse in a warmer, hypoxic ocean. As P_c are thought to represent the lowest PO_2 under which organisms can survive in nature, raised P_c may limit the availability of viable habitats for marine species (Seibel, 2011).

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Fig. 1.3 Predictions from the OCLTT hypothesis on the effect of temperature on P_c. An optimal thermal range (T_{opt}) exists for aerobic performance and internal PO_2. As temperatures increase/decrease towards "pejus" values (T_p), the internal PO_2 starts to decline reaching a minimum at critical temperatures (T_c) (solid red line). P_c is lowest at T_{opt} but starts to increase at T_p due to reductions in internal PO_2 reaching a maximum at T_c (dashed red line). However, the effects of sub-optimally cold temperatures on critical oxygen tensions remain largely unknown (indicated by question mark) (adapted from Pörtner, 2012).

1.4 Will hypoxia thresholds remain impaired in a chronically warmer ocean?: the role of thermal acclimation

While using acute temperature changes has been ubiquitous and useful as a probe of the mechanistic relationship between temperature and hypoxia, there has been comparatively little exploration of the possibility for longer term physiological remodelling, known as thermal acclimation (Prosser, 1973). Many

studies have investigated the effects of thermal acclimation under normoxic conditions (Kingsolver and Huey, 1998; Somero, 2004; Schulte *et al.*, 2011) dealing with both the physiological mechanisms of acclimation and the evolutionary consequences of such responses, i.e. beneficial or detrimental effects on organismal fitness (Kingsolver and Huey, 1998). Broadly, physiological acclimation is thought to stabilise physiological rates, e.g. MO_2, across variable environmental conditions and may reduce the acute thermal sensitivity of a physiological process, measured as a reduction in Q10 (Cossins and Bowler, 1987). Historically, all physiological responses were considered adaptive but this paradigm was heavily criticised (Gould and Lewontin, 1979). Leroi *et al.*, (1994) later termed this the “beneficial acclimation hypothesis (BAH)”, stating that organisms acclimated to a specific temperature would adjust their physiology and perform better at that temperature compared to organisms which had not had the opportunity to acclimate. There is mixed support for the BAH (Angiletta, 2009) but beneficial acclimation can at least be explicitly tested if appropriate experimental designs are used (Huey and Berrigan, 1996; Huey *et al.*, 1999).

As marine animals are likely to experience chronic warming under climate change, it has become essential to critically analyse what thermal acclimation does to hypoxia thresholds. I first outline the mechanisms of thermal acclimation under normoxic conditions which are well characterised, with a focus on metabolic physiology to understand the potential for interaction with low oxygen. The methodology for testing for acclimation is also presented before I critically analyse studies which have empirically tested the effects of thermal acclimation on hypoxic performance.

1.4.1 Thermal acclimation of metabolic physiology under normoxia and testing for acclimation

The loss of hypoxic performance (increased P_c) under rapid warming is thought to be mediated through the acute rise in MO_2 (Herreid, 1980). However, investigations of the time-course of thermal acclimation of MO_2 under normoxic conditions have long demonstrated different effects of chronic compared to acute warming (Prosser, 1973) which may create a different physiological starting point from which to react to hypoxia.

Under acute warming, an initial rise in MO_2 is typically supplied by hyperventilation and increased heart rate, but over time can be compensated to differing degrees by acclimation (Prosser, 1973). Precht *et al.*, (1973) proposed a framework depicting the time course of acclimation of physiological rates including MO_2 (Fig. 1.4). This proposes that following the initial acute rise in MO_2 caused by transfer from a cooler temperature (T_1) to a warmer temperature (T_2), MO_2 can either (1) continue to rise (inverse acclimation), (2) remain constant (no acclimation) (3) be partially compensated (partial acclimation) (4) be fully compensated returning to the rate observed at T_1 (complete acclimation) or (5) be overcompensated (supraoptimal acclimation). Acclimation of MO_2 may be achieved through a variety of mechanisms such as changes to the concentrations, isoforms and activities of metabolic enzymes, alterations to mitochondrial activity, and changes to oxygen transport by respiratory pigments

(Johnston and Dunn, 1987; Somero, 2004; Pörtner, 2010; Chung and Schulte, 2015).

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Fig. 1.4 Precht's framework for the time course of thermal acclimation. Physiological rates, such as MO_2, typically rise with an acute temperature increase (transfer from T_1 to T_2) supplied by hyperventilation and increased heart rate. Over time, MO_2 can continue to rise (inverse acclimation), not be acclimated (none), or can be partially (partial), fully (complete), or over compensated (supraoptimal). This can be achieved through adjustments to various metabolic parameters e.g. changes to enzyme activities, concentrations or isoforms, mitochondrial activity or oxygen transport by respiratory pigments (adapted from Precht *et al.*, 1973).

In addition to prediction of the time-course of thermal acclimation, Precht's framework also provided methodology for modelling these changes caused by acclimation with the key component being that individuals must be compared at standardised acute test temperatures (T_t) following acclimation to different acclimation temperatures (T_a) (Fig. 1.5A). Acute rate-temperature (RT) curves,

also referred to as thermal performance curves (TPCs) (Schulte *et al.*, 2011), can be constructed for each T_a for responses measured at different T_t which represents the acute thermal sensitivity/dependency of a physiological process (Cossins and Bowler, 1987). The different types of acclimation response (e.g. inverse, none, partial, full, supraoptimal) can shift the position of the rate-temperature curve, known as translation (Cossins and Bowler, 1987) (Fig. 1.5B). However, the effects of T_a may not necessarily be uniform across all T_t as there can be interactions between the two. Precht *et al.*, (1973) noted these interactions where the orientation of the curve (representing the acute thermal dependency of a physiological process or T_t effects) can also be altered by acclimation (T_a effects) (Fig. 1.5C). Prosser (1973) suggested that acclimation may, in addition to translation, also cause rotations and, in most cases, a complex combination of rotation and translation of RT curves (Cossins and Bowler, 1987) (Fig. 1.5C). Irrespective of the precise pattern of acclimation response displayed, this approach allows explicit testing of whether acclimation responses are beneficial for fitness (or a proxy for fitness such as MO_2) as improved/impaired performance can be identified through comparison of performance at each T_t between different T_a (Huey and Berrigan, 1996; Huey *et al.*, 1999) (Fig. 1.5A).

The approach of comparing responses at standardised T_t post-acclimation has now been widely used by evolutionary biologists explicitly testing the BAH (Angiletta, 2009), and continues to be advocated by physiologists to better understand acclimation of physiological performance (Schulte *et al.*, 2011). Using this approach, Seebacher *et al.*, (2015) carried out the most comprehensive meta-analysis to date of the effects of thermal acclimation on

physiological rates, including MO_2, for over two hundred ectothermic species from marine, terrestrial and freshwater environments. They estimated that physiological rates have risen by up to 20 % over the past 20 years, but marine ectotherms have been able to partially acclimate MO_2 resulting in a more reduced thermal sensitivity than present in individuals exposed to acute temperature change. Whether this acclimation of MO_2 is beneficial under normoxic conditions is somewhat subjective but could be interpreted to reduce energetic demand at warmer temperatures and stabilise metabolism across varying environmental temperatures (Prosser, 1973; Seebacher *et al.*, 2015).

Partial acclimation of MO_2 could perhaps be predicted to be more beneficial for hypoxic performance if it offsets the rise in MO_2 which drives the loss of hypoxic performance under acute warming (Herreid, 1980). Furthermore if, as the OCLTT suggests, increased temperature is first and foremost an issue of internal hypoxia, the physiological changes induced by warm acclimation, which may alleviate internal oxygen limitation, could improve the ability of organisms to deal with future hypoxia (Pörtner, 2010). There is therefore the potential for different subsequent effects of acclimation upon physiological hypoxia thresholds compared to acute warming, which need to be critically analysed.

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Fig. 1.5 Testing for acclimation of physiological rates such as MO_2. (A) Experimental design for acclimation studies where individuals are acclimated (T_a) to different temperatures ($T_a = T_1$ (solid lines = cold temperature) or T_2 (dashed lines = warm temperature)) before responses of acclimated individuals are measured acutely at multiple standardised test temperatures ($T_t = T_1, T_2 \ldots T_n$). (B) Thermal performance curves for a given response can then be constructed for both acclimated animals ($T_a = T_1$ or T_2) compared at standardised acute test temperatures ($T_t = T_1$ or T_2). From the Precht framework, this enables identification of the effects of thermal acclimation (T_a effects) dependent upon how the RT curve shifts. Five types of acclimation can occur: inverse, none, partial, complete or supraoptimal. Acute thermal change

within each acclimation treatment can also be identified (T_t effects). (C) Prosser also expanded on Precht's framework to include a greater range of test temperatures (T_t) in order to identify further changes caused by acclimation (T_a) other than shifts in the position of the RT curves, known as translation. Acclimation can also cause changes in the orientation of the RT curve (rotation) modifying the effect of acute test temperature (T_t) on a given physiological rate. A complex mix of translations and rotations are common with a physiological rate at any given time point being a complex combination of current temperature (T_t) and previous thermal history (T_a) (adapted from Cossins and Bowler, 1987).

1.4.2 The effects of thermal acclimation on hypoxia thresholds in marine species

Multiple studies exposing marine animals, particularly fish, for several weeks to months to different acclimation temperatures, have identified raised P_c when individuals are tested solely at the temperature to which they have been acclimated ($T_a = T_t$) (Butler and Taylor, 1975; Al-Wassia and Taylor, 1977; Schurmann and Steffensen, 1997; Barnes *et al.*, 2011; Collins *et al.*, 2013). This could be interpreted as chronically impaired performance under environmental hypoxia. Alternatively, other studies have shown that raised P_c may not persist following acclimation (Yamanaka *et al.*, 2013). In four species of freshwater fish, summer acclimated individuals exposed to acutely declining PO_2 at 20 °C surprisingly had a lower P_c than winter acclimated individuals tested at 10 °C, despite the significantly lower metabolic rate observed at 10 °C (Ultsch *et al.*, 1978). For these freshwater fish species, acclimation appears to have overridden the detrimental effects of acute warming on metabolic performance.

Thermal acclimation may therefore have unpredictable effects on hypoxic performance and needs to be fully quantified using appropriate experimental designs. Whilst measuring responses at an organism's respective acclimation temperature ($T_a = T_t$) is important to generate greater ecological realism, it might not necessarily capture the full mechanistic effects of acclimation (Schulte *et al.*, 2011). It is possible that raised P_c at an organism's respective acclimation temperature is driven primarily by the different T_t, making it almost impossible to distinguish an effect of T_a. Perhaps an exception where acclimation effects can possibly be inferred from studies where $T_a = T_t$, is a relatively recent study of crucian carp which did not focus on the absolute values of P_c but the magnitude of change in P_c between the fish's respective acclimation temperatures ($T_a = T_t$). Sollid *et al.* (2005) acclimated crucian carp where T_a = 10, 15, 20 and 25 °C and found both MO_2 and P_c increased when tested at their respective T_a (Fig. 1.6A). Between T_t = 10 - 20 °C, MO_2 and P_c scaled approximately linearly. However, the slope plateaued between T_t = 20 - 25 °C demonstrating a much smaller increase in P_c despite the continued increase in MO_2 (Fig. 1.6B). This has been attributed to an effect of T_a (gill remodelling in this case) occurring solely between T_t = 20 - 25 °C (Sollid *et al.*, 2005).

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Fig. 1.6 Effect of thermal acclimation on hypoxia thresholds from measurements at respective acclimation temperatures ($T_t = T_a$). Crucian carp were acclimated to different T_a before MO_2 and P_c were tested at the respective temperature to which organisms had been acclimated ($T_t = T_a$). (A) MO_2 and P_c of crucian carp rise almost linearly with increasing temperature but a reduction in the rate of increase in P_c occurs after 20 ºC (B) P_c rises almost linearly with MO_2 but then plateaus as a result of acclimation in gill area occurring only between T_a = 20 - 25 ºC (adapted from Sollid *et al.*, 2005)

Adoption of classic methodology (Precht *et al.*, 1973; Prosser, 1973; Huey *et al.*, 1999) involving determination of hypoxic responses at standardised T_t following exposure to different T_a may perhaps provide the best method to distinguish what effect acclimation has on hypoxia thresholds. The most convincing support for using this approach comes from a study of the zebra mussel *Dreissena polymorpha,* exposed to three T_a (T_a = 5, 15 and 25 ºC)

before hypoxic responses (survival time under severe hypoxia in this case) were measured at three acute T_t (T_t = 5, 15 and 25 ºC) in a fully factorial experimental design (Matthews and McMahon, 1999). Survival time under hypoxia appeared to decrease with increasing temperature when comparing hypoxic survival time at the individuals' respective acclimation temperatures (T_a = T_t). While this could be taken to mean that warm acclimation is detrimental, the opposite was true for individuals acclimated to different T_a tested at the same T_t. Individuals acclimated to T_a = 25 ºC showed greatest hypoxia tolerance at T_t = 15 and 25 ºC, while individuals where T_a = 5 ºC were the least tolerant at these T_t. Hypoxic survival time was unaffected by T_a at T_t = 5 ºC (Fig. 1.7). Warm acclimation, therefore, had no detrimental effect at any T_t and may have even improved hypoxia tolerance under the majority of test conditions. This provides the clearest example of how measurement of hypoxic responses solely where T_a = T_t can be misleading when trying to test whether acclimation is beneficial or not.

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Fig. 1.7 Effects of temperature acclimation (T_a) and acute test temperature (T_t) on survival time under severe hypoxia in the zebra mussel, *Dreissena polymorpha*. Individuals acclimated to T_a = 5, 15 and 25 °C were exposed to severe hypoxia (< 3 % a.s.) at T_t = 5, 15 and 25 °C in a fully factorial experiment. Declines in survival occur with increases to T_t in individuals incubated at the same T_a (marked by red arrows for animals incubated at T_a = 15 °C). Warmer T_a improved hypoxic performance at T_t = 15 and 25 °C but T_a has no significant effect at T_t = 5 °C (black arrows) (adapted from Matthews and McMahon, 1999)

The number of studies that have used the approach testing for beneficial acclimation is limited and, to my knowledge, restricted to the studies outlined in

this review. These provide evidence to suggest that warm acclimation can be beneficial for hypoxic performance. Warm acclimation has only been found to be directly detrimental for one tested mollusc species, where the ability to oxyregulate was impaired by warm acclimation across multiple T_t (Alexander and McMahon, 2004). In contrast, warm acclimation has been noted to improve hypoxic performance in several fish species. In killifish, time to loss of equilibrium under hypoxia increased in warm acclimated animals compared to individuals acutely exposed to warm T_t (McBryan *et al.*, 2016). This was attributed to gill remodelling similar to crucian carp and appears to be an important mechanism in fish for dealing with the combined challenge of chronic warming and hypoxia (Sollid *et al.*, 2003, 2005). Time to loss of equilibrium also increases in warm acclimated compared to cold acclimated salmon at standard T_t and may be associated with changes to cardiac performance (Anttila *et al.*, 2015), When T_t = 25 ºC, triplefin fish *Bellapiscis lesleyae*, kept at T_a = 20 ºC displayed a lower P_c compared to individuals where T_a = 15 ºC (Hilton *et al.*, 2008). These studies could also indicate some support for the OCLTT, which predicts some alleviation of oxygen limitation following warm acclimation (Pörtner, 2010).

In addition, species which exhibit a limited physiological capacity for thermal acclimation do not appear to benefit from an improvement in hypoxic performance, e.g. oxyregulation in two species of tropical fish, *Pomacentrus moluccensis* and *Apogon* (as *Ostorhinchus) doederleini*, and the brown mussel *Perna perna* (Hicks and McMahon, 2002; Nilsson *et al.*, 2010). For these species, changes to hypoxia tolerance and performance were attributable entirely to T_t. The absence of acclimation capacity may be detrimental in the

long-term and render them vulnerable to future climate change as their current physiological responses are insufficient to deal with change (Stillman, 2003). However, some species which show an ability to acclimate to increased temperature may not undergo changes to hypoxic performance. In the Asian clam *Corbicula fluminea*, a species which can acclimate to raised temperature, warm acclimation neither improved or impaired hypoxic survival time (Matthews and McMahon, 1999).

1.4.3 Will thermal acclimation benefit hypoxic performance of marine ectotherms in a chronically warming world?

Due to the paucity of data and studies using appropriate experimental designs, it is currently difficult to draw any broad conclusions of what thermal acclimation does to hypoxia thresholds. The few available studies reviewed here seem to suggest that some fish species show beneficial effects on hypoxic performance if given time to acclimate to raised temperatures, which may be mediated through changes to enhance oxygen uptake at the respiratory surfaces (Sollid *et al.*, 2005; McBryan *et al.*, 2016). Interestingly, fish have been identified as the most sensitive marine phylum to hypoxia in isolation (Vaquer-Sunyer and Duarte, 2008) but not to the combination of warming and hypoxia, which may perhaps be associated with acclimation capacity (Vaquer-Sunyer and Duarte, 2011). While crustacean species are thought to be the most sensitive marine taxa to hypoxia and warming, as they show the greatest increase in median lethal O_2 concentration with warming (Vaquer-Sunyer and Duarte, 2011), no studies to date have used the appropriate methodological framework (Huey *et al.*, 1999) to explicitly test for beneficial or detrimental effects of warm

acclimation on hypoxia thresholds. For molluscs, there is mixed evidence for beneficial acclimation of hypoxia thresholds with effects ranging from beneficial to detrimental, including no effect, observed in the species tested so far (Matthews and McMahon, 1999; Hicks and McMahon, 2002). The mechanisms driving these differing effects of thermal acclimation on hypoxic performance are largely unknown. Despite a relatively good understanding of the physiological and biochemical mechanisms of thermal acclimation under normoxic conditions (Prosser, 1973), empirical evidence is lacking of how these mechanisms are subsequently affected by hypoxia when tested at standardised T_t post-acclimation. Such an understanding will aid prediction of when warm acclimation will positively or negatively affect hypoxia thresholds.

Although studies are limited, it is clear that acclimation can be beneficial for hypoxic performance in some marine species, which is in stark contrast to the relatively consistent detrimental effects of acute warming (Herreid, 1980). This provides some support for the BAH, but it is not clear if acclimation will be able to fully compensate for the detrimental effects of raised acute thermal conditions on hypoxic performance (Herreid, 1980). MO_2 under normoxic conditions is typically only partially compensated by acclimation (Seebacher *et al.*, 2015), but warm acclimation may, at least, allow some recovery of hypoxic performance in a warming ocean. The degree of recovery will likely vary considerably between species (McBryan *et al.*, 2013) and may have significant fitness implications determining so called ‘winners’ (survivors) and ‘losers’ (Somero, 2010). However, the ability for thermal acclimation to improve hypoxia thresholds remains to be empirically tested for the majority of marine animals (McBryan *et al.*, 2013) and is urgently required to assess the vulnerability of marine

ectotherms to predicted expansions in hypoxic regions under future climate change (Breitburg *et al.*, 2018).

1.5 Conclusions

There is a good understanding of the physiological and biochemical effects of hypoxia in isolation but the underpinning molecular mechanisms are still unexplored for most marine animals, particularly invertebrates (Spicer, 2014), and largely limited to severe hypoxia (Spicer, 2016). Perhaps as a result, the range of responses measured under the combination of temperature and hypoxia are more limited in scope, being almost exclusively restricted to P_c and mortality. The effects of acute warming are fairly consistent, tending to raise P_c for many organisms (Herreid, 1980). The physiological effects of thermal acclimation under normoxic conditions and methodology for testing for acclimation effects are well established (Prosser, 1973; Huey *et al.*, 1999; Schulte *et al.*, 2011). Acclimation may offset the effects of acute temperature change and stabilise physiological rates such as MO_2 in thermally variable environments (Seebacher *et al.*, 2015). However, the effects of thermal acclimation on hypoxic performance remain unclear due to insufficient experimental work and appropriate experimental design. When disentangled from the effects of acute warming, warm acclimation tends to improve hypoxic performance of some, but not all, species. The hypoxic response of marine ectotherms in the wild cannot be predicted solely from the effects of acute warming and the modifying effects of thermal acclimation need to be more rigorously tested before any attempt can be made to predict the consequences of chronic warming and hypoxia on marine ecosystems.

1.6 Thesis aims and objectives

Given the scarcity of data on how thermal acclimation impacts the responses to low oxygen highlighted in this chapter, the aim of this thesis is to investigate the effects of thermal acclimation on hypoxia thresholds. The objectives in order to meet this aim are as follows:

1. To characterise the integrated mechanisms underpinning the response to hypoxia in isolation to provide a greater understanding of the systems upon which thermal acclimation could act. In Chapter 2, the metabolic (resting and active MO_2, aerobic scope), respiratory (ventilation, circulation), biochemical (anaerobic metabolism), and transcriptomic responses to different levels of hypoxia (40 % and 20 % a,s.) are investigated.

2. Secondly, to directly test if thermal acclimation is beneficial or detrimental for physiological hypoxia thresholds. In Chapter 3, individuals belonging to closely related species were exposed to different T_a (T_a = 10 or 20 °C) before determination of P_c under acutely declining PO_2 in individuals compared at multiple standardised T_t (T_t = 10 or 20 °C). Beneficial or detrimental effects of warm acclimation on hypoxia thresholds are investigated within species and compared across species.

3. Lastly, to characterise the underlying mechanisms by which thermal acclimation modifies hypoxic performance. In Chapter 4, the transcriptomic mechanisms supporting metabolic performance under hypoxia (30 % a.s.) are compared between individuals at a standardised T_t (T_t =10 °C) following acclimation to different T_a (T_a = 10 or 20 °C).

Gammarid amphipods are the models used throughout this thesis as their physiological responses to hypoxia (Sutcliffe, 1984; Agnew and Taylor, 1985; Toman and Dall, 1998; Spicer *et al.*, 2002) and temperature (Dorgelo, 1973; Bulnheim, 1979; Whiteley *et al.*, 2011; Rastrick and Whiteley, 2017) in isolation are reasonably well known. This includes metabolic responses (MO_2) under declining oxygen tensions (Sutcliffe, 1984; Verberk *et al.*, 2018) and in response to thermal acclimation under normoxic conditions (Bulnheim, 1979; Rastrick and Whiteley, 2011). They have similar morphologies and their phylogeny has received attention (Costa *et al.*, 2009; Hou and Sket, 2016). Their genomic responses to environmental stress have only received limited attention due to a paucity of transcriptomic and genomic data with assembled transcriptomes only existing for a small number of species (Truebano *et al.*, 2013; Bossus *et al.*, 2014; Trapp *et al.*, 2014; Gismondi and Thomé, 2016). The genomic responses of amphipods to environmental conditions warrant further attention given they are often abundant and ecologically important ”shredder” species/detritivores and prey for other invertebrates and fish (Lincoln, 1979). They are also widely distributed latitudinally and can be found in a range of habitats from fresh to fully marine conditions (Whiteley *et al.*, 2011).

Chapter 2 The integrated responses to hypoxia in isolation: is there a threat to coastal species from modest reductions in environmental oxygen?

Sections of this chapter have been published in:

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Collins, M., Tills, O., Turner, L.M., Clark, M.S., Spicer, J.I. and Truebano, M. (2019) Moderate reductions in dissolved oxygen may compromise performance in an ecologically-important estuarine invertebrate, 693, 133444 doi: 10.1016/j.scitotenv.2019.07.250

Please note that the RNA extractions, transcriptome assembly, annotation and counts data were generated by Dr Oliver Tills and Dr Manuela Truebano. I performed downstream transcriptome analyses (assembly statistics generation, differential expression, functional enrichment), biochemical and physiological analyses.

Abstract

Coastal ecosystems are increasingly pressured by expanding hypoxic regions. Ecologically, hypoxia is defined as < 2 mL O_2 L^{-1}, based upon avoidance behaviour and mass mortalities of benthic fauna. However, the use of such a 'limit' is contentious, given that taxa vary markedly in their sensitivity to environmental hypoxia, with physiological effects and life history consequences already apparent at higher dissolved oxygen levels. The physiological and cellular responses of the brackishwater amphipod *Gammarus chevreuxi* was investigated in response to an ecologically-relevant level of moderate hypoxia experienced in its natural habitat (40 % a.s. or ~ 2.6 mL O_2 L^{-1}) or severe hypoxia (20 % a.s. or ~ 1.3 mL O_2 L^{-1}), consistent with ecological thresholds. While moderate hypoxia elicited widespread, complex changes at the molecular level, these did not offset a significant decline in aerobic scope. Under severe hypoxia, marked physiological responses associated with maintenance of aerobic performance were accompanied by a dramatic reduction in changes at the molecular level, most of which indicate recourse to hypometabolism and/or physiological impairment. Adverse effects of hypoxia may therefore occur above a threshold value of 2 mL O_2 L^{-1}. Therefore, the severity of environmental oxygen reduction experienced *in situ* should be considered to understand and predict the effects of hypoxia on coastal invertebrates.

2.1 Introduction

Shallow coastal ecosystems commonly experience natural episodes of reduced oxygen (hypoxia) (Diaz and Rosenberg, 1995) but are pressured by increasing severity and duration of hypoxia driven by nutrient pollution (eutrophication) and climate change (Breitburg *et al.*, 2018). Ecologically, hypoxia has been defined by 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, there has been considerable discussion about the use and magnitude of such a 'limit' given that taxa vary markedly in their sensitivity to reduced environmental oxygen levels (Vaquer-Sunyer and Duarte, 2008). Therefore, the case has been made for the incorporation of physiological evaluations in hypoxia studies to directly characterise the effects of oxygen depletion at a species-specific level (Farrell and Richards, 2009; Seibel, 2011).

For almost all animal life, oxygen is important in sustaining essential metabolic processes (Semenza, 2007) and aerobic capacity is a critical factor in the maintenance of animal performance, not only for physiological factors such as growth and reproduction, but also activity levels and hence outcomes of ecological interactions (Pörtner, 2010). Hypoxia has been defined for deep-sea species by Seibel (2011) based upon the inhibition of resting rates of oxygen consumption (MO_2), marked by a critical oxygen tension (P_c), which correlate relatively well with the lowest partial pressure of oxygen (PO_2) experienced by species in nature. However, both ecological and physiological approaches have characterised hypoxia using rather severe thresholds at the levels of the individual and community, respectively. More 'moderate' levels of low oxygen above 2 mL O_2 L^{-1} and/or an organism's P_c, which would not be considered

hypoxic under current definitions, are prevalent in nature (Farrell and Richards, 2009; Spicer, 2016).

Mass mortality related to severe hypoxia in nature (Diaz and Rosenberg, 1995) is thought to be driven by disruption of MO_2 at an individual level, which compromises essential cellular energy stores (ATP) and leads to 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 limited number of crustacean species appears to support this paradigm but requires assessment for a wider variety of species (Richards, 2009; Rathburn *et al.*, 2013). 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 efficiency and oxygen transport by respiratory pigments, increase anaerobic (glycolytic) ATP production and prevent cellular damage (Nikinmaa and Rees, 2005; Larade and Storey, 2009; Richards, 2009).

While the physiological effects of severe hypoxia have been relatively well characterised in coastal species, the responses to moderate hypoxia are more widespread but less understood (Spicer, 2016). Many taxa experience sublethal effects above 2 mL O_2 L^{-1} including altered activity, growth, reproduction and ecological interactions such as changes to predation rates (Vaquer-Sunyer and Duarte, 2008). Furthermore, differences in species' physiological tolerance of moderate hypoxia has been suggested to drive changes in estuarine

assemblages (Froehlich *et al.*, 2015). A greater understanding of the mechanisms used by coastal organisms to deal with moderate hypoxia is required to predict how individuals, species, communities and ecosystems will be affected by the broad spectrum of chronically reduced oxygen levels predicted under climate change (Keeling *et al.*, 2010; Breitburg *et al.*, 2018). Moderate hypoxia does not typically disrupt resting MO_2 and the limited evidence available at the molecular level indicated that it may elicit a minimal response in terms of global gene expression (Brouwer *et al.*, 2007). However, the threat of moderate hypoxia should not be disregarded as it will directly impact aerobic scope which underpins many facets of fitness and ecological performance (Pörtner, 2010). Aerobic scope may become limited by the increased challenge of sustaining resting MO_2 (Farrell and Richards, 2009), achieved through raised ventilation rates and/or longer term adjustments to oxygen transport by respiratory pigments (McMahon *et al.*, 1974; Pan *et al.*, 2017). The effects of hypoxia on aerobic scope and resulting consequences for ecosystem function have received considerable attention in fish (Farrell and Richards, 2009; Pörtner, 2010) but have not been well characterised for most ecologically-important coastal invertebrates.

Given the prevalence of moderate hypoxia in estuarine ecosystems and the predicted increase in both its severity and duration (Breitburg *et al.*, 2018), this multidisciplinary study investigated the physiological and cellular 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 an assembled transcriptome is available for this species but

for embryonic stages (Truebano *et al.*, 2013). 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}) and severe hypoxia (20 % a.s., ~ 1.3 mL O_2 L^{-1}). Responses to different levels of hypoxia at the organism level were characterised by measurement of resting and active MO_2, 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, in order to determine what mechanisms are differentially regulated by moderate and severe hypoxia, and impact physiological performance.

2.2 Methods

2.2.1 Sampling site and pre-exposure conditions in the laboratory

Gammarus chevreuxi were collected using a hand-held net from the Plym estuary, Devon (50.391290 N, -4.084795 W). Dissolved oxygen levels were measured on one day at low tide using a hand-held dissolved oxygen probe (ProDO 2030, YSI Inc., Ohio, USA). 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 = 7.8 ± 1.7 mg, mean ± s.e.m.) were used in the experiments described below.

2.2.2 Exposure to different levels of hypoxia

Exposure to different levels 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, mean ± s.e.m), moderate hypoxia (40 % a.s.: 39.1 ± 0.7 % a.s, mean ± s.e.m) 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, mean ± s.e.m) consistent with ecological hypoxia thresholds. Other environmental factors were kept constant (T = 14.2 ± 0.1 °C, S = 14.7 ± 0.1, mean ± s.e.m, 12 h L: 12 h D).

Different levels of hypoxia were produced by aspirating a gas mixture, constructed from nitrogen and “carbon dioxide-scrubbed” air (air that had been previously bubbled through 1 M NaOH) 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, USA). Temperature and PO_2 in aquaria waters were recorded daily using an oxygen microsensor (Pm-Pst7, Presens, Germany) and temperature probe (Pst 100, Presens, Germany) coupled to a dissolved oxygen meter (Microx 4, Presens, Germany). Salinity was measured every 1 - 2 d using a refractometer (HI96822 Digital Refractometer, Hanna Instruments Ltd., 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.

2.2.3 Physiological responses to different levels of hypoxia

Upon removal from the different oxygen regimes, key metabolic and physiological responses of individuals were quantified. Aspects of organismal performance (resting and active MO_2, aerobic scope) were characterised and the supporting oxygen supply systems (ventilation by beating of the pleopods and circulation by heart rate). Anaerobic metabolism was also investigated by measuring the concentration of the anaerobic metabolite, L-lactate.

Animals were starved for 12 h prior to MO_2 measurements. To measure MO_2, 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, in common with many amphipod species, are least active (J.I. Spicer, pers. obs.). Each envelope was then transferred to a holding aquarium (vol. = 5 L), containing sea water at the appropriate PO_2 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 PO_2 (% a.s.) within the chamber was recorded using a needle-type oxygen micro-sensor (NTH-PSt7, Presens, Germany) connected to an oxygen meter (Microx 4, Presens, Germany). The chamber was then sealed, gently transferred to a water bath (T = 15 °C) and the individuals were kept for 2 h, after which period chambers were mixed by inversion and the PO_2 within the chamber were measured again as described. The start and end PO_2 within the respirometers was as follows: 100% a.s.: ~ 96% - 81%, 40% a.s.: 39 - 27% a.s.,

20% a.s.: ~ 22 - 10% a.s., MO_2 under resting conditions was calculated from the difference between water PO_2 at the beginning and at the end of the experiment. Data are expressed as $\mu L\ O_2\ g\ wet\ mass^{-1}\ h^{-1}$ STP.

To estimate MO_2 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 PO_2 within the chamber was then remeasured as previously described and the aerobic scope was calculated by subtracting resting from active MO_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, 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 quiescent 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 (n = 2 for 15 s, in each case) under low power magnification (x 10) using a light microscope (MZ15, Leica Microsystems Ltd, UK).

2.2.4 Biochemical responses to different levels of hypoxia

Frozen individuals (wet mass = 7.7 ± 1.8 mg, mean ± s.e.m) were sonicated (60 % amplification for 60 s) in 50 µL of 10 % TCA (Fisher Scientific Ltd., UK). The

concentration of L-lactate was quantified using a commercially-available lactate assay kit (Lactate Kit 735-10, Trinity Biotech, Bray, Ireland). The kit relies upon the conversion of lactate by the enzyme lactate oxidase to pyruvate and hydrogen peroxide. The hydrogen peroxide causes oxidative condensation of chromogen precursors, producing an increase in absorbance at λ = 540 nm. 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, USA) and calibrated against standards (Lactate Standard Solution 826-10, Trinity Biotech, Ireland).

2.2.5 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 ($P > 0.05$). Eight one-way ANOVA were utilised to test for the effect of oxygen regime (100, 40 and 20 % a.s.) on (1) resting MO_2, (2) resting pleopod rate, (3) resting heart rate, (4) active MO_2, (5) active pleopod rate, (6) active heart rate, (7) lactate concentration of active animals and (8) aerobic scope. 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.

2.2.6 Transcriptomic responses to different levels of hypoxia

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 animals per treatment, 10 individuals per

pool, using the PureLink RNA Mini Kit (Ambion, USA) with a TRIzol step. RNA integrity was determined using a Bioanalyzer (Agilent Technologies, USA). TruSeq RNA libraries (Illumina, San Diego, USA) were synthesised and sequenced 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. Sequencing produced 227.1 M 100 base paired-end reads. *De novo* transcriptome assembly was performed using the Trinity pipeline (v 2.2.0, with the parameters –trimmomatic, for adapter trimming, and –normalise reads, for digital normalisation) (Haas *et al.*, 2013). Contigs were annotated using Trinotate (v 3.0.0, www.trinotate.github.io) with an e-value cut-off of 1e-05.

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 focused upon and genes which had received attention in other hypoxia studies of crustaceans (Brouwer *et al.*, 2007). 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)).

2.3 Results

2.3.1 Oxygen levels at sample site

The site from which *G. chevreuxi* were collected experiences considerable variation in PO_2 from normoxia (102 – 106 % a.s.) within the main river channel to hyperoxia (up to 134 % a.s.) in areas of high algal density. Different levels of hypoxia are present in small pools isolated from the river channel at low tide (18 – 35 % a.s.) and regions of the main channel of low flow (13 – 55 % a.s.).

2.3.2 Physiological and biochemical responses to different severities of low oxygen

For quiescent individuals, there was no significant effect of exposure to either moderate (40 % a.s) or severe (20 % a.s.) hypoxia on MO_2 compared to normoxia ($F_{2,19} = 1.51$, $P = 0.246$, Fig. 2.1A). Ventilation rate only increased during exposure to severe hypoxia ($F_{2,19} = 5.79$, $P = 0.011$, Fig. 2.1B) but heart rate decreased significantly upon exposure to both hypoxia treatments for 7 d ($F_{2,16} = 11.60$, $P < 0.001$, Fig. 2.1C). For active individuals, MO_2 was significantly lower in individuals exposed to both moderate ($P = 0.013$) and severe ($P < 0.001$) hypoxia compared to those under normoxic conditions ($F_{2,17}$

= 15.82, P < 0.001, Fig. 2.1D). For active individuals, there was no effect of hypoxia exposure on either ventilation rate ($F_{2,16}$ = 1.40, P = 0.275, Fig. 2.1E) or heart rate ($F_{2,16}$ = 0.04, P = 0.966, Fig. 2.1F). Significant reductions in aerobic scope ($F_{2,17}$ = 17.25, P < 0.001, Fig. 2.1G) occurred under both moderate (P = 0.009) and severe (P < 0.001) hypoxia and were associated with a significant increase in L-lactate concentration ($F_{2,13}$ = 5.28, P = 0.021, Fig. 2.1H) in individuals exposed to moderate (P = 0.026), but not severe hypoxia (P = 0.726). The calculated negative value for aerobic scope observed under 20 % a.s. was not significantly lower than zero (one sample T-test, T_6 = -0.65 , P = 0.268) and so it was concluded that there was zero aerobic scope under severe hypoxia (Claireaux and Chabot, 2016).

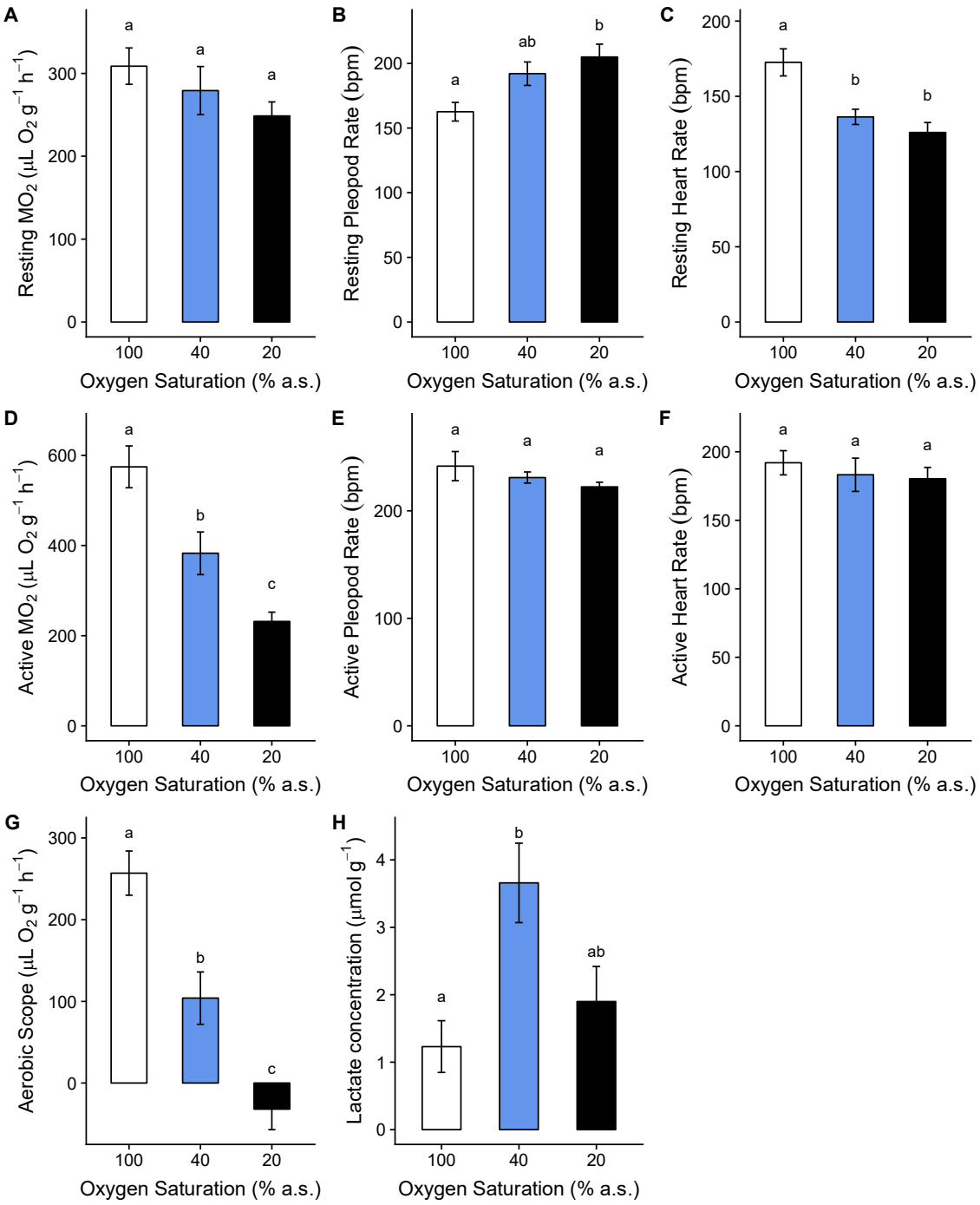

Fig. 2.1 The physiological effects of 7 d exposure of *G. chevreuxi* to normoxia (100 % a.s.), moderate hypoxia (40 % a.s.) or severe hypoxia (20 % a.s.). (A) resting MO_2 (n = 7 – 8 animals per treatment) (B) resting pleopod rate (n = 7 – 8 animals per treatment) (C) resting heart rate (n = 6 – 8 animals per treatment) (D) active MO_2 (n = 5 – 8 animals per treatment) (E) active pleopod rate (n = 5 – 8 animals per treatment) (F) active heart rate (n = 5 – 8 animals per treatment) (G) aerobic scope (n = 5 – 8 animals per treatment) (H)

lactate concentration of active individuals (n = 4 – 7 animals per treatment) (mean values ± s.e.m). Letters indicate significant differences between treatments identified by ANOVA and *post-hoc* Tukey test ($P < 0.05$).

2.3.3 The assembled transcriptome of *G. chevreuxi*

The assembled transcriptome consisted of 291,934 transcripts belonging to 218,558 genes (Trinity genes). Of the assembled transcripts, 24,030 were annotated using the gene ontology (GO) database and corresponded to 12,400 unique GO terms. The top fifteen GO terms and the percentage of transcripts mapped to each term are presented (Fig. 2.2). For further assembly and annotation statistics, see Appendix 1 (Table A1.1 and A1.2).

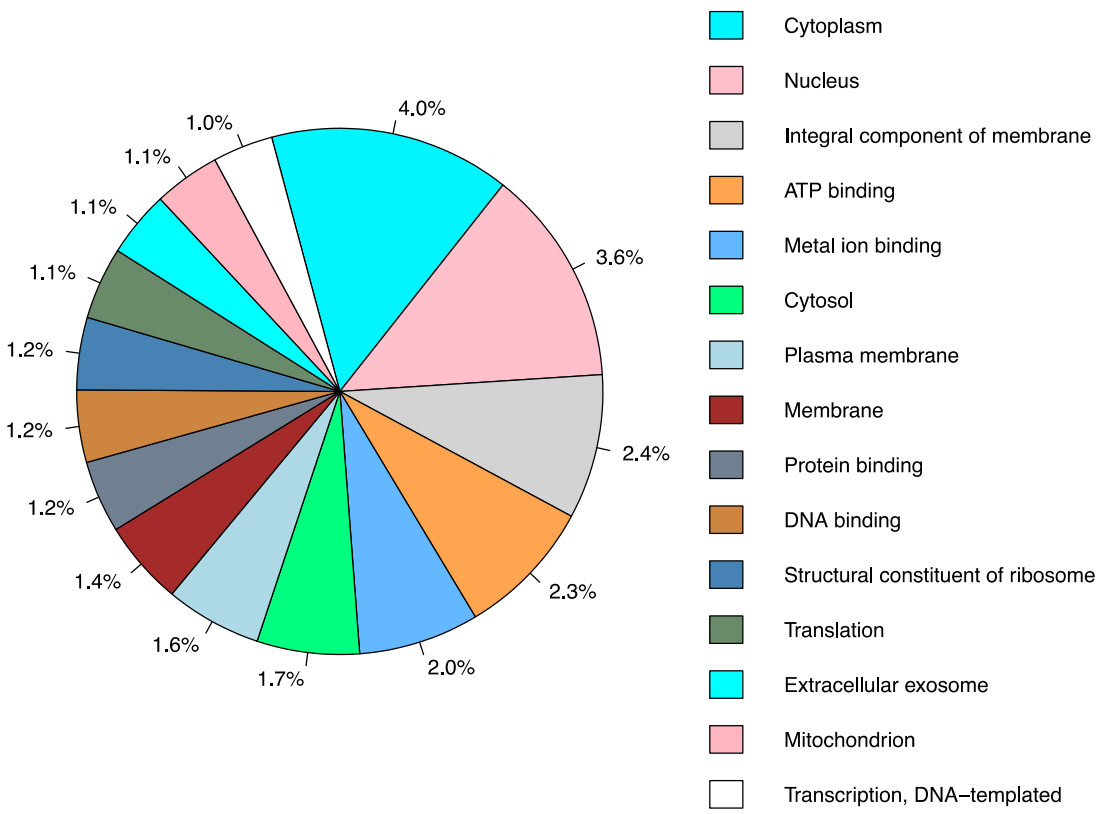

Fig. 2.2 Top fifteen GO terms of the *G. chevreuxi* transcriptome. Transcripts mapping to the top fifteen GO terms expressed as a percentage of all transcripts generated by the assembly

2.3.4 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. 2.3A). 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 upregulated. In comparison, a more limited transcriptional response was observed in individuals exposed to 20 % a.s. compared to the normoxic controls, with 1,721 significantly differentially expressed unique genes, 52 % of which were upregulated (Fig. 2.3B).

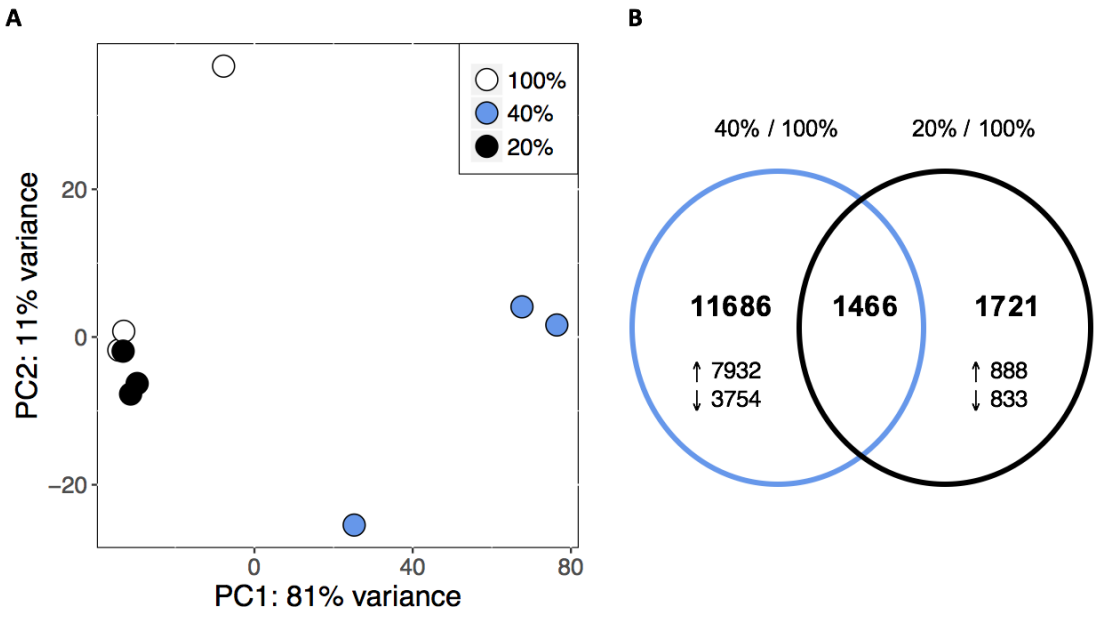

Fig. 2.3 Transcriptomic responses to moderate (40 % a.s.) and severe hypoxia (20 % a.s.). (A) Principal components 1 and 2 from principal component analysis performed using variance stabilised counts of all tested

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-regulation and down-regulation respectively in each treatment compared to the normoxic control.

Functional enrichment analysis of significantly upregulated genes following exposure to moderate hypoxia (40 % a.s.) compared to normoxia identified 23 significantly affected KEGG pathways ($P_{adj} < 0.05$) (Fig. 2.4). 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 transport by respiratory pigments, amongst others (Fig. 2.5A, Table A1.3). Downregulated genes under moderate hypoxia compared to normoxia were significantly enriched for GO terms involved in muscle structure (Fig. 2.5B, Table A1.3).

In response to severe hypoxia, upregulated DEGs were significantly enriched for multiple GO terms involved in chitin metabolism (Fig. 2.6A, Table A1.4). Coagulation was the only KEGG pathway significantly enriched in this group. Downregulated DEGs under severe hypoxia were significantly enriched for chitin metabolism, protein degradation and glucose metabolism GO terms (Fig. 2.6B, Table A1.4). Ribosomal pathways were the only significantly affected KEGG pathway for downregulated genes under 20 % a.s ($P_{adj} < 0.05$).

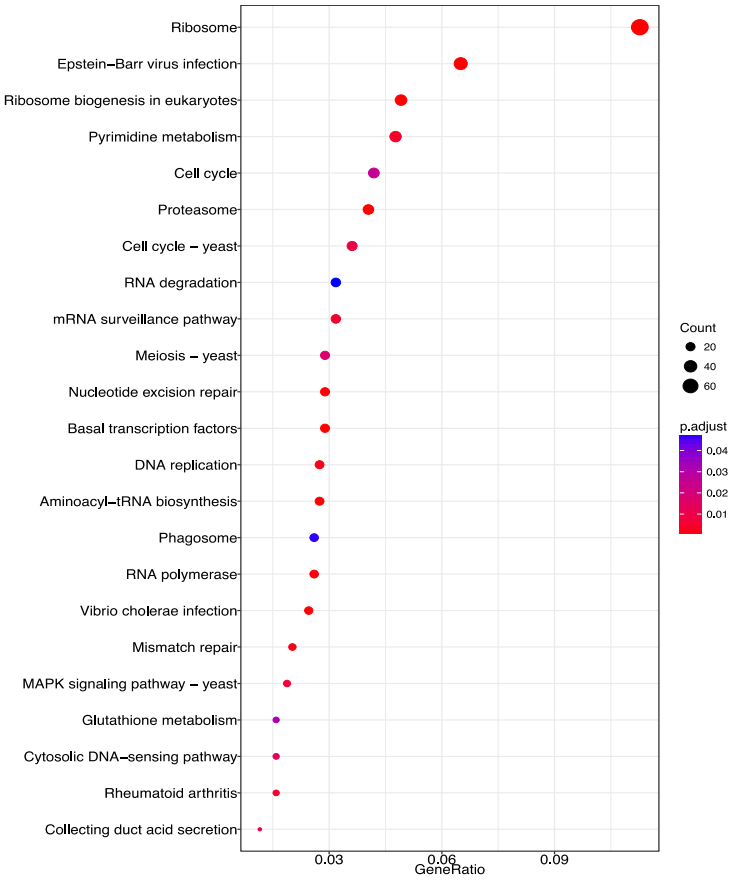

Fig. 2.4 Dotplot showing the 23 significantly enriched KEGG pathways in upregulated DEGs for *G. chevreuxi* exposed to 40 % a.s. for one week in comparison to normoxic controls. Gene ratio indicates the ratio of DEGs mapping to the pathway out of all DEGs. Diameter of the dot indicates the number of genes assigned to that pathway, colour (red to blue) represents the significance of enrichment (P_{adj}).

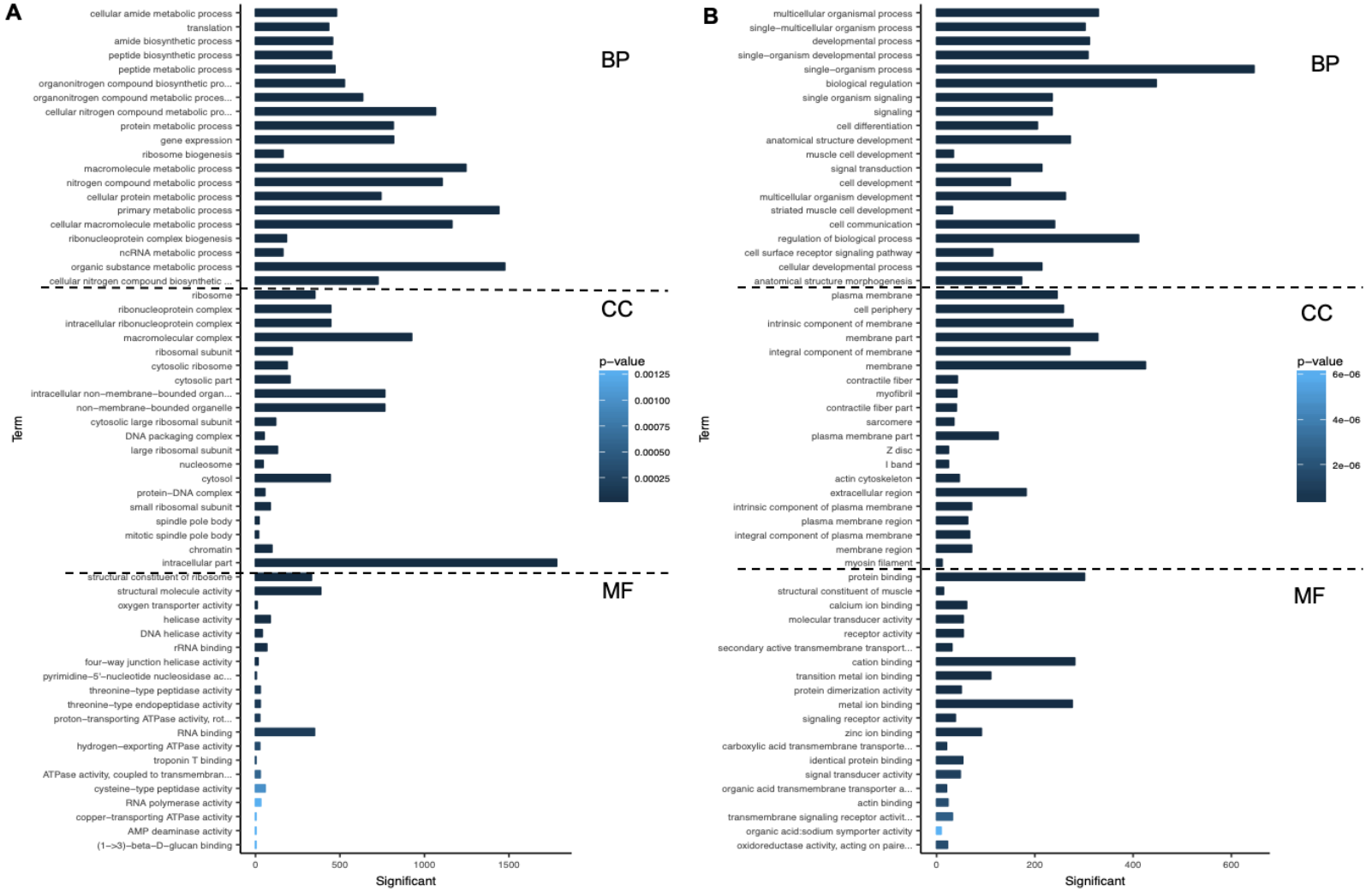

Fig. 2.5 The top 20 significantly enriched GO terms following exposure of *G. chevreuxi* to 40 % a.s. compared to 100 % a.s. Enriched GO terms are shown for (A) upregulated DEGs ($P_{adj} < 0.01$, log-2 FC > 1). and (B) downregulated DEGs ($P_{adj} < 0.01$, log-2 FC < -1). Enriched GO terms are shown for biological processes (BP), cellular components (CC) and molecular function (MF). Data show the number of significant genes belonging to each GO category and significance of enrichment (P_{adj}) is indicated by bar colour.

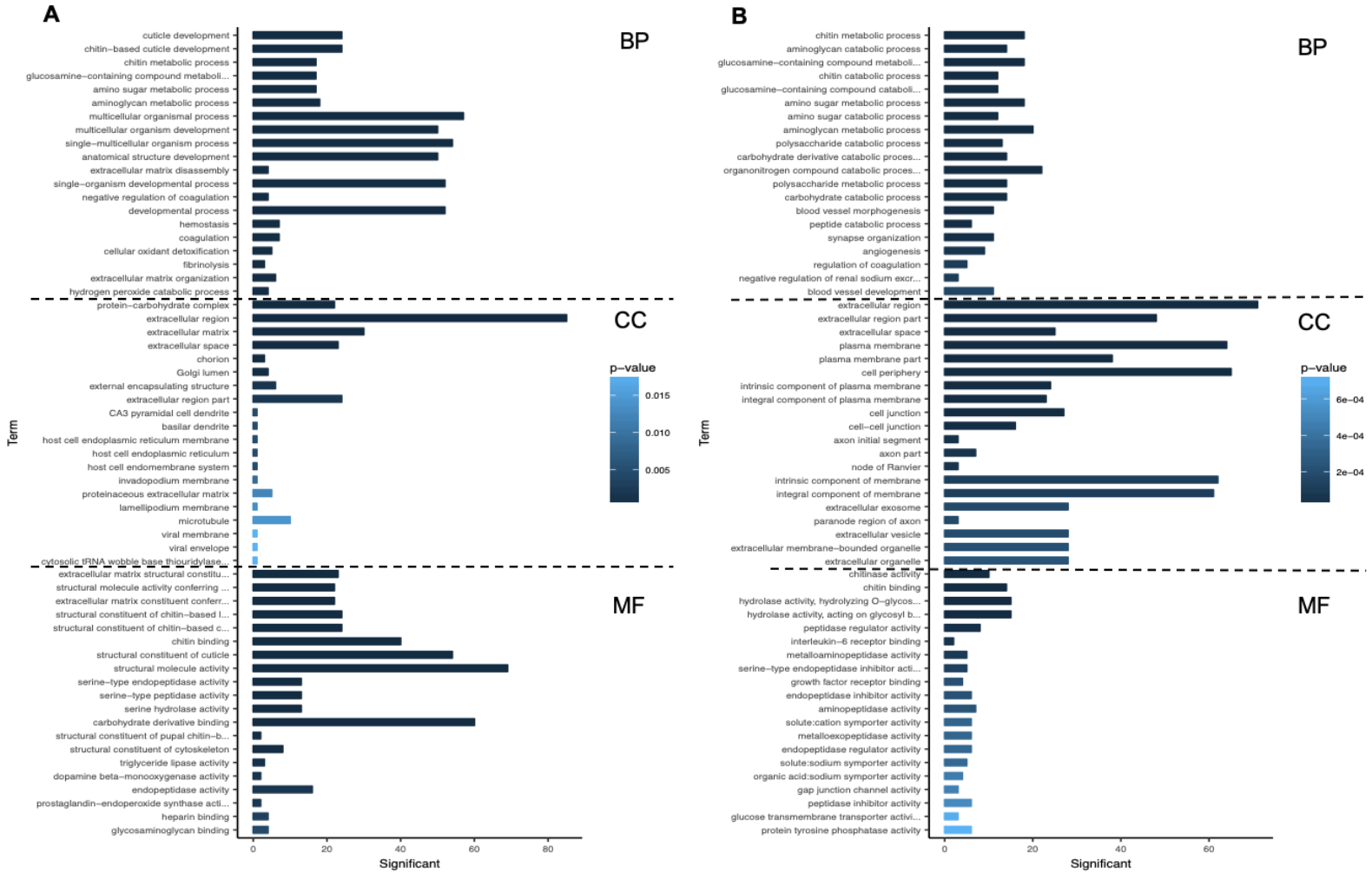

Fig. 2.6 The top 20 significantly enriched GO terms following exposure of *G. chevreuxi* to 20 % a.s. compared to 100 % a.s. Enriched GO terms are shown for (A) upregulated DEGs ($P_{adj} < 0.01$, log-2 FC > 1). and (B) downregulated DEGs ($P_{adj} < 0.01$, log-2 FC < -1). Enriched GO terms are shown for biological processes (BP), cellular components (CC) and molecular function (MF). Data show the number of significant genes belonging to each GO category and significance of enrichment (P_{adj}) is indicated by bar colour.

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

Metabolic genes including hemocyanin (Fig. 2.7A), multiple glycolytic enzymes (Fig. 2.7B), TCA cycle enzymes (Fig. 2.7C), and mitochondrial subunits (Fig. 2.7D) 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 upregulated under moderate hypoxia. Upregulated metabolic enzymes included five transcripts annotated as isocitrate dehydrogenase (IDH) and the glycolytic enzyme phosphofructokinase (PFK). Several mitochondrial ETC complexes were upregulated, 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 upregulated under 40 % a.s. including two contigs annotated as catalase and seven contigs annotated as superoxide dismutase isoforms (Fig. 2.7E). Under severe hypoxia, a significant reduction in the expression of one hemocyanin contig occurred (Fig. 2.7A). Metabolic genes such as PFK, IDH, ATPα and ATPß returned to baseline levels of expression in amphipods exposed to 20 % a.s. (Fig. 2.7B-D). Cellular antioxidants also mostly returned to a baseline level of expression but six glutathione-S-transferases were significantly downregulated in amphipods exposed to severe hypoxia (Fig. 2.7E). 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. 2.7E).

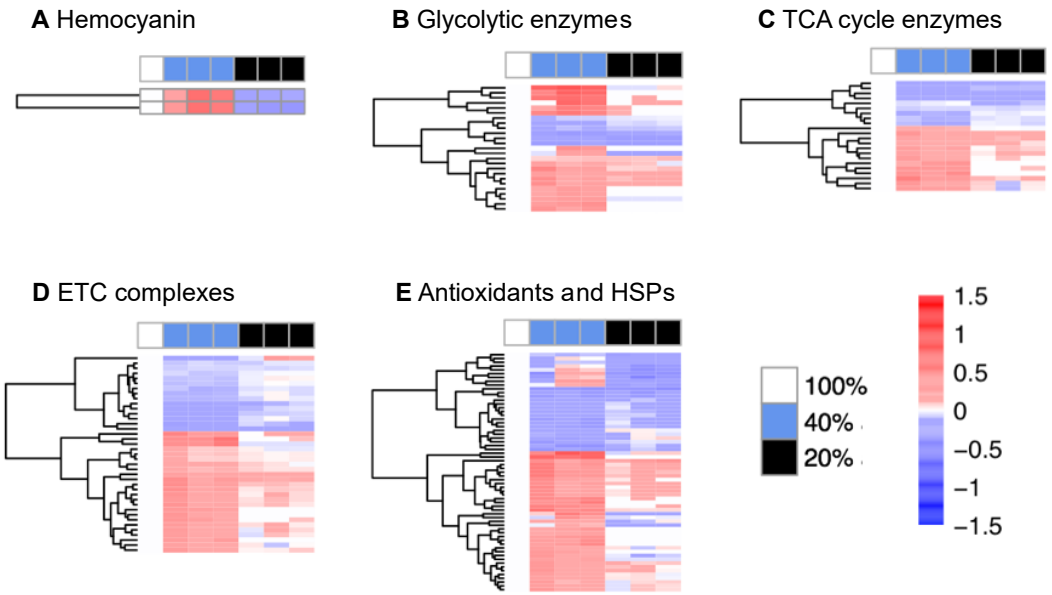

Fig. 2.7 Heat map of log-2 fold changes of DEGs (P_{adj} < 0.05) for moderate and severe hypoxia (*n* = 3 pools of 10 animals per treatment) in comparison to the mean of the normoxic control (100 % a.s.). Counts were subjected to variance stabilising transformation using the DESeq2 library prior to calculation 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. The full list of contigs contained within (A-E) can be found in Table A1.5.

2.4 Discussion

Hypoxia, of different severities, is ubiquitous in estuarine ecosystems (Diaz and Rosenberg, 1995). Many species experience moderate hypoxia but its effects remain poorly characterised (Spicer, 2016). In this study, the physiological and cellular responses of the brackishwater invertebrate *Gammarus chevreuxi* to moderate hypoxia, experienced seasonally in this species' estuarine habitat, to responses elicited by severe hypoxia were compared. Despite the limited effect

of moderate hypoxia on resting MO_2, there was a widespread reorganisation at the cellular level and a significant reduction in aerobic scope. The mechanisms differed markedly from those utilised under severe hypoxia. Although resting MO_2 was still regulated at the physiological level, there were biochemical and molecular responses observed which may indicate a hypometabolic strategy, characterised by limited recourse to anaerobic metabolism and a significant down-regulation of genes involved in protein synthesis.

Hypoxic responses have been shown to be dependent upon the type of hypoxia encountered *in situ*, e.g. cyclic vs static (Brown-Peterson *et al.*, 2008; Peruzza *et al.*, 2018), or acute vs prolonged exposure (Li and Brouwer, 2009; Truebano *et al.*, 2018). The current study emphasizes that hypoxic severity should also be considered when assessing the effects of hypoxia. This study demonstrated that coastal invertebrates rely on markedly different multilevel responses (molecular, biochemical and physiological) upon encountering different levels of hypoxia in the wild and the current accepted ecological threshold value of 2 mL O_2 L^{-1} means that it is possible to overlook the consequences of responses to less extreme and ecologically-relevant levels of moderate hypoxia in coastal species.

Moderate hypoxia did not disrupt the ability to regulate resting MO_2 despite a significant bradycardia and without recourse to a significant hyperventilatory response as previously observed in Truebano *et al.* (2018). 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). 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 next generation sequencing approach. The data presented here reveals a far more complex cellular response than previous studies on crustaceans (Brouwer *et al.*, 2007) with significant changes in the expression of over 13,000 genes compared to normoxia. Molecular changes included up-regulation of transcription and translation, suggesting that amphipods are having 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 in *G. chevreuxi* may be associated with the up-regulation of multiple genes involved in aerobic metabolism (TCA cycle enzymes and mitochondrial subunits), which could help maintain aerobic ATP generation in the mitochondria (Brouwer *et al.*, 2007). 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). In some species, hypoxia induces the production of respiratory pigment subunits of a higher oxygen affinity, lower P_{50}, which may aid oxygen loading at the respiratory surface (Pan *et al.*, 2017).

Despite an apparent cellular attempt to meet energetic demands aerobically, amphipods may be compromised by even fairly moderate levels of hypoxia. An up-regulation of glycolytic enzymes was observed, including the rate-limiting enzyme phosphofructokinase (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. Animals may be oxygen limited as

there was a significant decline in aerobic scope which may be compromised as a result of oxidative stress reflecting enhanced maintenance costs (Sokolova, 2013). This observation was supported by the enhanced expression of several key antioxidant enzymes, also observed in other marine invertebrates exposed to hypoxia (Clark *et al.*, 2013). 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 of moderate hypoxia on coastal invertebrates. For example, reduced fitness of *G. chevreuxi* under moderate hypoxia has been directly observed where the F_1 generation of hypoxia-treated parents displayed reduced size at hatching and impaired hypoxic performance (Truebano *et al.*, 2018).

Studies describing how coastal organisms respond to severe hypoxia at the physiological level predict limitation of resting MO_2 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 MO_2 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 MO_2 could be interpreted to mean that *G. chevreuxi* is fairly hypoxia tolerant and may be resilient to future increases in the severity of coastal hypoxia. However, unlike the situation in moderate hypoxia, regulation of resting MO_2 under severe hypoxia did not appear to be supported by changes at the molecular level, as indicated by the down-regulation of one hemocyanin gene and baseline levels of metabolic enzymes. In combination

with an extremely limited transcriptomic response, the gene expression profile may suggest the beginning of an alternate hypometabolic strategy under severe hypoxia. Molecular signatures of hypometabolism can arise even though resting MO_2 still appears to be regulated. This could reflect that 20 % a.s is approaching P_c for the species (approximately 12 % a.s. / 2.4 kPa) (Truebano *et al.*, 2018). Alternatively, it has been noted that at oxygen tensions approaching P_c, an increasing proportion of oxygen is utilised by ventilatory pumps leaving less oxygen to support cellular metabolism (McMahon, 1988; Wood, 2018). It is possible that this might occur in *G. chevreuxi* which displayed a hyperventilatory response by the pleopods potentially contributing to molecular indicators of suppressed cellular metabolism.

As gene expression was only measured at a singular time point (one week), it is possible that changes to gene expression could have occurred earlier on within the exposure before returning to a baseline level of expression. Molecular responses to hypoxia can occur rapidly within crustaceans under severe hypoxia being dynamic over hourly to daily scales (Li and Brouwer, 2009). Very few studies have investigated the temporal dynamics of exposure to different levels of hypoxia. Only in one crustacean species, *Palaemon* (as *Palaemonetes*) *pugio* has the time course of gene expression under moderate and severe hypoxia been investigated in the same study (Brouwer *et al.*, 2007). In *P. pugio,* only severe hypoxia but not moderate hypoxia elicited a marked transcriptional response (Brouwer *et al.*, 2007) in contrast to *G. chevreuxi*. It is possible there may be taxonomic differences in the transcriptional responses to hypoxia between amphipods and decapods but this is difficult to assess due to a paucity of molecular data for amphipod species.

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 (Spicer, 2014; Seibel *et al.*, 2018). 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. Active MO_2 was extremely limited/suppressed by severe hypoxia compared to normoxia despite an increase in heart rate resulting in zero aerobic scope. A similar response has been observed in fish where aerobic scope also declines 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 with no significant change occurring. However, this may reflect the limited changes to glycolytic enzyme expression in individuals exposed to severe hypoxia compared to those under moderate hypoxia. 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 downregulated 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 a muted antioxidant response was observed. 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 at the physiological level 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 (Abehsera *et al.*, 2015; Peruzza *et al.*, 2018). Although it can be difficult to directly attribute changes to chitin genes to growth as they are involved in several processes. Chitin is continually being broken down and resynthesized to provide a source of glucose (Stevenson and Hettick, 1980). Also it could reflect changes to exoskeleton composition and integrity which can be affected by environmental stress (Gagné *et al.*, 2005). 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 extremely detrimental to the persistence of this species.

There has been a paucity of data regarding the effects of moderate hypoxia on ecologically-important estuarine invertebrates. Here, it is demonstrated, through the adoption of a multilevel approach, that even moderate levels of hypoxia above 2 mL O_2 L^{-1} have implications for coastal organisms. Using a key brackishwater invertebrate as a model, it was shown that moderate hypoxia elicited an extensive transcriptional response representing a significant rearrangement of cellular metabolism to accommodate life under the new conditions. Thus, physiological performance of coastal invertebrates may begin to be compromised even at fairly moderate PO_2, as indicated by pronounced up-regulation of cellular defences and a reduction in aerobic scope, with potential consequences for population dynamics in coastal systems (Pörtner, 2012). The amphipods subjected to severe hypoxia were still able to sustain resting MO_2 through hyperventilation but were characterised by a distinctly different transcriptomic response to that of moderate hypoxia. The widespread down-regulation of genes involved in protein synthesis and baseline levels of expression for metabolic genes may mark the onset of hypometabolism. Alternatively, individuals under severe hypoxia may be at their physiological limit for survival as indicated by severely constricted aerobic scope (Sokolova, 2013). Hence, unique strategies underlie the responses to different severities of hypoxia. This means that the level 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 (Spicer, 2014). Future increases in the frequency of fairly moderate hypoxia (Breitburg *et al.*, 2018)

may threaten the future performance and resilience of coastal species with significant ecological consequences for coastal habitats.

Chapter 3 Is thermal acclimation beneficial for hypoxic performance in closely-related gammarid amphipods?

Abstract

Aquatic ectotherms are increasingly threatened by multiple anthropogenic drivers including increasing episodes of reduced oxygen (hypoxia) in conjunction with chronically raised temperatures. Our knowledge of how temperature impacts performance under environmental hypoxia is largely limited to acute warming, excluding the possibility for physiological plasticity (acclimation). Of the studies testing for the effects of longer-term thermal acclimation on hypoxic performance, appropriate experimental designs to test the possibility of beneficial acclimation have not always been used. Studies have typically measured hypoxic responses solely at the test temperature (T_t) to which individuals have been acclimated ($T_a = T_t$) in order to understand performance at different T_a encountered seasonally in nature. Hypoxic responses have rarely been measured for individuals directly compared at standardised T_t following acclimation to different T_a, which is key to explicitly identifying beneficial acclimation. It is the beneficial or detrimental effects of thermal acclimation on hypoxia thresholds that urgently need to be critically assessed at a time when chronic warming is predicted to drive increases in the extent, severity and duration of hypoxia. Consequently, this study tests if thermal acclimation is beneficial or detrimental to hypoxic performance in closely-related gammarid amphipods. Amphipods acclimated to different T_a (T_a = 10 or 20 °C) were compared at two T_t (T_t = 10 and 20 °C) to determine effects

on resting oxygen consumption (resting MO_2) and critical oxygen tensions (P_c) under acutely declining oxygen tensions (PO_2). Thermal acclimation was beneficial for hypoxic performance in some species at some T_t. There was evidence of a relationship across the tested species between the warm-acclimated change in resting MO_2 and warm-acclimated change in P_c but this was not statistically significant and subject to considerable interspecific variation. Some species with a capacity for thermal acclimation of MO_2 may benefit from increased resilience to hypoxia whilst those unable to acclimate may be rendered vulnerable to increasing episodes of hypoxia in a warming, coastal ocean.

3.1 Introduction

Coastal biodiversity is increasingly threatened by the combination of spreading hypoxic regions and rising temperatures, driven by eutrophication and climate change (Breitburg *et al.*, 2018). A predicted 2.7 °C rise in average sea surface temperature (Pörtner *et al.*, 2015) could result in a 7% decrease in oxygen dissolved in the world's oceans by 2100 (Keeling *et al.*, 2010). This increase in temperature will intensify coastal hypoxia through enhanced stratification, reduced oxygen solubility and enhanced rates of biological oxygen consumption (Breitburg *et al.*, 2018).

Spreading hypoxia and rising sea temperatures, are predicted to limit the availability of viable habitats for marine species (Deutsch *et al.*, 2015). A recent meta-analysis suggests this may result from increased temperature and hypoxia

making it difficult to sustain aspects of physiological performance such as the ability to sustain MO_2 (Deutsch *et al.*, 2015). Models suggest that both marine fish and invertebrates will experience reductions in performance under environmental hypoxia in a warming ocean marked by an increase in P_c (Herreid, 1980; Rogers *et al.*, 2016), key hypoxia thresholds which represent the lowest PO_2 at which resting MO_2 can be sustained under hypoxia (Grieshaber *et al.*, 1994). P_c's are thought to rise due to the increased challenge of sustaining raised resting MO_2 at increased temperatures when external oxygen levels are low (Herreid, 1980). The oxygen and capacity limited thermal tolerance (OCLTT) hypothesis also attributes the acute loss of hypoxic performance to increased internal hypoxia caused by the rise in resting MO_2, which exceeds the capacity of internal oxygen delivery systems (Pörtner, 2010). As survival time is thought to be limited below P_c (Seibel, 2011), any increase in P_c could be interpreted as being detrimental to the fitness of marine organisms (Rogers *et al.*, 2016).

However, predictions of the impacts of future hypoxia and warming on marine animals come largely from acute studies (hours to days) excluding the possibility for longer-term physiological plasticity, i.e. acclimation (Prosser, 1973; McBryan *et al.*, 2013). Acclimation can be considered to be the longer term remodelling of physiological systems in response to a single abiotic factor, as opposed to acclimatisation, in response to multiple environmental variables (Prosser, 1973). Acclimation effects were historically interpreted as always being adaptive (Huey and Berrigan, 1996), an assumption later tested formally in the form of the "beneficial acclimation hypothesis (BAH)" (Leroi *et al.*, 1994). This states that an organism acclimated to a particular environment should

show greater fitness compared to an organism that has not had the chance to acclimate (Leroi *et al.*, 1994). An established framework for determining beneficial (or detrimental) effects of acclimation exists (Precht *et al.*, 1973; Huey and Berrigan, 1996) stipulating that organisms acclimated to different T_a must be directly compared post-acclimation under standardised T_t to determine fitness (or a proxy for fitness e.g. metabolic performance) (Huey *et al.*, 1999).

Tests of the BAH have largely been restricted to the effects of single stressors in isolation, particularly the effect of thermal acclimation on thermal performance (Cossins and Bowler, 1987; Johnston and Dunn, 1987; Schulte *et al.*, 2011). These provide some but not unequivocal support for the hypothesis (Wilson and Franklin, 2002; Woods and Harrison, 2002; Angiletta, 2009). Perhaps as a result, many investigators no longer set out to specifically test the original definition of the BAH but instead whether acclimation is "beneficial" in a looser sense (i.e. acclimation may improve performance under some but not all conditions). This is now commonly referred to as "adaptive plasticity" (Seebacher *et al.*, 2015; Hildebrandt *et al.*, 2018). In comparison, the consequences of thermal acclimation for hypoxic performance are poorly understood (McBryan *et al.*, 2013). Given the predicted expansion of hypoxic regions (Breitburg *et al.*, 2018), thermal acclimation may only confer improved fitness in the wild if it does not impair performance under hypoxia. While the methodological approach to test the BAH could easily be extended to incorporate hypoxic performance, most studies have not adopted this approach, instead opting to measure hypoxic responses solely at the same temperature to which organisms have been acclimated ($T_a = T_t$). While this may move toward greater ecological realism, it may not fully characterise the mechanistic effects

of acclimation as noted for thermal acclimation under normoxic conditions (Schulte *et al.*, 2011). Therefore there is only limited evidence that thermal acclimation explicitly benefits hypoxic performance (Anttila *et al.*, 2015; McBryan *et al.*, 2016), although, predictions do exist from theoretical models (Pörtner, 2010). The OCLTT hypothesis predicts that warm acclimation could be beneficial by induction of longer-term mechanisms to alleviate internal oxygen limitation caused by an acute temperature increase (Pörtner, 2010). In addition, studies from normoxic conditions demonstrate that the initial rise, and sometimes overshoot, in resting MO_2 caused by acute warming (associated with raised P_c (Herreid, 1980)) can in some species be partially or fully offset by acclimation (Precht *et al.*, 1973; Prosser, 1973; Seebacher *et al.*, 2015) which may potentially lower MO_2 and P_c under hypoxia. However, these are only predictions, and empirical studies are required to test if thermal acclimation can improve hypoxic performance using appropriate experimental designs that compete individuals under hypoxia at standardised T_t post-acclimation. An extremely limited number of studies have adopted this methodology but have revealed that in some fish species, warm acclimated fish show greater hypoxia tolerance than cold acclimated fish at standardised T_t (Anttila *et al.*, 2015; McBryan *et al.*, 2016). Only one study of triplefin fish has investigated the effect of thermal acclimation on P_c at standard T_t post-acclimation (Hilton *et al.*, 2008). Responses were species specific with one fish species benefiting from a reduction in P_c following warm acclimation whilst no effect of acclimation occurred in the other species (Hilton *et al.*, 2008). The effects of thermal acclimation on hypoxic performance requires testing in a greater number of species.

Therefore, the aim of this study was to directly test if thermal acclimation is beneficial for hypoxic performance. Individuals belonging to closely-related species were acclimated to one of two different temperatures (T_a = 10 or 20 °C) for one week before resting MO_2 and hypoxic performance, measured as P_c, were determined at two standardised T_t (T_t = 10 and 20 °C). Both individual and cross-species effects of thermal acclimation on resting MO_2 and P_c were tested. The responses for four gammarid species were compared (*Gammarus chevreuxi* (Sexton, 1913), *Gammarus duebeni* (Lilljeborg, 1852), G*ammarus zaddachi* (Sexton, 1912) and *Echinogammarus marinus* (Leach, 1913*)*. These gammarid amphipods were used as models as they are closely-related, have similar morphologies, and experience considerably different environmental regimes with congeners occurring from brackishwater to fully marine habitats (Lincoln, 1979).

3.2 Methods

3.2.1 Sampling sites and pre-exposure conditions in the laboratory

Amphipods were collected using a hand-net from various locations on the Plym and Tamar estuaries, Plymouth, UK around winter/early spring when average water temperatures are usually close to 10 °C (Dec 9.9 °C ± 0.98 – Apr 9.3 ± 0.63 °C , mean ± s.d.) (Joyce, 2006). *G. chevreuxi* was collected from a brackishwater stream feeding the Plym Estuary Plymouth (50.391290 N, -4.084795 W), *G. zaddachi* and *G. duebeni* both from a brackishwater stream near Kingsmill Lake, Saltash (50.426192 N, -4.215582 W)*, E. marinus*, a marine species, from intertidal mudflats on the Tamar Estuary, Saltash (50.415392 N, -4.210981 W). Animals were transferred to the laboratory within 2 h of collection

and kept in a number of small aquaria (Vol. = 10 L, T = 10 °C, 12 h L:12 h D regime) containing dilute sea water (S = 15 PSU) for *G. chevreuxi*, *G. zaddachi* and *G. duebeni*, and undiluted seawater (S = 32 PSU) for *E. marinus*. They were all fed carrot *ad libitium*. Only adult males (wet mass: *G. chevreuxi* = 10.5 ± 0.3 mg, *G. zaddachi* = 29.8 ± 1.1 mg, *G. duebeni* = 62.7 ± 1.7 mg, *E. marinus* = 93.7 ± 2.4 mg, mean ± s.e.m) identified according to Lincoln (1979) were used in the experiments described below.

3.2.2 Experimental design and exposure conditions

Individuals were acclimated for at least 1 week (7 d for *G. chevreuxi, G. duebeni*, and *E. marinus* and 10 d for *G. zaddachi*), to one of two different environmental temperatures: T_a = 10 °C (control) or 20 °C. This was deemed a sufficient period to allow full thermal acclimation of gammarid amphipods which are typically thought to thermally acclimate within several hours (Bulnheim, 1979). However, there was no evidence of acclimation in *G. chevreuxi* after 1 week. Consequently, the effect of 6 weeks acclimation on this species was also tested.

All acclimations were carried out using a mesocosm system constructed from sealed aquaria (Vol. = 1.4 L) (n = 6 - 8 replicates per treatment, 5 - 8 individuals in each). Each aquarium was partially immersed in one of a number of plastic trays filled with deionised water (acting like a water bath). Each sealed aquarium was directly supplied with an airstone, connected to an air pump (Mistral 2000, Aqua Medic GmbH, Germany). This kept the water within each

aquarium fully aerated. To minimise the possibility of hypercapnia, air was scrubbed for CO_2 by passing it through a trap filled with NaOH (1mol.L^{-1}, Sigma-Aldrich, Germany), which was replaced every two to three days as required. Deionised water was circulated around the tray outside of the sealed aquaria using a Koralia pump to ensure even temperature distribution across the tray (Koralia Nano Evolution 900 Circulation Pump, Hydor, Italy). The entire mesocosm was located within a controlled temperature environment (T = 15 °C). The water in the control treatment (T = 10 °C, 10.1 ± 0.7, mean ± s.e.m) was kept at constant temperature using a water chiller (L-350 Water Chiller, Guangdong Boyu Group Co.,Ltd., China) and that for the higher temperature treatment (T = 20 °C, 20.4 ± 0.9, mean ± s.e.m) was supplied with an adjustable water heater (100 W aquarium heater, EHEIM GmbH & Co KG, Germany). Complete changes of water within the aquaria were carried out every 3 - 4 d. Other environmental factors were kept constant (O_2 = 90.7 ± 1.9 % a.s., S = 32.0 ± 1.5 for *E. marinus* and S = 14.2 ± 1.3 for the other species, pH = 8.1 ± 0.2, mean ± s.e.m). Water temperature and PO_2 were measured daily using an oxygen microsensor (Pm-Pst7, Presens, Germany) and temperature probe (Pst 100, Presens, Germany) with a dissolved oxygen meter (Microx 4, Presens, Germany). Salinity and pH were measured every 1 - 2 d using a refractometer (HI96822 Digital Refractometer, Hanna Instruments, USA) and bench top pH meter (S400 SevenExcellence pH/mV meter, Mettler-Toledo International Inc., USA).

3.2.3 Effects of thermal acclimation on resting MO_2 and P_c

The changes in resting MO_2 during exposure to acutely declining PO_2 were measured for individuals at one of two test temperatures (T_t = 10 °C or 20 °C) and for amphipods acclimated to two different temperatures (T_a = 10 °C or 20 °C) using a well-established closed bottle respirometry technique (Mangum and Van Winkle, 1973) and employing a fully-factorial design. Individuals were starved for 12 h prior to MO_2 measurements.

After acclimation, individuals were transferred to a controlled temperature environment (T_t = 10 °C or 20 °C) and allowed to settle for 1 h following the thermal shift (Dorgelo, 1973) before any measurements of whole organism rates of oxygen uptake (as a proxy for resting MO_2) were carried out. Individuals were carefully introduced singly into submerged respirometry chambers (blacked out, Vol. = 1.5 mL for all species except *E. marinus* Vol. = 5 mL because of its larger size). Each chamber was fitted with a non-invasive oxygen sensor spot (SP-PSt3-NAU-D3-YOP, PreSens, Germany). For the 1.5 mL bottles, ventilatory activity of the amphipod was sufficient to ensure mixing within the chamber but, for the 5 mL chambers, a magnetic flea rotated by a magnetic stir plate was used (MIX 15 eco; 2mag AG, Germany). Amphipods were separated from the magnetic flea by a square of plastic mesh (mesh size = 2 mm) The movement of animals was limited by a combination of the small size of the respirometer and the plastic mesh which kept animals confined to one end of the respirometer. The plastic mesh also served as a substrate for the amphipod to cling to. Bottles were sealed using a gas-tight lid, and the individuals allowed to acutely reduce the oxygen content of the water. The PO_2

within the chamber was monitored manually applying an optical sensor (POF-L2.5-1SMA, Presens, Germany) connected to an oxygen meter (Fibox 4, Presens, Germany) to each of the sensor spots within the chambers. Individuals were allowed to deplete the oxygen to approx. PO_2 = 3-5 % a.s. (~ 10% a.s. for *E. marinus* at T_t = 20 °C as P_c = ~ 18 – 25 % a.s. / 5.18 – 3.74 kPa and MO_2 dropped rapidly to almost zero below this point) before the bottle was opened, the individual removed and gently blotted dry using tissue paper. Wet mass was determined using a microbalance (MSA225P-000-DA, Göttingen Sartorius AG, Germany, ± 0.01 mg). Two empty chambers were run to estimate, and where necessary account for, background microbial respiration. Separate individuals were tested in each experimental condition to minimise any confounding effects and stress resulting from repeat exposure to hypoxia and different temperatures. Resting MO_2 were expressed as $\mu L\ O_2\ g^{-1}\ h^{-1}$ and were corrected for rates of background respiration, barometric pressure and water vapour pressure (Weiss, 1970). The point at which resting MO_2 began to fall (the P_c) was estimated as described below.

3.2.4 Statistical analysis of effects of thermal acclimation on resting MO_2 and P_c for individual species

All statistical analyses were performed using R statistical software (version 3.2.4, R Core Team, 2016). To determine the P_c for an individual, segmented regression analysis was performed on the relationship between oxygen saturation and time, similar to Spicer and El-Gamal, (1999), using the "segmented" package (Muggeo, 2008) to determine the PO_2 at which the slope of the line changed, i.e. P_c point. The first 20 – 30 mins of data were removed to

try to minimise the effects of handling stress. The slope of the regression line above the P_c was used to calculate resting MO_2 over the range of environmental PO_2 individuals were seen to oxyregulate. For *E. marinus* at T_t = 10 °C, data were collected from two repeats of the same experiment and were pooled as responses were not significantly different ($P > 0.05$). Levene's tests were used to test for variance homogeneity ($P > 0.05$). For each species, two-way ANOVA and *post-hoc* Tukey tests were used to detect any significant differences ($P < 0.05$) between T_a and T_t for (1) resting MO_2 and (2) P_c. For *G. chevreuxi*, duration of exposure (1 or 6 weeks) was included as a third fixed factor in the ANOVA model, in addition to T_a and T_t.

3.2.5 Statistical analysis of relationships between warm acclimation of resting MO_2 and P_c across species

It has been suggested that changes to MO_2 underpin the effects of temperature on P_c in invertebrates (Herreid, 1980). Firstly, to see if P_c may broadly be related to an individuals MO_2, P_c and MO_2 were pooled from all individuals in the study. MO_2 and P_c for each individual used in the experiments were first log transformed to normalise the data. The relationship between log MO_2 and log P_c was tested using an ANCOVA with species as a factor in the model.

Secondly, the specific effects of warm acclimation (T_a effects) on resting MO_2 and P_c were investigated. The relationship between the effects of warm acclimation of resting MO_2 and warm acclimation of P_c was tested across all species. To do this, average values for MO_2 and P_c were calculated for each species at each combination of T_t and T_a (mean values per species, displayed in Fig. 3.1: P_c and MO_2 at (1) T_a = 10 and T_t = 10 °C (2) T_a = 20 and T_t = 10 °C (3)

T_a = 10 and T_t = 20 °C (4) T_a = 20 and T_t = 20 °C). Then T_a effects on these mean values for resting MO_2 and P_c within each T_t was measured e.g. at T_t = 10, how mean MO_2 or P_c changes between T_a = 20 and T_a = 10 °C. The warm acclimated change in resting MO_2 (resting MO_2 at T_a 20 °C – resting MO_2 at T_a 10 °C) and warm acclimated change in P_c (P_c at T_a 20 °C – P_c at T_a 10 °C) were calculated for each T_t. (also see Fig 3.3. for explanatory diagram). Linear regression was then performed between the warm acclimated change in resting MO_2 and the warm acclimated change in P_c. For *G. chevreuxi* both the 1w and 6w experiments were included. As a relatively small number of species were investigated (n = 4), a formal phylogenetic approach was not used, but all the tested species were closely related, thus minimising any phylogenetic signal.

3.3 Results

3.3.1 Effect of thermal acclimation on resting MO_2 and P_c in individual species

G. chevreuxi showed no significant effect of T_a on resting MO_2 when measured at the same T_t ($P > 0.05$). Resting MO_2 was significantly affected by T_t when measured at the same T_a ($F_{1,46} = 145.70$, $P < 0.001$). There was not an interaction between T_a and T_t ($P > 0.05$) on resting MO_2 but there was an interaction between T_t and duration of exposure ($F_{1,46} = 5.62$, $P = 0.023$) (Fig. 3.1A). This may potentially reflect a laboratory effect. Although, there appears to be no difference in MO_2 based upon Tukey tests under control conditions ($P>0.05$). There is perhaps a slight but non-significant trend towards a reduction in MO_2 at warm T_t after 6 weeks compared to 1 week exposure. For *G. chevreuxi,* P_c was only significantly affected by T_a when measured at the same T_t ($F_{1,46} = 8.96$, $P = 0.004$) and exposure duration ($F_{1,46} = 21.28$, $P < 0.001$) (Fig.

3.1B). No significant effect of T_t was observed when measured at the same T_a and no interactive effects between T_a, T_t or exposure duration ($P > 0.05$).

For *G. zaddachi*, T_a in isolation had no significant effect on resting MO_2 when measured at the same T_t ($F_{1,20} = 1.52$, $P = 0.233$). A significant effect of T_t when measured at the same T_a was detected ($F_{1,20} = 10.15$, $P = 0.005$) and an interactive effect of T_a and T_t ($F_{1,20} = 5.41$, $P = 0.031$) (Fig. 3.1C). There were no significant effects of T_a or T_t in isolation on P_c for *G. zaddachi* ($P > 0.05$). A significant interaction of T_a and T_t was observed ($F_{1,19} = 5.46$, $P=0.031$) (Fig. 3.1D), although, *post-hoc* Tukey tests indicated no significant differences in P_c between the treatments for *G. zaddachi* ($P > 0.05$). There was a non-significant trend that cold acclimated ($T_a = 10$ °C) *G. zaddachi* displayed a higher P_c at $T_t = 20$ °C ($P = 0.08$). The discrepancies between the ANOVA and post-hoc tests may reflect high individual variability and possibly a limitation of small sample size.

For *G. duebeni*, there was no significant effect of T_a on resting MO_2 when measured at the same T_t ($F_{1,23} = 3.48$, $P = 0.075$) but a significant effect of T_t when measured at the same T_a ($F_{1,23} = 13.07$, $P = 0.001$) and their interaction ($F_{1,23} = 4.55$, $P = 0.044$) (Fig. 3.1E). P_c was not significantly affected by T_a, T_t or their interaction in this species ($P > 0.05$) (Fig. 3.1F).

For *E. marinus* at $T_t = 10$ °C, the response of resting MO_2 during exposure to declining PO_2 was slightly more curvilinear. Individuals displayed a low P_c at ~ 2

kPa (~ 10% a.s.) but MO_2 was not fully regulated at 100% of normoxic MO_2 in some individuals and showed a slight decrease in MO_2 (up to ~ 23 %) before P_c. Therefore, MO_2 across the range of tested PO_2 was estimated by fitting a segmented regression with either one or two breakpoints and selecting the fit with highest R^2. For individuals where a segmented regression with two breakpoints was fitted, two distinct regulatory phases for oxygen uptake (R_1MO_2 between normoxia and ~ 40 – 50% a.s. and R_2MO_2 between ~ 40 - 10% a.s.) and two breakpoints, i.e. two P_c (P_{c1} at approx. ~ 40-50% a.s. (~ 8.4 - 10.5 kPa) and P_{c2} at ~ 10% a.s (~ 2 kPa)) could be identified (Fig. 3.1G). At T_t = 20 °C, only one break point could be reliably estimated (i.e. one P_c and one regulatory phase of oxygen uptake, R_1MO_2) given that individuals were in the respirometer for a shorter duration due to higher MO_2 at higher T_t, thus reducing the number of PO_2 measurements from which breakpoints could be estimated. R_1MO_2 was significantly affected by T_t when measured at the same T_a ($F_{1,38}$ = 90.14, P < 0.001) but not T_a when measured at the same T_t or the interaction of T_a and T_t (P > 0.05) (Fig. 3.1G). At T_t = 10 °C, R_2MO_2 was significantly different between T_a being significantly lower in warm acclimated compared to cold acclimated individuals ($F_{1,22}$ = 6.76, P = 0.016) (Fig. 3.1G). Paired t-tests indicated that the reduction in MO_2 between R_1MO_2 and R_2MO_2 was only significantly lower for warm acclimated (t_{11} = -2.05, P = 0.032) but not cold acclimated individuals (t_{11} = -1.43, P = 0.090). Warm acclimated individuals took a significantly longer time to reach P_c (P_{c2} at ~2 kPa / 10% a.s.) from normoxic conditions ($F_{1,22}$ = 8.60, P = 0.008). There were significant differences observed in mean values of P_c between T_a when measured at the same T_t ($F_{1,38}$ = 4.33, P = 0.044), T_t when measured at the same T_a ($F_{1,38}$ = 92.20, P < 0.001), and there was an interactive effect of T_a and T_t ($F_{1,38}$ = 6.93, P = 0.012) (Fig. 3.1H).

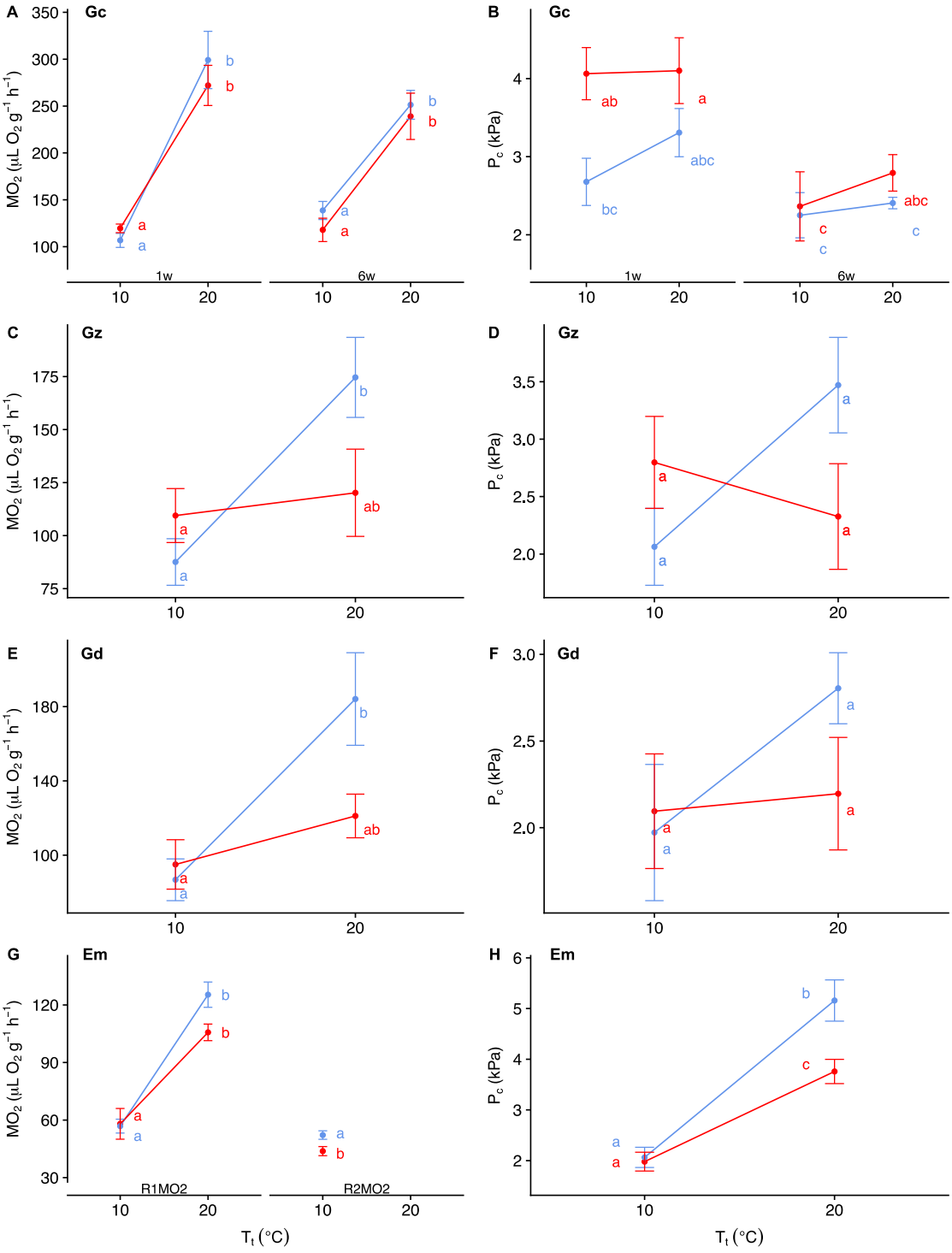

Fig. 3.1. The effect of 1 week acclimation to 10 and 20 °C (T_a °C) on resting MO_2 under normoxia (µL O_2 g^{-1} h^{-1}) and P_c (kPa) (mean values ± s.e.m) when tested at T_t = 10 °C (blue) and 20 °C (red). Responses were measured for the following gammarid amphipod species: *G. chevreuxi* (n = 6 – 8 animals

per treatment) (A,B), *G. zaddachi* (n = 5 – 7 animals per treatment) (C,D), *G. duebeni* (n = 6 - 7 per treatment) (E,F) and *E. marinus* (n = 8 – 12 animals per treatment) (G-H). For *E. marinus* at T_t = 10 °C, resting MO_2 is split into R_1MO_2 depicting resting MO_2 above ~ 40% a.s., and R_2MO_2 between 40% and ~ 10% a.s. (R_1MO_2 and R_2MO_2 analysed separately). R_1MO_2 and R_2MO_2 only characterised at cold T_t. For *E. marinus* P_c data at T_t = 10 °C, P_c is represented by P_{c2} (~ 2 kPa / 10% a.s.). (1 μL O_2 = ~ 0.04464 μmol O_2).

3.3.2 Relationships between resting MO_2 and P_c across species and the effects of warm acclimation

A significant relationship was observed between log P_c and log resting MO_2 ($F_{1,138}$ = 28.25, P = 4.18 E -07) and species ($F_{3,138}$ = 7.22, P = 1.56 E -04) and there was a significant interaction of log MO_2 and species ($F_{3,138}$ = 2.72, P = 0.047) (Fig. 3.2). There was some evidence of a relationship between warm-acclimated change in resting MO_2 and warm-acclimated change in P_c, but this was not statistically significant ($F_{1,8}$ = 5.30, P= 0.050) (Fig. 3.3).

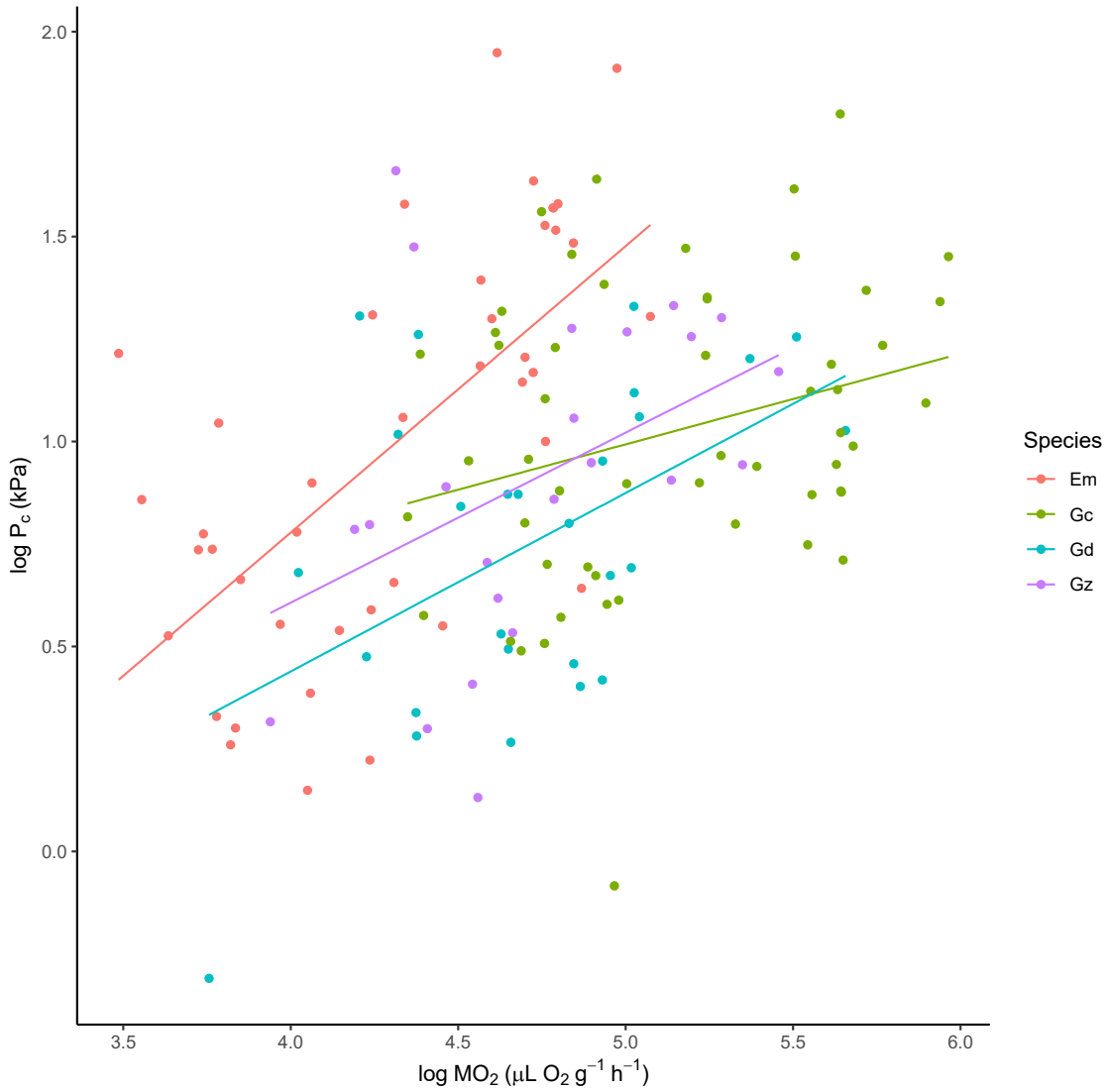

Fig. 3.2 The relationship between MO_2 and P_c across the tested *Gammarus* species. MO_2 and P_c data were first log transformed. Equations are as follows: *E. marinus* ($\ln P_c = -2.02 + 0.699 \ln MO_2$, $R^2 = 0.40$), *G. chevreuxi* ($\ln P_c = -0.11 + 0.221 \ln MO_2$, $R^2 = 0.06$), *G. duebeni* ($\ln P_c = -1.30 + 0.435 \ln MO_2$, $R^2 = 0.26$), *G. zaddachi* ($\ln P_c = -1.05 + 0.415 \ln MO_2$, $R^2 = 0.14$). Species are indicated as follows: *G. chevreuxi* (Gc), *G. duebeni* (Gd), *G. zaddachi* (Gz) and *E. marinus* (Em).

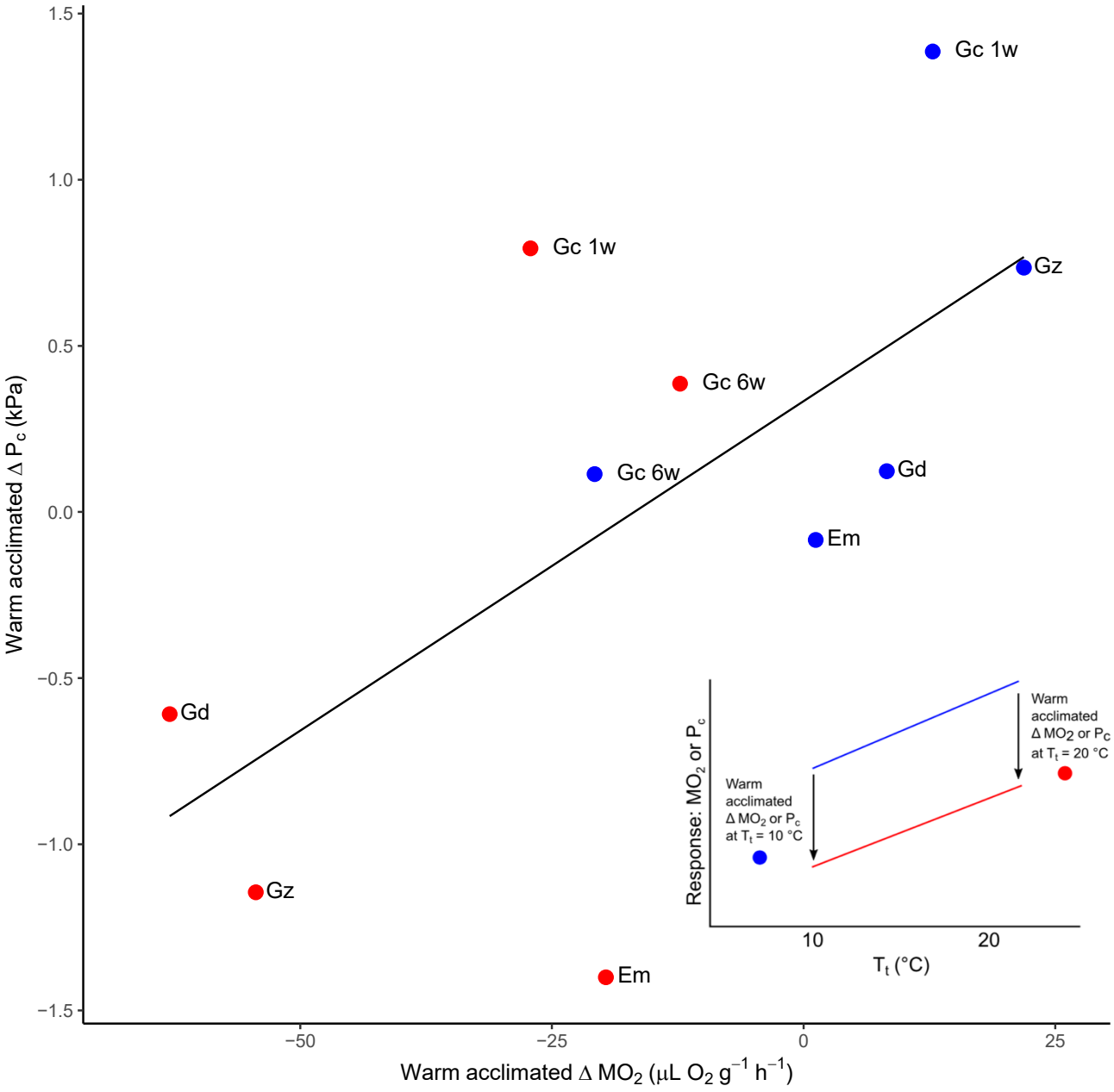

Fig. 3.3 The relationship between the warm-acclimated change in average resting MO_2 (μL O_2 g^{-1} h^{-1}) and acclimation of hypoxic performance, measured as the warm-acclimated change in the average P_c (kPa). (R^2 = 0.32, P = 0.050). For *E. marinus*, R_1MO_2 was utilised. Warm acclimated change in MO_2 or P_c calculated as depicted in the bottom right of figure. This involved subtraction of mean values for P_c or MO_2 of warm acclimated individuals from cold acclimated individuals (T_a = 20 – T_a = 10 °C) at each T_t (10 °C = blue

circles, 20 °C = red circles). Species are indicated as follows: *G. chevreuxi* (Gc), *G. duebeni* (Gd), *G. zaddachi* (Gz) and *E. marinus* (Em).

3.4 Discussion

This study tested the hypothesis that thermal acclimation will be beneficial (i.e. improve the performance of) amphipods during exposure to acutely declining PO_2. There was interspecific variation in whether thermal acclimation was beneficial for hypoxic performance. Improvements in performance were observed in some but not all species. In some species, an improvement in hypoxic performance, measured as a reduction in P_c, may be associated with a warm acclimated reduction in resting MO_2. Therefore, a reduction in oxygen demand during thermal acclimation could be an important mechanism by which some species buffer against the combined threat of hypoxia and climate change.

3.4.1 Effects of thermal acclimation on hypoxic performance in individual species

Warm acclimation affected resting MO_2 similarly for most species, but there were marked differences in effects of warm acclimation on P_c between species. At cold T_t (T_t = 10 °C), acclimation elicited very little effect on resting MO_2 under normoxic conditions in *G. chevreuxi, G. duebeni* and *G. zaddachi.* This was associated with limited changes to P_c in *G. duebeni*. For *G. zaddachi*, there was an interactive effect of T_a and T_t suggesting that T_a effects on P_c are dependent upon T_t. Although there only appears to be a small change in P_c between T_a at

cold T_t with perhaps evidence for greater magnitude of change between T_a at warm T_t.

An altered metabolic response to hypoxia was observed at cold T_t after acclimation in *E. marinus*. Warm acclimated *E. marinus* displayed a regulated reduction in resting MO_2 under hypoxia (at ~ 40 - 50 % a.s.) resulting in a lowered MO_2 under hypoxia (between ~ 50% to 10 % a.s.) compared to cold acclimated individuals (also see PO_2 trace in Fig 4.1). The phase-wise shift in resting MO_2 (hypometabolic shift) occurring between 50 % a.s. and P_c in warm acclimated individuals may seem unusual in comparison to models where MO_2 is usually depicted as being fully regulated down to P_c (Grieshaber *et al.*, 1994). However, other gammarid amphipod species can display a curvilinear relationship between MO_2 and declining PO_2 (Sutcliffe, 1984). For some *Gammarus* species, MO_2 is not fully regulated at the normoxic rate and undergoes a partial reduction before reaching P_c (Sutcliffe, 1984; Verberk *et al.*, 2018). Also, previous studies, which have measured hypoxic responses at standardised T_t following exposure to different T_a, have also observed unusual metabolic responses (Hicks and McMahon, 2002). Some species display several hypometabolic shifts under declining PO_2, making it difficult to identify a distinct, single P_c (Hicks and McMahon, 2002).

A regulated reduction in MO_2 in warm acclimated *E. marinus* could have been predicted to be beneficial and lower P_c as studies suggest that having a lower MO_2 contributes to hypoxia tolerance (Mandic *et al.*, 2009). Also, in other gammarids, partial reductions in MO_2 under declining PO_2 are thought to

promote lower P_c (Verberk *et al.*, 2018). The lack of reduction in P_c makes it difficult to assess if the hypometabolic response of warm acclimated *E. marinus* is beneficial or not. However, it may be beneficial given that the lowered MO_2 led to a significantly longer duration of oxyregulation (longer time to reach P_c because of the lower rate of oxygen depletion) in warm acclimated compared to cold acclimated individuals. Also, warm acclimation may be beneficial for this species as warm acclimated individuals displayed a significant reduction in P_c at the warm T_t compared to cold acclimated individuals.

In the other amphipod species examined, metabolic performance at the warm T_t was generally more sensitive to the effects of thermal acclimation. Warm-acclimated *G. duebeni* and *G. zaddachi* displayed a lowered resting MO_2 compared to cold-acclimated individuals. *G. duebeni* has been shown to display a capacity for thermal compensation as its metabolic rate was found to be relatively constant across latitudinal thermal gradients (Whiteley *et al.*, 2011). Acclimation did not result in a statistically significant improvement in P_c for either species. However, there was some indication of a reduction in P_c as a result of warm acclimation, although this may reflect a non-significant trend, perhaps due to considerable individual variation.

In contrast, *G. chevreuxi* did not display any ability to acclimate resting MO_2 after both 1 and 6 weeks. However, the effects of thermal acclimation on responses of *G. chevreuxi* to acutely declining PO_2 were more complex. Warm-acclimated individuals displayed a higher P_c at 20 °C after 1 week, which appears to partially return to control levels after 6 weeks. Surprisingly, acute

exposure of cold-acclimated individuals to 20 °C did not significantly raise P_c, despite the increase in resting MO_2. However, thermally-insensitive hypoxia thresholds have been observed for several other *Gammarus* species (Sutcliffe, 1984; Hoback and Barnhart, 1996). *G. chevreuxi* may possess the capacity to deal with acute warming and hypoxia, typical of shallow coastal environments (Truchot and Duhamel-Jouve, 1980; Diaz and Rosenberg, 2008), but warm acclimation may be detrimental for hypoxic performance in the longer term taking many weeks for recovery. The limited acclimation capacity of *G. chevreuxi* may render it vulnerable to future chronic warming if its current physiological limits are exceeded (Stillman, 2003).

3.4.2 Effects of thermal acclimation on hypoxic performance across species: implications in warming, hypoxic coastal habitats

The current study also attempted to investigate the effects of acclimation across species. Thermal effects on hypoxic performance are broadly thought to be driven by changes to oxygen supply and oxygen demand i.e. MO_2 (Anttila *et al.*, 2015). Therefore, it was investigated whether a relationship existed between MO_2 and P_c for all individuals in the study. This did not take into account T_a and T_t as factors, so was not treatment specific, but showed that a significant positive correlation between MO_2 and P_c existed. A relationship between MO_2 and P_c has previously been demonstrated in fish species where having a lower MO_2 is thought to contribute to hypoxia tolerance (Mandic *et al.*, 2009). A positive correlation between MO_2 and P_c also seems intuitive given the temperature-mediated rise in MO_2 has long been demonstrated to contribute to raised P_c in other invertebrates (Herreid, 1980).

The relationship between MO_2 and P_c observed in the current study was dependent upon species identity. Although all species showed a positive correlation between MO_2 and P_c, the relationship was weak (lowest R^2 of 0.07) in *G. chevreuxi*. Overall, the correlation between MO_2 and P_c may suggest that a factor which drives changes to MO_2 may contribute to changes in P_c but this effect will vary between species. This variation could be attributable to alternate mechanisms other than MO_2 affecting P_c. In fish, oxygen affinity of respiratory pigments and respiratory surface area are also related to P_c (Mandic *et al.*, 2009).

It is possible that the adoption of a closed respirometry technique in the study could have also contributed to the variation in the relationship between MO_2 and P_c. Different MO_2 between individuals will induce variability in the rate of induction of hypoxia within a respirometry chamber particularly with different temperatures (Rogers *et al.*, 2016). This potential limitation may be widespread in hypoxia studies where most determinations of P_c have been conducted using closed respirometry with little standardisation of the time of exposures (Rogers *et al.*, 2016). A recent study demonstrates that in one fish species, a slower rate of hypoxic induction allows physiological changes which lower P_c (Regan and Richards, 2017) but it remains largely unknown to what extent a rise in P_c at higher temperature is driven by an increased rate of hypoxic induction.

After identification of a relationship between MO_2 and P_c, the specific effects of T_a on the relationship between MO_2 and P_c across species was investigated. These T_a effects could only be identified by the adoption of a framework to test

for beneficial acclimation. Previous studies acclimating animals to increased temperature and only assaying hypoxic responses at the same acclimation temperature ($T_a = T_t$) have not facilitated identification of the potential benefits/detrimental consequences of thermal acclimation on hypoxia thresholds. As a result, the ability of acclimation to buffer against the combination of hypoxia and warming still remains largely undetermined (McBryan *et al.*, 2013). A limited number of studies that have used an approach comparing responses at standard T_t post-acclimation have shown that several fish species display greater hypoxia tolerance following warm acclimation (Anttila *et al.*, 2015; McBryan *et al.*, 2016). This was attributed to plasticity in oxygen supply systems such as increased respiratory surface area and aspects of cardiac performance (Anttila *et al.*, 2015; McBryan *et al.*, 2016).

Even fewer investigations have been conducted comparing whether beneficial effects are elicited in more than one species within the same study. Two recent studies on closely-related fish species show interspecific variation in whether hypoxic performance improves with warm acclimation (Hilton *et al.*, 2008; Anttila *et al.*, 2015) similar to the amphipod species tested here. The mechanisms underpinning variation between species in the effects of thermal acclimation on hypoxic performance remain unknown. It is important to understand what drives this variation as differences in thermal acclimation capacity between species under normoxic conditions is thought to be important in determining so called "winners" (survivors) and "losers" under future climate change (Somero, 2010). Therefore, this study tried to investigate whether changes to P_c across the species were potentially underpinned by acclimation of resting MO_2. There was some evidence for a relationship between the warm acclimated change in

resting MO_2 and warm acclimated change in P_c. However, this was not statistically significant, due to considerable interspecific variation in the data. This suggests that some species but not others may benefit from a reduction in P_c associated with a reduction in MO_2 following warm acclimation. This may be the case for *E. marinus*, *G. duebeni* and *G. zaddachi* at T_t = 20 °C where warm acclimation appeared to induce a reduction in MO_2 and P_c (Fig. 3.3, red circles for Em, Gz and Gd). Although, in the case of *G. zaddachi* and *G. duebeni*, the reduction in P_c following warm acclimation compared to cold acclimated individuals was not statistically-significant. Only *E. marinus*, showed a clear benefit of warm acclimation through a significant reduction in P_c. These differing responses between species could also possibly reflect differences in their ecology. *G. duebeni*, *G. zaddachi* and *G. chevreuxi* are all brackish species whilst *E. marinus* inhabits an intertidal estuarine mudflat. However, this is somewhat speculative given the small number of species and would require investigation using a larger number of species from different habitat types.

Regardless of whether acclimation has beneficial effects or not, the observation that T_a and T_t can have interactive effects on P_c, in some species, means that studies of acute warming may not accurately predict the consequences of long-term warming and hypoxia on marine species. The acclimation responses observed in this study, including a significant partial recovery of P_c in one species, may become particularly important for maintaining the fitness of taxa thought to be sensitive to the combination of hypoxia and warming, such as crustaceans (Vaquer-Sunyer and Duarte, 2011). Therefore, beneficial (or detrimental) changes to hypoxic performance resulting from thermal acclimation need to be identified in a greater number of marine species using appropriate

experimental designs. Understanding the ability of individual species to buffer against hypoxia through warm-acclimation will be essential if we are to accurately assess the ecological threat posed to coastal regions by expanding hypoxic regions under chronic climate change (McBryan *et al.*, 2013; Breitburg *et al.*, 2018).

Chapter 4 Mechanisms underpinning altered hypoxic performance following thermal acclimation

Abstract

Rising sea temperatures are predicted to drive reductions in performance under environmental hypoxia in many marine species. However, this prediction is based predominantly upon the effects of acute warming. Conversely, through interactions upon the same physiological trait (aerobic metabolism), there is the potential for longer-term warm acclimation to enhance performance under hypoxia, via cross-talk between the underpinning cellular mechanisms. However, the molecular basis for altered hypoxic performance following warm acclimation is poorly understood. Consequently, this chapter investigates the cellular mechanisms elicited by warm acclimation that drive the altered metabolic responses to hypoxia in the amphipod *Echinogammarus marinus* observed in Chapter 3. The mechanisms supporting metabolic performance under hypoxia at T_t = 10 °C following acclimation to T_a = 10 (cold) or 20 °C (warm) are explored. Warm acclimated *E. marinus* displayed a reduction in MO_2 (hypometabolic shift) between normoxia and hypoxia (30 % a.s.) which did not occur in cold acclimated individuals (Chapter 3). The reduction in MO_2 was correlated with downregulation of mitochondrial, ribosomal and cuticle genes. Alterations to metabolic function may also be associated with transcriptional frontloading during the acclimation period where frontloading of transcripts involved in the reduction of energetically-costly cellular processes such as protein synthesis, translation and ion transport resulted in a reduced inducible

stress response to hypoxia. Thermal acclimation may therefore prepare marine species for future hypoxia at a cellular level which may be important in maintaining physiological performance in an increasingly warm, hypoxic ocean.

4.1 Introduction

Marine organisms rarely experience stressors in isolation, being instead subjected to combinations of multiple stressors (Todgham and Stillman, 2013; Henson *et al.*, 2017). A mechanistic understanding of the interplay between stressors is needed in order to predict the ecological consequences of rapid environmental change (Pörtner, 2010; Boyd *et al.*, 2018). For the interaction of temperature and hypoxia, an integrative framework has been proposed under the oxygen and capacity limited thermal tolerance (OCLTT) hypothesis (Pörtner *et al.*, 2017). The model initially focussed on thermal limitation in isolation which may in some, but not all, species stem from internal oxygen limitation (Pörtner, 2002; Jutfelt *et al.*, 2018). However, the model has since been updated to explicitly consider the reverse role of warming on hypoxia thresholds, predicting reduced hypoxic performance through a greater degree of internal oxygen limitation at sub-optimal “pejus” temperatures (Pörtner, 2010). Numerous studies, both pre- and post- OCLTT, have demonstrated reductions in metabolic performance (raised critical oxygen tension (P_c)) and survival time under environmental hypoxia at acutely raised temperature (Herreid, 1980; Pörtner, 2010; Vaquer-Sunyer and Duarte, 2011; McBryan *et al.*, 2013).

However, studies of the acute interaction between stressors may not accurately predict the ecological consequences of future warming and hypoxia as they exclude the modifying effects of acclimation (McBryan *et al.*, 2016). Given that temperature and hypoxia act upon similar physiological traits (i.e. aerobic metabolism) (Pörtner, 2010), there is the potential for warm acclimation to elicit mechanisms which enhance hypoxic performance (Todgham and Stillman, 2013; McBryan *et al.*, 2016). In Chapter 3, the amphipod *E. marinus* displayed an altered metabolic response to declining oxygen tensions following warm acclimation (T_a = 10 or 20 °C) when tested at a singular standardised test temperature (T_t = 10°C). Briefly, amphipods displayed similar rates of aerobic metabolism (MO_2) regardless of acclimation temperature between 100 % a.s. and ~40-50 % a.s. (R_1MO_2). However, under hypoxia between ~40-50% down to the P_c of ~10 % a.s., warm acclimated individuals displayed a significantly lower, but regulated, metabolic rate (R_2MO_2), compared to cold acclimated individuals. Warm acclimated individuals displayed a hypometabolic shift not present in cold acclimated individuals. This reduction in MO_2 in warm acclimated individuals did not lower P_c compared to cold acclimated individuals. The lower rate of oxygen consumption resulted in a significantly longer time to reach P_c. Thus animals may be able to oxyregulate for a longer duration before reaching P_c which may be beneficial. Particularly, as P_c represents the transition to anaerobiosis/hypometabolism and typically time-limited survival (Seibel, 2011).

Improvements to hypoxic performance resulting from thermal acclimation in other species has largely been restricted to investigating physiological mechanisms that enhance oxygen supply, e.g. altering respiratory surface area

or cardiac function (Sollid *et al.*, 2005; Anttila *et al.*, 2015; McBryan *et al.*, 2016). However, the underpinning cellular mechanisms remain poorly understood. “Cross-talk” between cellular mechanisms (Todgham and Stillman, 2013) elicited by temperature and hypoxia may occur, although evidence for such cross-talk is restricted to a small number of genes and/or proteins in several fish species (Todgham *et al.*, 2005; Fraser *et al.*, 2006; Rissanen *et al.*, 2006). For example, increased hypoxia tolerance in the tidepool sculpin following pre-exposure to an acute heat shock may be associated with changes to the expression of heat shock proteins (Todgham *et al.*, 2005). In carp species, thermal acclimation has been shown to modify the expression of hypoxia-inducible factors (Rissanen *et al.*, 2006) but not the expression of respiratory pigments upon exposure to hypoxia (Fraser *et al.*, 2006).

Global gene expression profiling may provide a thorough assessment of the effects of environmental stressors on marine animals as they allow identification of changes to thousands of genes (Gracey, 2007; Logan and Buckley, 2015). However, transcriptomic approaches have not yet been applied to understand how the hypoxic response is altered by temperature for any aquatic species. In one study, a microarray consisting of ~13,000 genes was constructed to investigate the hypoxic response at different acclimation temperatures in carp but analysis focussed on respiratory pigment genes (Fraser *et al.*, 2006; Gracey, 2007) and analysis of the entire global gene expression profile has yet to be carried out (Williams *et al.*, 2008).

Therefore, our current understanding of cellular responses to hypoxia under chronic warming has been almost entirely inferred from studies dealing with the effects of these stressors in isolation. For species where the OCLTT model applies, thermal acclimation could be predicted to modify the hypoxic response by eliciting responses at all levels of organisation which serve to reduce internal oxygen limitation, including alterations to cellular energy consumption (ATP), oxygen supply pathways and mitochondrial function (Pörtner, 2010). Global molecular approaches have also revealed considerable overlap between the functional groups of genes affected by thermal acclimation and hypoxia in isolation such as aerobic (TCA cycle) and anaerobic (glycolytic) metabolic enzyme activity, mitochondrial function, protein turnover and cellular defences (Richards, 2009; Logan and Somero, 2010; Jayasundara *et al.*, 2013; Johnson *et al.*, 2015; Logan and Buckley, 2015). A more recently defined mechanism by which thermal acclimation could alter performance is “transcriptional/constitutive frontloading” where changes to constitutive gene expression elicited during acclimation prepare for subsequent stress and reduce the requirement for an inducible cellular stress response (Barshis *et al.*, 2013; Kenkel *et al.*, 2013). However, studies to date which have investigated constitutive frontloading have been restricted to the effects of thermal acclimation on subsequent acute thermal stress responses (Kenkel *et al.*, 2013) and have not yet investigated its consequences for performance under additional stressors of a different nature.

While these studies highlight the potential for interaction between hypoxia and thermal acclimation, stressors in combination have been shown to elicit unpredictable effects on marine species in many cases (Crain *et al.*, 2008; Todgham and Stillman, 2013; Boyd *et al.*, 2018) as was previously observed for

the physiological responses of *E. marinus* in Chapter 3. Also, the limited number of studies dealing with cellular responses have already highlighted the potential for the hypoxic response to be altered by other environmental variables (Todgham *et al.*, 2005; Rissanen *et al.*, 2006; Johnson *et al.*, 2015) but the modifying effects of thermal acclimation remain largely undetermined. Consequently, this chapter investigates the cellular mechanisms accompanying thermal acclimation which may lead to altered metabolic performance under hypoxia. Transcriptomic responses of individuals exposed to hypoxia (30 % a.s., normoxic control = 80 % a.s.) following 7 d warm acclimation (20 °C, control = 10 °C) were identified using RNA-Seq. The amphipod *Echinogammarus marinus* was used as a model as its physiological performance under hypoxia following warm acclimation was characterised in Chapter 3.

4.2 Methods

4.2.1 Animal collection and maintenance

E. marinus were collected from an intertidal mudflat at Saltash, UK (50°24’51.57” N, 4°12’41.70” W). Physico-chemical parameters (temperature and PO_2) of the mudflat which this species inhabits, were measured in summer (July). Temperature was measured using a temperature logger (iButton DS1921G, Thermochron, Australia) every 10 mins over ~ 12 days. Spot measurements of oxygen levels were measured on one day at low tide using a needle-type oxygen micro-sensor (NTH-PSt7, Presens, Germany) connected to an oxygen meter (Microx 4, Presens, Germany). Animals were maintained in the laboratory exactly as described in Chapter 3. Once again, only adult males

identified according to morphological criteria (Lincoln, 1979) were used in experiments to control for life cycle and gender differences. Males were distinguished from females by the presence or absence of oostegites.

4.2.2 Experimental acclimation to different temperatures

Individuals were acclimated for 7 d to one of two different temperatures (T_a = 10 °C (control) or 20 °C) exactly as described in Chapter 3. Briefly, amphipods were kept in sealed aquaria (vol. = 1.4 L) (n = 5 per treatment, 2 - 5 individuals in each) each partially immersed in plastic trays filled with deionised water at the appropriate T_a. The deionised water for the control temperature was reduced to T = 10 °C (9.9 ± 0.24, mean ± s.e.m) using a water chiller (L-350 Water Chiller, Guangdong Boyu Group Co.,Ltd., China) and that for the higher temperature was supplied with an adjustable water heater (100 W aquarium heater, EHEIM GmbH & Co KG, Germany) set at 20 °C (20.9 ± 0.3, mean ± s.e.m). A Koralia pump (Koralia Nano Evolution 900 Circulation Pump, Hydor, Italy) circulated water around the tray. Each aquarium was supplied with an airstone, connected to an air pump (Mistral 2000, Aqua Medic GmbH, Germany) and in order to minimise hypercapnia, air was scrubbed for carbon dioxide by passing it through a trap filled with NaOH (250-1000 $mmol.L^{-1}$, Sigma-Aldrich, Germany). This was replaced every two to three days as required and water changes were carried out every 3-4 days. Other environmental factors were kept constant (O_2 = 91.0 ± 1.7 % a.s., S = 32.0 ± 1.5, pH = 8.1 ± 0.15, mean ± s.e.m). Water temperature and oxygen partial pressure were measured daily using an oxygen microsensor (Pm-Pst7, Presens, Germany) and temperature probe (Pst 100, Presens, Germany) with a

dissolved oxygen meter (Microx 4, Presens, Germany). Salinity and pH were measured every 1 - 2 d using a hand held refractometer (HI96822 Digital Refractometer, Hanna Instruments, USA) and bench top pH meter (S400 SevenExcellence pH/mV meter, Mettler-Toledo International Inc., USA) respectively.

4.2.3 Hypoxic exposure conditions of amphipods for transcriptomic analysis

In order to test the cellular mechanisms underpinning altered metabolic responses to hypoxia at T_t = 10 °C identified in Chapter 3 (Fig. 4.1A), particularly, the hypometabolic shift under hypoxia in warm acclimated individuals, the exact same closed respirometry method was utilised from Chapter 3 at T_t = 10 °C but in the present study with one modification: individuals were only allowed to deplete the oxygen to 80 % a.s. or 30 % a.s. Individuals which were allowed to deplete oxygen down to 80 % a.s. were treated as normoxic controls for each acclimation temperature (T_a = 10 or 20 °C) (10NO or 20NO). R_1MO_2 was calculated for each individual. Individuals were allowed to deplete oxygen down to 30 % a.s. to allow identification of the hypometabolic shift and represented the hypoxia treatment for each acclimation temperature (10HY or 20HY). The depletions took approximately 1 hour for the normoxia treatments (10NO: 64 ± 6.8 mins, 20NO: 68 ± 11.6 mins, mean ± s.e.m). The hypoxia treatments took approximately 4 - 5 hours. The warm acclimated individuals took slightly longer due to the hypometabolic shift and slower rate of oxygen depletion (20HY: 300 ± 28.8 mins, mean ± s.e.m) than cold acclimated individuals (10HY: 248 ± 11.6 mins, mean ± s.e.m). This physiological approach meant that there was some variation in the time spent in

the respirometer between individuals from different treatments. The option was taken to standardise by end oxygen tension (80 % or 30% a.s.) rather than time spent in the respirometer due to the results of Chapter 2, where the transcriptomic responses of a gammarid amphipod to hypoxia varied drastically between different severities of hypoxia.

A segmented regression was once again fitted as described previously in Chapter 3 to calculate R_1MO_2 and R_2MO_2 for each individual. Once individuals had depleted the oxygen to 80 % a.s. for the normoxia treatments or 30% a.s. for the hypoxia treatments, the respirometry chamber was then opened, the individual rapidly removed and blotted dry before the wet mass was determined using a microbalance (MSA225P-000-DA, Göttingen Sartorius AG, Germany, ± 0.01 mg). Amphipod samples (wet mass = 87.3 ± 4.1 mg, mean ± s.e.m) were rapidly frozen in liquid nitrogen and stored at -80 °C for RNA-seq analysis (n = 5 individuals per treatment). For hypoxic individuals (10HY and 20HY), paired t-tests (performed in R v. 3.3.1, alternative hypothesis = “less”) between normoxic and hypoxic rates of aerobic metabolism (R_1MO_2 vs R_2MO_2) indicated a significantly lower MO_2, i.e. a hypometabolic shift, in warm acclimated individuals (20HY) (t_4 = -2.14, P = 0.049) but not cold acclimated individuals (10HY) (t_4= 2.82, P = 0.97) (Fig. 4.1B). In order to test the cellular mechanisms associated with altered hypoxic performance following warm acclimation, an RNA-seq experiment was performed using individuals exposed to the four conditions previously described: (1) 10 °C acclimated under normoxia (10NO) (2) 10 °C acclimated under hypoxia (10HY) (3) 20 °C acclimated under normoxia (20NO) and (4) 20 °C acclimated under hypoxia (20HY).

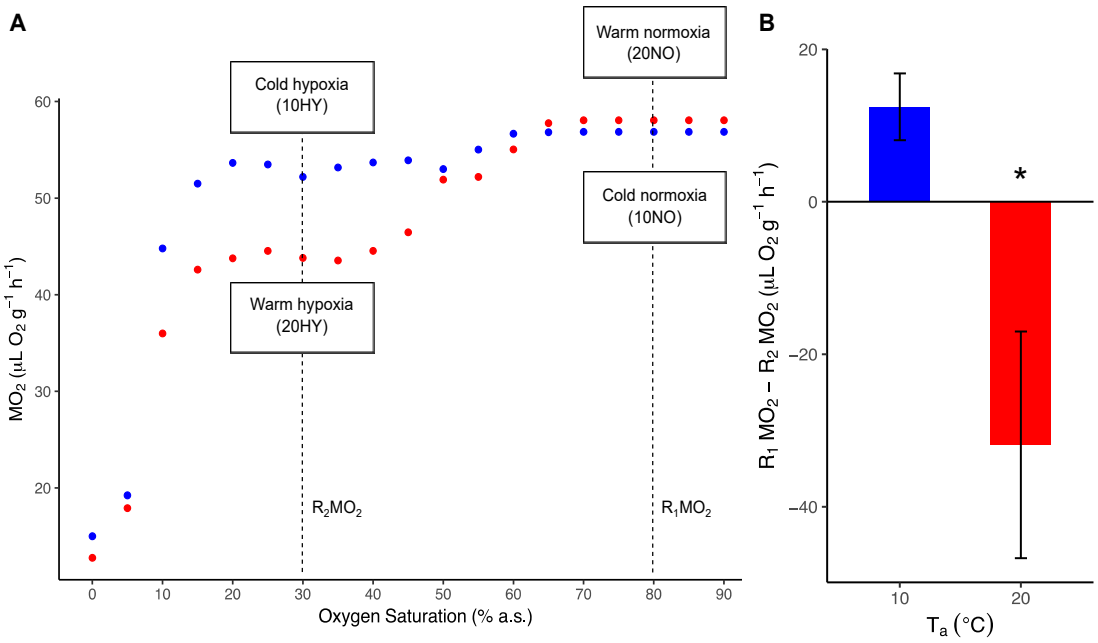

Fig. 4.1 Physiological response of *E. marinus* to hypoxia following thermal acclimation. (A) Representation of the metabolic response (MO_2) to acutely declining oxygen (% a.s.) at T_t = 10 °C in the amphipod *Echinogammarus marinus* following 7 d acclimation to different temperatures identified from Chapter 3. Metabolic rate was similar between acclimation temperatures between warm (T_a = 20 °C, red line) and cold acclimated (T_a = 10 °C, blue line) individuals between 100 % a.s. down to ~40-50 % a.s. (R_1MO_2). Between ~ 40-50 % a.s. and 10 % a.s., warm acclimated individuals display a significant reduction in MO_2 or hypometabolic shift (R_2MO2) not found in cold acclimated individuals. White boxes indicate the treatments utilised in the current study for the samples utilised for transcriptomic analysis (n = 5 per treatment). Individuals were allowed to deplete the oxygen within the respirometry chamber to 80 % a.s. for the normoxia (10NO and 20NO) treatment and 30 % a.s. for the hypoxic individuals (10HY and 20HY) following thermal acclimation. (B) Changes to metabolic rate between R_1MO_2 and R_2MO_2 (R_2MO_2 – R_1MO_2) for individuals allowed to deplete oxygen down to 30 % a.s. in the current experiment for warm

acclimated (20HY, red bars) compared to cold acclimated individuals (10HY, blue bars). Paired t-tests indicated warm acclimated, but not cold acclimated individuals underwent a significant reduction in MO_2, i.e. a hypometabolic shift between R_1MO_2 and R_2MO_2 ($P < 0.05$, indicated by asterisk, for further details, see Fig. A2.2)

4.2.4 Transcriptomic responses to hypoxia following thermal acclimation

4.2.5 Library preparation and RNA sequencing

Total RNA was isolated using the GeneJet RNA Purification Kit (Thermo Scientific, USA) for individuals in each treatment (n = 5). RNA integrity was assessed using Agilent Bioanalyser 2100 (Agilent Technologies, USA). Library preparation and RNA sequencing was performed at the Beijing Genomics Institute, Hong Kong. A total of 20 TruSeq RNA libraries (Illumina, San Diego, USA) were prepared and sequenced on two lanes of an Illumina HiSeq 4000 sequencer using 100 bp paired-end sequencing (Illumina, San Diego USA). Sequencing produced 862.4 M “clean” 100 bp paired-end reads (adapter-trimmed, removal of low quality reads (PHRED score < 7 across > 40 % of bases). Transcriptome assembly was performed using Trinity v.2.5.1 (with the parameter *–normalize_by_read_set* for *in silico* normalisation and *-min_kmer_cov* = 2 due to memory requirements) (Haas *et al.*, 2013). The assembly was annotated using Trinotate v.3.1.1.

4.2.6 Differential expression analysis and functional enrichment

Transcript expression was quantified using kallisto v0.44.0 (Bray *et al.*, 2016) ran as a part of Trinity. Transcript expression was imported into R and

summarised to the “gene” level using tximport v1.0.3 (Soneson *et al.*, 2015). Differential expression analysis was performed using DESeq2 v1.12.4 (Love *et al.*, 2014). Functional enrichment of significantly differentially expressed genes (DEGs) ($P_{adj} < 0.05$, log-2 fold change < -1 or >1) for GO terms was identified using GOSeq v1.24.0 (Young *et al.*, 2010). GOSeq corrects for annotation bias generated by different lengths of assembled transcripts. “Gene” lengths were generated from the assembled transcripts using the ‘TPM_weighted_gene_length.py’ script ran as part of Trinity.

4.2.7 Genes supporting altered metabolic performance under hypoxia

Gene expression was correlated with the metabolic responses of individuals to hypoxia using the method of Veilleux *et al.*, (2015). Briefly, MO_2 of each individual was standardised by transforming MO_2 for each individual to a difference from the mean MO_2 of the control group (10NO) (Fig 4.2B). This approach means that there will be some co-variation of MO_2 with treatment but the individuals for the warm-acclimated hypoxia treatment (20HY) did display a marked hypometabolism. A treatment-specific approach was not utilised given the small sample size per experimental condition (n=5). DEGs were used from pairwise comparisons between the following treatments: (1) 10 NO vs 10HY (2) 20 NO vs 20HY (3) 20 NO vs 10 NO (4) 20HY vs 10HY. Statistically significant correlations ($P < 0.05$) between these DEGs and physiological responses were identified using Pearson correlations between variance stabilised counts of DEGs (i.e. counts of transcript expression for each individual in the study which have then been subjected to variance stabilising transformation as part of the DESeq2 package) and the standardised MO_2 of individuals. DEGs found to be

significantly correlated with standardised MO_2 ($P < 0.05$) then underwent functional enrichment analysis as described previously for standard differential expression analysis (Section 4.2.6). Frontloaded DEGs were identified according to (Barshis *et al.*, 2013). Briefly, DEGs significantly affected by hypoxia were compared between the control group (10HY vs 10NO) against the warm acclimated group (20HY vs 20NO). Genes unique to the cold acclimated group (10HY vs 10NO) were then compared against DEGs resulting from prior warm acclimation (20NO vs 10NO). Up-regulated and down-regulated hypoxic DEGs in cold acclimated individuals (10HY vs 10NO) were considered separately. For upregulated genes, they were considered to be frontloaded following warm acclimation only if they met both of the following criteria: (1) expression of gene under normoxic conditions greater in warm acclimated than cold acclimated treatment (20NO > 10NO) (2) expression of gene displays a reduced degree of upregulation under hypoxia in warm acclimated individuals compared to cold acclimated individuals under hypoxia (the increase in expression (fold change) between 20HY vs 20NO < increase in expression (fold change) between 10HY vs 10NO). Downregulated transcripts can also be frontloaded and must meet similar criteria: (1) expression of gene under normoxic conditions reduced in warm acclimated compared to cold acclimated treatment (20NO < 10NO) (2) expression of gene displays a reduced degree of downregulation under hypoxia in warm acclimated individuals compared to cold acclimated individuals under hypoxia (the decrease in expression (fold change) between 20HY vs 20NO < decrease in expression (fold change) between 10HY vs 10NO).

4.3 Results

4.3.1 Environmental conditions at field site

The site which *E. marinus* inhabits experiences considerable variation in both temperature and oxygen levels. *E. marinus* can typically be found inhabiting mud beneath boulders and decaying seaweed, *Ascophyllum nodosum*. During summer months, water temperatures remain fairly stable at high tide at ~ 20 °C but can range between ~ 12 - 33 °C at low tide (Fig. A2.1). Hypoxia occurred at low tide within the moist mud and shallow pools (a few centimetres in depth), which this species inhabits, ranging from 3 – 40 % a.s.

4.3.2 Transcriptome assembly

The transcriptome assembly for *E. marinus* consisted of 386, 291 transcripts corresponding to 253, 499 genes (Trinity “genes”). For the resultant assembly, N50 equalled 1112 bp based upon the longest isoform per gene whilst the Ex90N50 equalled 2496 bp (N50 based upon transcripts accounting for 90 % of total normalised gene expression). Of the assembled transcriptome, 115,089 transcripts (29.8 %) received annotations (BlastX/BlastP) against the Uniprot/Swissprot database using an e-value cut-off of $1x10^{-5}$ and 112,552 (29.1%) against the GO database.

4.3.3 Effects of thermal acclimation and hypoxia on global gene expression

Thermal acclimation (20NO vs 10NO) elicited significant differences in the expression ($P_{adj} < 0.05$) of 24,189 genes. Upregulated DEGs (4981 genes)

were significantly enriched for multiple GO terms associated with carbohydrate metabolism, glycolipid metabolism and oxygen transporter activity (Table A2.1). Downregulated DEGs (19208 genes) were enriched predominantly for GO terms associated with protein synthesis, unfolded protein binding, the TCA cycle and ion transport (Table A2.2). Also, there were considerable changes to metabolic genes such as upregulation of anaerobic enzymes (lactate dehydrogenase) and hemocyanins (Table A2.3) but downregulation of aerobic metabolic enzyme genes belonging to the TCA cycle (citrate synthase) and mitochondrial electron transport chain (cytochrome c oxidase) (Table A2.4). Cellular heat shock proteins (HSPs) were also predominantly downregulated (Table A2.4). Exposure to hypoxia in 10 °C acclimated individuals (10HY vs 10NO) elicited significant changes in the expression of 267 genes but no significant GO enrichment was observed in upregulated DEGs (49 genes). Significant GO enrichment was only observed for downregulated DEGs (218 genes) in this group and was linked to protein synthesis and ion transport (Table A2.5). For 20 °C acclimated individuals exposed to hypoxia (20HY vs 20NO), 118 DEGs were found but no significant functional enrichment was observed for either upregulated (11 genes) or downregulated DEGs (107 genes).

4.3.4 Genes supporting metabolic performance under hypoxia following warm acclimation

Of the DEGs, 591 were significantly correlated ($P < 0.05$) (Fig 4.2A) with changes to MO_2 (Fig 4.2B). However, the majority of correlated genes arose from differences between acclimation temperatures rather than the transition

from normoxia to hypoxia (588 of the total 591 correlated genes were only significantly differentially expressed ($P < 0.05$ identified by DESeq2) between acclimation temperatures under normoxic conditions, 20NO and 10NO, and not hypoxic conditions 10HY vs 10NO or 20HY vs 20NO). Genes clustered into two groups (Clusters 1 and 2) but only Cluster 1 (consisting of 297 genes), which was primarily downregulated following warm acclimation (Fig. 4.2C), showed any significant GO enrichment. Cluster 1 consisted of genes that were significantly enriched for GO terms predominantly associated with the extracellular region and transmembrane transport (Table A2.6). These included genes involved in structural constituent of cuticle (e.g. cuticle proteins) (Fig. 4.2D). and structural molecule activity (e.g. ribosomal subunits) (Fig. 4.2E). Genes involved in transmembrane transport were involved in a variety of process such as ion transport and mitochondrial function (e.g. several electron transport chain complexes) (Fig. 4.2F). Cluster 2 (294 genes) was not significantly enriched for any GO terms ($P_{adj} > 0.05$) but the top three were associated with steroid, nucleic acid and nucleobase compound metabolic processes ($P_{adj} > 0.25$).

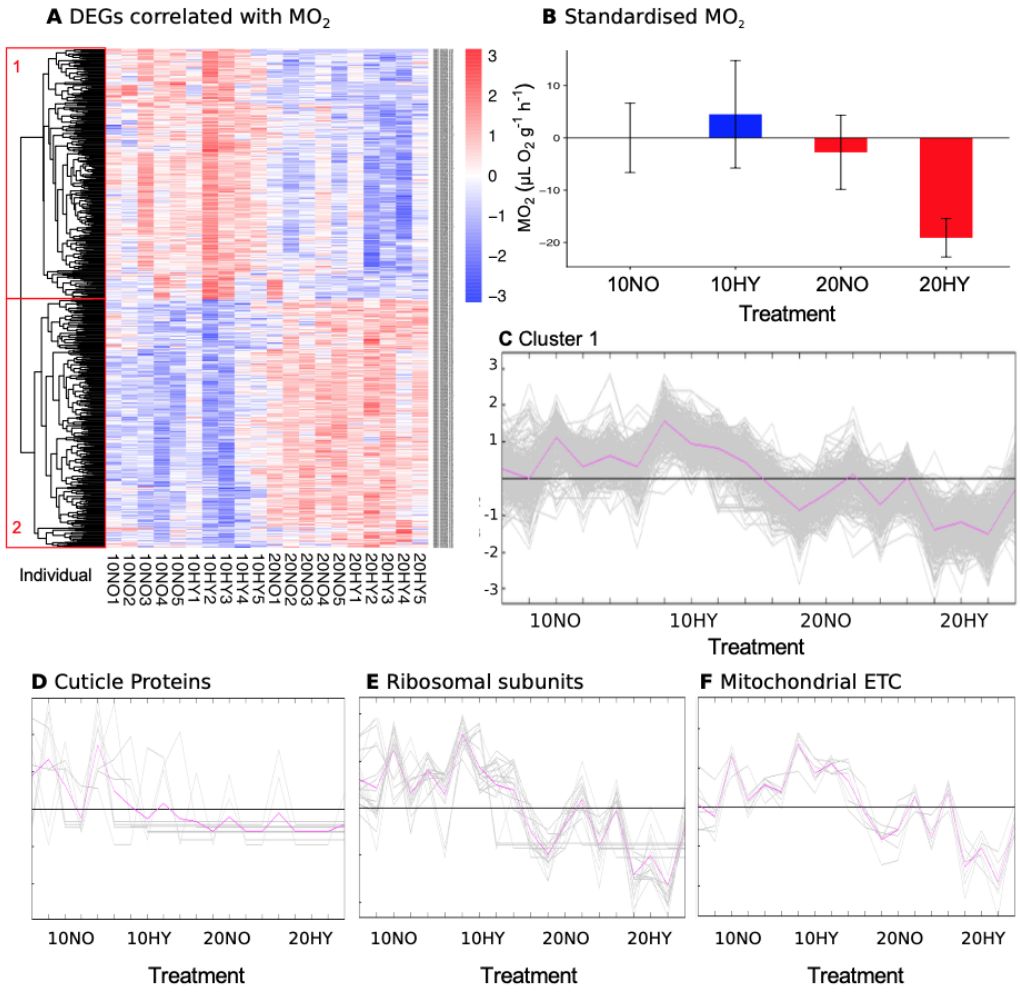

Fig. 4.2. Expression of DEGs significantly correlated with MO_2 in *E. marinus*. (A) Heatmap of gene expression (variance stabilised counts) in each individual (x-axis) for DEGs which were significantly correlated ($P < 0.05$) with MO_2. Colour scale indicates upregulated (red) and downregulated expression (blue). Genes were clustered by cutting the dendrogram at different heights following hierarchical clustering of genes producing two to five clusters of genes dependent upon cut height. However, clustered genes received similar functional annotations (GO terms) regardless of cut height and were therefore assigned to two larger clusters as smaller clusters may reflect redundancy (red boxes) (B) Genes were correlated against standardised MO_2 (difference to mean MO_2 of the control group (10NO)) (mean values ± s.e.m shown for each

treatment) (C) Expression of all genes (y-axis represents scaled variance stabilised counts) belonging to Cluster 1 (297 genes) for all twenty samples (x-axis represents treatment, tick marks represent replicates, n = 5 per treatment) associated with (D) cuticle proteins (E) ribosomal protein synthesis (F) mitochondrial ETC complexes. Grey lines represent individual genes while the purple line displays average expression. Data for Cluster 2 are not shown as no significant functional enrichment was observed.

4.3.5 Frontloaded transcripts via warm acclimation upon hypoxic exposure

A ~ 56 % reduction in the number of DEGs elicited by hypoxia was observed in warm acclimated (20HY vs 20NO) compared to cold acclimated individuals (10HY vs 10NO). All the annotated DEGs elicited by hypoxic exposure were explored further (116 of 267 DEGs for the cold acclimation treatment, 57 of 118 DEGs for the warm acclimation treatment). The responses to hypoxia were unique in each acclimation temperature with no overlap observed. Of the 116 unique DEGs in the cold acclimated hypoxia group, 15 of the 29 (51.7 %) upregulated DEGs (Fig. 4.3A) and 75 of the 87 (86.2 %) downregulated DEGs (Fig. 4.3B) were already significantly affected by previous warm acclimation (20NO vs 10NO) and could be considered to be “frontloaded” transcripts. Frontloaded upregulated DEGs were not significantly enriched for any GO terms. Downregulated transcripts frontloaded during warm acclimation showed a “reduced reaction” (Barshis *et al.*, 2013) upon exposure to subsequent hypoxic stress. These “reduced reaction” genes were significantly enriched for GO terms involved in protein synthesis (e.g. GO terms for ribosomal protein

subunits), translation, and calcium ion binding (e.g. caltractin/centrin genes) (Table A2.7).

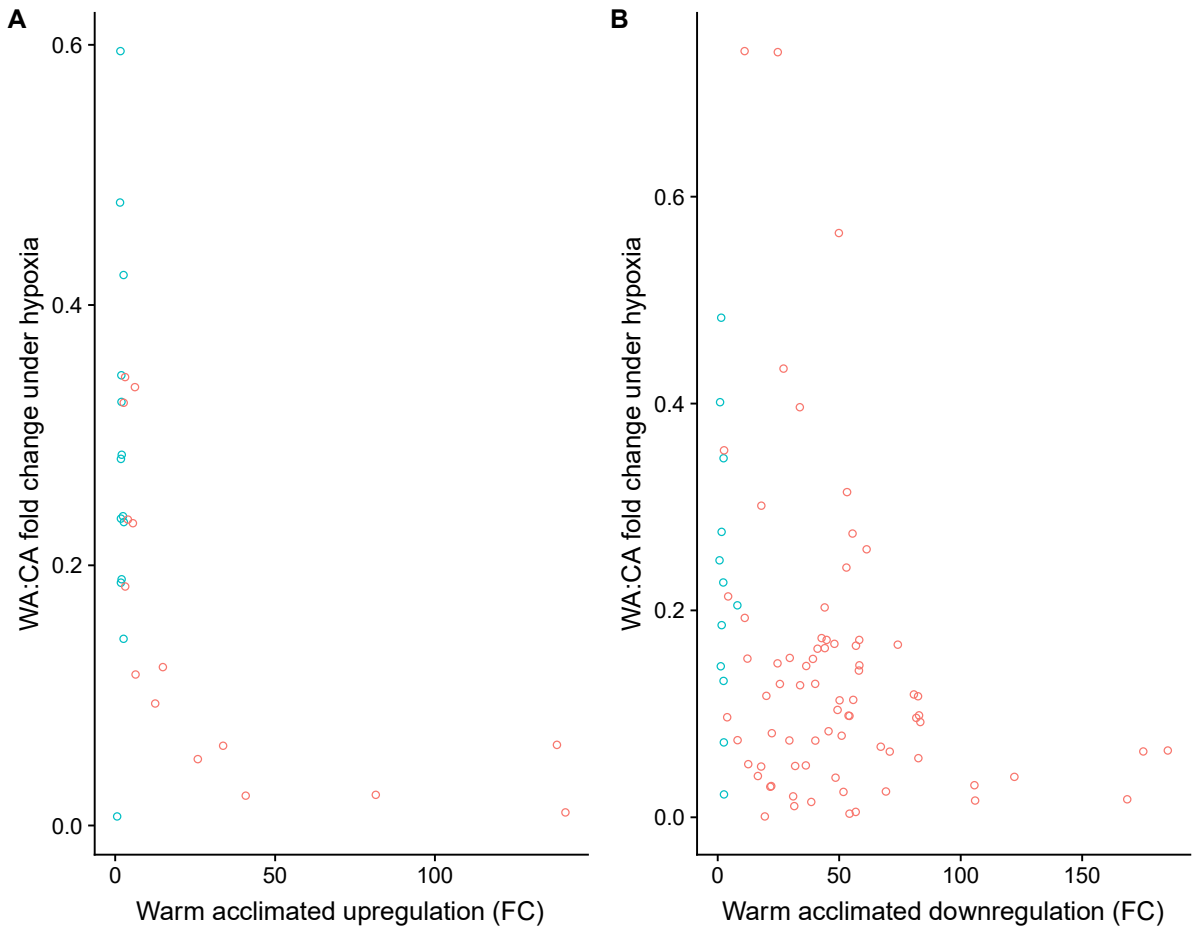

Fig. 4.3 Relationship between changes to gene expression resulting from warm acclimation and ratio of fold changes under hypoxia between warm and cold acclimated treatments. (A) Upregulated genes under hypoxia (n = 29) and (B) Downregulated genes (n = 87). Fold changes (FC) of genes resulting from warm acclimation under normoxic conditions (20NO vs 10NO) are portrayed along the x-axis. The y-axis deals with comparison of hypoxic responses between cold and warm acclimated individuals. The ratio of fold changes of genes under hypoxia between cold and warm acclimated individuals was calculated (fold change between 20HY vs 20NO: fold change between

10HY vs 10NO). Values < 1 for the warm acclimated: cold acclimated (WA:CA) fold change under hypoxia indicate a “reduced reaction” to hypoxia in the warm acclimated individuals (fold change between 20HY vs 20NO < fold change between 10HY vs 10NO). Genes which were significantly affected by previous warm acclimation indicated by red circles ($P_{adj} < 0.05$).

4.4 Discussion

This study investigated the molecular mechanisms accompanied by thermal acclimation which contribute to altered metabolic responses to hypoxia in the amphipod *Echinogammarus marinus*. Previous studies have only investigated a small number of genes and/or proteins (Todgham *et al.*, 2005; Fraser *et al.*, 2006; Rissanen *et al.*, 2006) while this present study provides the first insights into the global transcriptional mechanisms for any aquatic species experiencing hypoxia following warm acclimation. Correlation of gene expression profiles with metabolic responses implicated reductions in protein synthesis, mitochondrial function and cuticle gene expression with the hypometabolic response displayed by warm acclimated *E. marinus* exposed to declining PO_2. Gene expression profiles suggest that transcriptional frontloading arising from warm acclimation may be an important mechanism by which organisms may deal with the challenge of increasing frequencies of hypoxic events, as pre-exposure to elevated temperatures lead to the constitutive, frontloaded expression of multiple genes that are important in the reaction to hypoxia. These genes were putatively identified as being involved in translation, protein synthesis and calcium ion binding. Previous studies using fish have attributed an enhanced ability to deal with hypoxia following acclimation to mechanisms which increase

oxygen supply (Fraser *et al.*, 2006; Anttila *et al.*, 2015; McBryan *et al.*, 2016). However, it appears that mechanisms which reduce energetically-costly cellular processes may be more important for *E. marinus* and may contribute to the reduced metabolic demand observed at the level of the whole organism.

4.4.1 Warm acclimation creates a novel starting point to react to hypoxia

Whilst, it has long been recognised that thermal history can modify physiological responses to hypoxia in marine species (Fry, 1971; Herreid, 1980; Pörtner, 2010), the underpinning molecular mechanisms remain largely uncharacterised. At the physiological level in *E. marinus*, warm acclimation had no significant effect on MO_2 under normoxic conditions but resulted in a significantly lower, but regulated, MO_2 under hypoxia, when compared to cold acclimated individuals. It was initially unclear whether this warm acclimated hypometabolic shift was beneficial or detrimental given that interpretation of changes to MO_2 resulting from acclimation are somewhat subjective (Seebacher *et al.*, 2015). Although, it did significantly increase the duration of oxyregulation which may possibly contribute to the ability of *E. marinus* to inhabit a warm and, at times, severely hypoxic intertidal mudflat. The current findings at the transcriptional level appear to be consistent with the idea of an apparently adaptive hypometabolic strategy rather than a detrimental loss of oxyregulatory capacity under hypoxic stress.

An increased ability to deal with acute stress may be represented at the transcriptional level by a reduction in the number of significantly affected genes (i.e. less cellular reorganisation may be required to cope with a stressor)

(Barshis *et al.*, 2013). A reduced cellular response to acute thermal stress has been demonstrated following warm acclimation and acclimatisation in fish and corals (Logan and Somero, 2011; Barshis *et al.*, 2013) but has rarely been tested in a multistressor context. Reduced sensitivity to hypoxia may be indicated for the warm acclimated individuals by the observed 56 % reduction in the number of genes significantly affected by hypoxic exposure following warm acclimation. Alternatively, the reduced response could represent a detrimental effect and an inability to mount a response upon subsequent exposure to hypoxia.

Warm acclimation may have created a different physiological starting point from which to react to low oxygen through widespread cellular reorganisation involving significant changes in the expression of > 24,000 genes. Temperature has long been known to be a pervasive stressor at all levels of organisation (Somero, 2004). In fish and crustacean species, thermal acclimation has been shown to significantly affect a considerable proportion of the transcriptome (up to ~ 46 % of all tested genes in some species) (Ronges *et al.*, 2012; Jayasundara *et al.*, 2013). For *E. marinus*, these changes indicated widespread metabolic reorganisation involving a reduction in aerobic metabolic processes, such as downregulation of citrate synthase and cytochrome c oxidase, which may represent reduced capacity of the TCA cycle and mitochondrial electron transport chain respectively (Seebacher *et al.*, 2015). There may be an attempt to increase oxygen supply as indicated by upregulation of several hemocyanin genes (Johnson *et al.*, 2016) but recruitment of anaerobic metabolism may be indicated by increased expression of the glycolytic enzyme lactate dehydrogenase (Soñanez-Organis *et al.*, 2012). Despite their key role in

protein repair (Feder and Hofmann, 1999), heat shock proteins were surprisingly predominantly downregulated. However, a similar response has been observed in several species of warm acclimated fish and may reflect a unique effect of acclimation compared to acute heat stress (Logan and Somero, 2010; Jayasundara *et al.*, 2013). Also, acclimation can increase the temperature at which the heat shock response occurs (Tomanek and Somero, 1999) so potentially may have increased the temperature at which upregulation of heat shock proteins may begin in *E. marinus*.

4.4.2 Cellular mechanisms correlated with hypoxic performance following warm acclimation

The interpretation of transcriptomic data and integration of cellular responses with physiological performance remain a key challenge for marine animals exposed to hypoxia and temperature (Spicer, 2014; Logan and Buckley, 2015). There currently is no consensus method for correlating genes and physiological traits for RNA-Seq data (Seo *et al.*, 2016) and correlation analyses are typically hindered by the small sample sizes typical of RNA-Seq experiments (Ballouz *et al.*, 2015; Seo *et al.*, 2016). However, correlations were still attempted, accepting sample size as a potential limitation of our study. The method of Veilleux *et al.*, (2015) was utilised as it has previously been used to correlate metabolic performance with NGS expression data in a marine species using a similar sample size (n = 5 per treatment).

Whilst thermal acclimation affected a broad suite of genes in *E. marinus*, many of these cellular responses were not directly implicated in metabolic function in response to hypoxia when gene expression profiles were correlated with MO_2. Genes downregulated following warm acclimation that were significantly correlated with metabolic performance were functionally enriched for protein synthesis, ion transport, mitochondrial function and cuticle genes. Mitochondrial genes are key controllers of aerobic metabolism and known to be subject to modification by thermal acclimation (Seebacher *et al.*, 2010; Healy *et al.*, 2017) so their downregulation may be associated with the hypometabolic shift under hypoxia in warm acclimated *E. marinus*. Furthermore, reductions in ion transport and protein synthesis have long been implicated in hypometabolic states (Storey, 1988; Storey and Storey, 2004) but the transcriptional mechanisms coordinating hypometabolism are poorly characterised in most crustaceans, outside of selected models such as the brine shrimp *Artemia* (Hand, 1997; Hand *et al.*, 2011), due to a paucity of assembled genomes (Stillman and Hurt, 2015). However, downregulation of genes involved in ribosomal protein synthesis have been implicated in an increasing number of crustaceans in response to both severe hypoxia (Brouwer *et al.*, 2007; Rathburn *et al.*, 2013) and thermal acclimation (Yampolsky *et al.*, 2014). Genes involved in cuticle synthesis were also downregulated which may reflect a reduction in the energetic demand for moulting and growth (Abehsera *et al.*, 2015) and contribute to the lowered set point of metabolic regulation. Alternatively, the downregulation of these groups of genes may reflect a negative effect of acclimation to higher temperature. The shutting down of cellular processes may be needed as increased temperature can cause metabolic disturbance and oxidative stress (Sokolova, 2013)

4.4.3 Alterations to the hypoxic response may be driven by transcriptional frontloading during warm acclimation

Almost all of the genes correlated with changes to metabolic function were from changes elicited by thermal acclimation rather than the acute exposure to hypoxia. Hypoxia elicited an extremely limited transcriptional response affecting only several hundred genes regardless of acclimation temperature, which was in stark contrast to the widespread changes elicited by thermal acclimation. Responses were measured acutely over several hours and it is possible that greater magnitudes of response could have occurred if exposed to hypoxia over a longer time frame. Some hypoxia tolerant intertidal species, such as tidepool sculpins, display a minimal response to acute hypoxia typical of their variable intertidal environment but then much greater magnitudes of change under chronic hypoxic conditions (Mandic *et al.*, 2014). A muted transcriptional response of *E. marinus* may occur acutely to weather short periods of hypoxia typical of highly variable intertidal habitats (Mandic *et al.*, 2014).

Initially, the differing magnitudes of response elicited by warm acclimation and hypoxia may make it seem that there is little cross-talk between the underpinning mechanisms contrary to the OCLTT hypothesis that temperature is a problem of oxygen limitation (Pörtner *et al.*, 2017). However, upon further inspection ~ 75 % of DEGs elicited by hypoxia under control conditions were also significantly affected by prior warm acclimation. This is in agreement with previous studies on crustaceans where considerable similarity in microarray

profiles have been observed for temperature and hypoxia in isolation (De La Vega *et al.*, 2007).

This also suggest that transcriptional frontloading may have occurred which may have contributed to the observed differences in the metabolic responses to hypoxia between cold and warm acclimated individuals. For the cold acclimated treatment, the transcriptional response to hypoxia was characterised by significant downregulation of ribosomal pathways suggesting a reduction in costly protein synthesis similar to other marine species exposed to hypoxia (Gracey *et al.*, 2001; Brouwer *et al.*, 2007). In comparison, no biological functions were significantly enriched upon hypoxic exposure in the warm acclimated group which may be attributable to frontloading of genes during the acclimation period. Genes involved in protein synthesis and translation had already been significantly downregulated following warm acclimation and thus showed a non-significant “reduced reaction” (Barshis *et al.*, 2013) to hypoxia in warm acclimated individuals. Hypoxia-related reductions in the expression of protein synthesis genes have previously been shown to be subject to modification by other environmental factors for crustacean species, but only for hypercapnia (Rathburn *et al.*, 2013). However, changes to protein synthesis in some gammarid amphipod species have received attention in response to warm acclimation in isolation (Rastrick and Whiteley, 2013) and may contribute to a reduction in the energetic costs of life at warmer temperatures (Cossins and Bowler, 1987). Frontloaded calcium ion binding genes may also contribute to lowered energetic costs through downregulation of caltractin/centrin genes involved in the cell cycle (Salisbury *et al.*, 2002) and may also be associated with the greater degree of hypoxic hypometabolism observed in the warm

acclimated group. Whilst hypometabolism is a key strategy to survive environmental stress (Storey and Storey, 2004; Gorr, 2016), it is only a short-term solution and may not be beneficial in the long term with significant costs for fitness (Pörtner, 2010).

These marked alterations to hypoxic responses resulting from thermal acclimation, and expressed at the cellular level, highlight that molecular responses of marine animals to hypoxia may only be accurately predicted if the modifying effects of other environmental stressors are included. This notion has received attention for estuarine invertebrates exposed to hypoxia in combination with hypercapnia (Rathburn *et al.*, 2013; Johnson *et al.*, 2015) but this study highlights that thermal history needs to be carefully considered in future studies.

In conclusion, cellular reorganisation during warm acclimation, including transcriptional frontloading, may prepare for hypoxia in an intertidal amphipod and be associated with both an altered metabolic response and a muted transcriptional response to hypoxia compared to cold acclimated individuals. The thermally-regulated hypometabolic shift under hypoxia in warm acclimated *E. marinus*, may be beneficial and contribute to its ability to inhabit the high intertidal zone where hypoxia and high temperature are common (Agnew and Taylor, 1985). Cross-talk between cellular mechanisms which serve to reduce energetic costs during hypoxia and transcriptional frontloading may be a key mechanism by which marine species will deal with the threat of rapidly spreading hypoxic regions in a warming ocean (Breitburg *et al.*, 2018).

Chapter 5 General Discussion

5.1 Thesis aims: a recap

The aim of this thesis was to investigate the effects of thermal acclimation on hypoxia thresholds. Previous studies of the effects of temperature on the responses of aquatic animals to environmental hypoxia have centred on characterising the effects of acute warming (Herreid, 1980; McBryan *et al.*, 2013, 2016). Of those studies that examine the effects of acclimation, few explicitly test whether or not acclimation is beneficial. Previous studies have tested hypoxic performance at the temperature to which organisms have been acclimated ($T_a = T_t$). While this has been valuable in keeping the experiments focussed on physiological function, doing so does not necessarily forward our understanding of whether acclimation is beneficial/detrimental to hypoxic performance. This current thesis adopted classic and well-established methodology to directly test if warm acclimation can improve the ability to cope with hypoxia (i.e. individuals kept at different T_a compared at standardised T_t) (Precht *et al.*, 1973; Huey and Berrigan, 1996). Using this approach demonstrated that for species with thermally-sensitive hypoxia thresholds, it is predominantly an acute increase in temperature which is detrimental to oxyregulatory capacity under acutely declining oxygen tensions whilst warm acclimation may improve performance in some species or have no effect. This contrasts with a number of studies which predict reduced hypoxic performance in a warming ocean based predominantly upon studies of acute warming and acclimation studies where $T_a = T_t$ (Herreid, 1980; Rogers *et al.*, 2016). This thesis builds upon a comparatively limited number of recent studies on individual species demonstrating that warm acclimation may, in some cases,

offset the detrimental effects of acute warming on hypoxic performance at the physiological level (Anttila *et al.*, 2015; McBryan *et al.*, 2016). However, this thesis expanded upon these studies by incorporation of responses at multiple levels of organisation to characterise the responses to hypoxia in isolation (Chapter 2) and in combination with thermal acclimation across a range of species (Chapter 3) in order to identify inter and intraspecific mechanisms associated with beneficial changes to hypoxic performance (Chapters 3 and 4). The findings and implications of these investigations are explored in more detail below (Sections 5.2 - 5.4) but highlight that given the potential for acclimation to produce altered responses to hypoxia, acclimation capacity needs to be included in any future attempt to predict the consequences of future climate change-driven hypoxia on marine species.

5.2 What is hypoxia?: insights from the gammarids

In order to characterise the effects of thermal acclimation on hypoxic performance, the integrated responses to hypoxia in isolation were first characterised in the brackishwater amphipod *Gammarus chevreuxi* (Chapter 2). This was necessary given that we do not currently have an integrative understanding of the hypoxic responses of amphipods, amongst many other ecologically important marine invertebrates (Spicer, 2014).

While the physiological and biochemical responses of gammarid amphipods to low oxygen are well documented (Sutcliffe, 1984; Agnew and Taylor, 1985; Agnew and Jones, 1986; Hervant *et al.*, 1999), a truly integrative approach is

hindered by a lack of assembled genomes for this ecologically-important crustacean group (Stillman and Hurt, 2015). Published transcriptomes only exist for one species belonging to the genus *Echinogammarus* (Bossus *et al.*, 2014) and three *Gammarus* species (Truebano *et al.*, 2013; Trapp *et al.*, 2014; Gismondi and Thomé, 2016). For crustacean species, NGS sequencing has only fairly recently started to be applied to assess the effects of hypoxia (Sun *et al.*, 2014; Johnson *et al.*, 2015, 2016) with the majority of evidence coming from studies utilising microarrays (Brouwer *et al.*, 2004, 2007; Rathburn *et al.*, 2013). However, no studies utilising either microarrays or NGS sequencing have investigated the responses of amphipods to low oxygen.

Therefore, the assembled transcriptome for *G. chevreuxi* provided a first look into the global transcriptional response to low oxygen for any amphipod species. In an attempt to interpret the functional consequences of changes to global gene expression, the assembled transcriptome was integrated with biochemical analysis (anaerobic metabolites) and metabolic physiology (resting MO_2 and aerobic scope) to identify mechanisms affected by different levels of hypoxia (moderate = 40 % a.s. and severe = 20 % a.s.). Perhaps, the key finding of this study was that the hypoxic responses of this species was revealed to be highly complex once multiple levels of organisation were considered. A minimal physiological response to moderate hypoxia masked widespread molecular changes including upregulation of stress proteins, metabolic enzymes and hemocyanin genes. Whilst, conversely, a marked physiological response to severe hypoxia including hyperventilation by the pleopods was accompanied by an extremely limited transcriptional response indicating downregulation of genes involved in protein synthesis (ribosomal

subunits) (Sections 2.3.2 - 2.3.5). However, the minimal transcriptional response may represent a shutdown of the transcriptional machinery to conserve energy under severe hypoxia (Mandic *et al.*, 2014).

It was difficult to make meaningful comparisons of the integrated responses of *G. chevreuxi* with other marine invertebrates and identify whether amphipods display unique responses. The main reason for this was the paucity of comparable studies. While there is considerable evidence of multilevel responses to hypoxia for selected marine invertebrates, typically aquaculture species, these are largely restricted to relatively short exposures (< 24 hours) to severe hypoxia (Sussarellu *et al.*, 2012; Sun *et al.*, 2014, 2018; Johnson *et al.*, 2015). Studies which have investigated integrated responses of marine invertebrates to different levels of hypoxia chronically are extremely limited. The only comparable study for crustacean species comes from Brouwer *et al.*, (2007) where molecular responses to moderate and severe hypoxia over a period of several weeks were measured in the grass shrimp *Palaemon* (as *Palaemonetes*) *pugio*. However, the findings for *G. chevreuxi* (Section 2.3.4 – Section 2.3.5) are not in agreement this previous study of *P. pugio* which found minimal changes to gene expression under moderate hypoxia with widespread molecular changes only occurring under severe levels of hypoxia below P_c when resting MO_2 became disrupted (Brouwer *et al.*, 2007).

It is possible that this difference represents an increase in the resolution of NGS sequencing (~250, 000 genes) compared to previous studies which utilised microarray analysis (~ 76 - 691 genes) (Brouwer *et al.*, 2007, 2008). However, it

could also indicate taxonomic differences between decapods and amphipods in their response to hypoxia. The genes investigated previously in decapods (Brouwer *et al.*, 2007) belonged to similar functional categories as the study of *G. chevreuxi* such as hemocyanin, mitochondrial genes, metabolic enzymes and stress proteins, yet showed limited change in expression under moderate hypoxia (above P_c) compared to their widespread upregulation in *G. chevreuxi* (Section 2.3.5).

In this thesis it is tentatively suggested that amphipods may display unique transcriptional responses to hypoxia compared to other invertebrate taxa given that the second transcriptome assembled during this thesis for the amphipod *Echinogammarus marinus* (Chapter 4) displayed similar responses to *G. chevreuxi*. The transcriptomic response of *E. marinus* was investigated in response to hypoxia (30 % a.s.) following thermal acclimation (T_a = 10 or 20 °C). However, focussing solely on the molecular responses of *E. marinus* to hypoxia in isolation (i.e. under control conditions T_a = 10 °C), molecular changes associated with a reduction in ribosomal protein synthesis was once again observed under fairly moderate hypoxia (30 % a.s.) far above P_c (~ 10 % a.s. / 2 kPa) (Section 4.3.3). However, whether amphipods truly display unique responses will only become clear once more studies of marine invertebrates focus on the integrated effects of different levels of hypoxia under chronic conditions.

For *G. chevreuxi* and *E. marinus*, the adoption of an integrative approach also challenges current definitions of what degree of oxygen reduction may

constitute “hypoxia”. An ecological approach defines hypoxia based upon a threshold oxygen concentration of < 2 mL O_2 L^{-1} (Diaz and Rosenberg, 1995) but as demonstrated for *G. chevreuxi* by the reduction in aerobic scope (Section 2.3.2) and wide-ranging cellular response under 40 % a.s. (~2.6 mL O_2 L^{-1}), may overlook the consequences of more ‘moderate’ hypoxia (Sections 2.3.4 – 2.3.5). This thesis is not the first to question the applicability of the 2 mL O_2 L^{-1} definition of hypoxia with detrimental physiological and life-history consequences documented in many species above this threshold (Vaquer-Sunyer and Duarte, 2008) but may provide some insight into the molecular mechanisms driving reduced performance in ecologically-important marine invertebrates.

Given that challenges associated with the universal applicability of the ecological definition of hypoxia have been noted (Vaquer-Sunyer and Duarte, 2008), both ecologists and physiologists alike have since suggested that hypoxia should be context-specific either by ecosystem (Rabalais *et al.*, 2014) or by species (Seibel, 2011). Both *E. marinus* and *G. chevreuxi* are shallow coastal amphipods and a more recent ecological threshold has been suggested for “typically normoxic coastal regions” of ~ 1.4 mL O_2 L^{-1} (Rabalais *et al.*, 2014). However, it seems somewhat counter-intuitive to redefine the threshold at an even lower oxygen concentration given the numerous studies which have already demonstrated physiological reorganisation and life-history effects of hypoxia above 2 mL O_2 L^{-1} for coastal species (Butler *et al.*, 1978; Wiklund and Sundelin, 2001; Froehlich *et al.*, 2015).

The view taken in this thesis is that a physiological approach to define hypoxia should be used based upon the responses low oxygen elicits from individuals or species (Farrell and Richards, 2009; Seibel, 2011; Nikinmaa, 2013). Hypoxia has been defined based upon inhibition of MO_2 i.e. hypoxia begins at PO_2 below P_c (Seibel, 2011). While P_c is an important measure of aerobic capacity and indicator of hypoxia tolerance (Herreid, 1980; Mandic *et al.*, 2009), this thesis adopts the view that it may be too severe a threshold to mark the "beginning" of hypoxia for coastal species given the observed responses of *G. chevreuxi* and *E. marinus*. Both tested levels of hypoxia (40 % and 20 % a.s.) lie above the P_c for *G. chevreuxi* of ~ 12 % a.s. / 2.4 kPa (Truebano *et al.*, 2018), yet already elicited wide-ranging effects at multiple levels of organisation (Section 2.3.2 – 2.3.5). Similarly, *E. marinus* also demonstrated molecular changes at partial pressures of oxygen above P_c. Interestingly both *E. marinus* and *G. chevreuxi* displayed transcriptional responses associated with hypometabolism above P_c such as reduced expression of protein synthesis genes (Storey and Storey, 2011). This response may be associated with the observation that many gammarid amphipod species may not regulate metabolic rate under declining oxygen tensions at 100 % of the normoxic rate and instead can depress metabolism by up to 60 % before P_c is reached (Verberk *et al.*, 2018). *G. chevreuxi* shows good oxyregulatory capacity with a low P_c but may undergo slight, but not significant, reductions in metabolic rate (~ 10 - 20 %) before P_c (Section 2.3.2, Fig. 2.1A). There may be a different set point of metabolic regulation somewhere between more "moderate" hypoxia and P_c which remains to be fully characterised for gammarid amphipods.

Current definitions of hypoxia, both physiological and ecological, may therefore focus too much on the lower limits of what marine organisms can tolerate. P_c typically sets the lower limit of where individuals can survive (Boutilier and St-Pierre, 2000; Seibel, 2011) and the 2 mL O_2 L^{-1} threshold sets the lower limit of where healthy, diverse aquatic communities can exist (i.e. defaunation occurs below 2 mL O_2 L^{-1}) (Diaz and Rosenberg, 1995). Thresholds should perhaps not focus on the lowest limits of survival (for individuals or ecosystems) but when the sublethal effects of hypoxia begin. Given the findings for *G. chevreuxi* and *E. marinus*, there is much merit in the definitions of Farrell and Richards (2009) who suggest hypoxia should be defined as any reduction in oxygen that elicits a physiological effect and Nikinmaa (2013) who explicitly includes lower levels of organisation where hypoxia constitutes any 'reduction in oxygen tension below that normally experienced by an organism or cell'. Whilst the exact PO_2 at which hypoxic effects began was not identified due to the considerable costs of RNA-Seq, it is still important to consider that moderate hypoxia could have wide-ranging effects and should not be disregarded, which has largely been the case to date (Spicer, 2014). For gammarid amphipods, hypoxia may begin at PO_2 above current definitions through reductions in aerobic scope and the requirement for widespread transcriptional changes to maintain cellular viability, which may be associated with long term reductions in fitness (Truebano *et al.*, 2018).

5.3 Hypoxia in a chronically warming world: is acclimation beneficial?

Following assessment of the hypoxic response of *G. chevreuxi*, the modifying effects of thermal acclimation on hypoxic performance was investigated across

four species of closely related gammarid amphipods (*G. chevreuxi*, *G. duebeni*, *G. zaddachi* and *E. marinus)* (Chapter 3). It is unclear why the majority of previous studies have not attempted to extend methodology which tests for beneficial acclimation (Huey *et al.*, 1999) to include hypoxic performance, particularly as it has been used ubiquitously to test for the consequences of thermal acclimation for marine animals under normoxic conditions (Precht *et al.*, 1973; Prosser, 1973; Schulte *et al.*, 2011; Seebacher *et al.*, 2015). An understanding of acclimation capacity has repeatedly been deemed important in determining resilience to future climate change (i.e. increased temperature in isolation) (Stillman, 2003; Seebacher *et al.*, 2015) yet it has been recently noted that the ability to buffer against the combination of hypoxia and temperature via acclimation still “remains an open question” (McBryan *et al.*, 2013).

In crustaceans, metabolic responses to hypoxia have been investigated when $T_a = T_t$, typically demonstrating reduced performance at higher T_t (Al-Wassia and Taylor, 1977). The study of the four gammarid species in this thesis provides the first direct test of whether warm acclimation benefits hypoxic performance for any crustacean species. The effects of temperature, both acutely and chronically, on hypoxia thresholds are poorly characterised for amphipods and have only been characterised in a few freshwater *Gammarus* species (Sutcliffe, 1984; Hoback and Barnhart, 1996). Amphipods acclimated to different temperatures (T_a = 10 or 20 °C) were exposed to declining oxygen tensions at multiple acute T_t and P_c determined (T_t = 10 or 20 °C) to facilitate identification of beneficial acclimation (Huey and Berrigan, 1996) but in the context of dealing with hypoxia (Section 3.3.1). There was interspecific variation

in the responses elicited. Warm acclimated (T_a = 20 °C) *E. marinus* displayed a lower P_c than cold acclimated (T_a = 10 °C) individuals at warm T_t (T_t = 20 °C) (Section 3.3.1, Fig. 3.1H). This species showed the clearest beneficial effect of warm acclimation on hypoxic performance. *G. duebeni* and *G. zaddachi* may have displayed a somewhat similar response but this reflected a non-significant trend (Section 3.3.1, Fig. 3.1 D and F). Identification of significant effects was perhaps hindered by high levels of individual variation and would perhaps benefit from either an individual approach or larger sample size. *G. chevreuxi* displayed thermally insensitive P_c after 6 weeks thermal acclimation (Section 3.3.1, Fig. 3.1 B, 6w data) but such a response is not unheard of for *Gammarus* species (Hoback and Barnhart, 1996). It is unclear why hypoxic performance of some *Gammarus* species is seemingly unaffected by increased temperature, contrary to the majority of organisms where increased temperature raises P_c (Herreid, 1980). There is some evidence that larger species are more oxygen-limited in response to the combination of warming and hypoxia compared to smaller species (Verberk and Bilton, 2011; Verberk *et al.*, 2018). Amphipod species such as *G. duebeni*, *G. chevreuxi* and *G. zaddachi* are relatively small (< ~ 60 mg wet mass) and may perhaps not be as oxygen limited when exposed to hypoxia at 20 °C perhaps explaining the relatively small increases in P_c (~ 3-5 % a.s. / 0.62 – 1.04 kPa). Although not formally tested, the largest amphipod species tested, *E. marinus* (~ 90 mg wet mass), interestingly showed the greatest reduction in hypoxic performance at 20 °C (P_c increased by ~ 8 - 15 % a.s. / 1.66 – 3.11 kPa) (Section 3.3.1, Fig. 3.1H). Alternatively, it may be that the upper test temperature (T_t = 20 °C) was simply not high enough to elicit a reduction in hypoxic performance for some of these species. For the amphipod *Gammarus pseudolimnaeus,* oxyregulatory capacity was found to be

similar between 10 - 25 °C (Hoback and Barnhart, 1996). Additionally, the intertidal copepod *Tigriopus brevicornis* displays thermally insensitive P_c between 15 - 25 °C with an increase only observed at 30 °C (McAllen *et al.*, 1999).

Overall, the observation that warm acclimation allows for a recovery of hypoxic performance in some species suggests that investigation of the effects of acute temperature change, typical of laboratory studies, may not accurately predict subsequent future hypoxic performance. As seen for *E. marinus*, acute thermal conditions and thermal acclimation had a significant interactive effect on P_c (i.e. the effects of warm acclimation were not consistent across T_t, with a significant reduction in P_c only noted at some (T_t = 20 °C) but not all (T_t = 10 °C) T_t (Section 3.3.1). Testing responses at the temperature to which organism have been acclimated may be ecologically relevant ($T_a = T_t$), as noted for the effects of temperature in isolation (Schulte *et al.*, 2011), but organisms may not always experience hypoxia at the temperature to which they have been acclimated. The responses elicited by hypoxia in the wild may be influenced by a complex combination of both the acute thermal conditions and previous thermal history of an organism. Particularly, as demonstrated for the tested species in this thesis and previously for molluscs, acclimation can modify hypoxic responses at none, some or all acute T_t (Hicks and McMahon, 2002; Alexander and McMahon, 2004).

5.4 Mechanisms driving altered hypoxic performance in a chronically warmer ocean

Whilst adoption of methodology to test the BAH allowed the consequences of thermal acclimation for hypoxic performance to be tested, a key question remained of which mechanisms affected by thermal acclimation drove altered hypoxic performance across these species. These underpinning mechanisms are important to understand as they "represent the how of acclimation" (Mayr, 1961; Kingsolver and Huey, 1998). In an attempt to identify a potential underpinning mechanism, the warm acclimated change in MO_2 was correlated against the warm acclimated change in P_c across the tested species (Section 3.3.2). If more species had been tested, a formal phylogenetic approach would have been utilised given that phylogenetic trees are available for gammarid amphipods (Costa *et al.*, 2009; Hou and Sket, 2016). Unfortunately, there were too few species tested in the study (n = 4) to produce a reliable correlation between the two traits. However, data were pooled from T_t across species to give a larger sample size (n = 10). A relationship was observed between the warm acclimated change to MO_2 and P_c, but this was not statistically significant (Section 3.3.2). This may be associated with interspecific differences in the relationship between MO_2 and P_c. *E. marinus*, *G. zaddachi* and *G. duebeni* showed a positive correlation between MO_2 and P_c but for *G. chevreuxi,* the relationship between MO_2 and P_c was weak (lowest R^2) (Section 3.3.2, Fig. 3.2). Interpretation of whether a warm acclimated reduction in MO_2 is beneficial under normoxic conditions has been considered to be subjective (Seebacher *et al.*, 2015) but from this thesis, the reduction in MO_2 may have benefits for some (but not all) species for hypoxic performance through a reduction in P_c (Section 3.3.2, Fig. 3.3).

Finally, this thesis set out to assess the mechanisms underpinning altered hypoxic performance within a single species. The molecular mechanisms underpinning altered metabolic responses to hypoxia following thermal acclimation in the amphipod *E. marinus* were investigated (Chapter 4). *E. marinus* displayed an interesting metabolic response to declining oxygen tensions at cold test temperatures (T_t = 10 °C). Warm acclimated individuals (T_a = 20 °C) displayed a hypometabolic shift between ~40 – 50 % and 10 % a.s. not present in cold acclimated individuals (T_a = 10 °C). This shift did not represent a precipitous decline in MO_2 but was regulated at a lower set point until the P_c at ~ 10 % a.s. / 2kPa (Section 3.3.1 and 4.2.3 Fig. 4.1A). The reduction in MO_2 does not necessarily represent a reduction in performance and may contribute to oxyregulation in *Gammarus* species (Verberk *et al.*, 2018). While the subtle reduction in metabolism did not have the benefit of lowering P_c, it may reflect enhanced performance by letting individuals oxyregulate for significantly longer periods of time compared to cold acclimated individuals (Section 3.3.1).

The effects of thermal acclimation in isolation on MO_2 and cellular mechanisms (protein synthesis) have received attention in *Gammarus* species (Bulnheim, 1979; Rastrick and Whiteley, 2011, 2017). However, how those affected mechanisms subsequently perform under hypoxia has received little attention in amphipods and aquatic organisms generally.

For the study of *E. marinus*, a similar methodological approach was utilised to that for *G. chevreuxi* (Chapter 2) combining RNA-Seq with metabolic physiology. Of particular interest were the mechanisms which coordinated the

hypometabolic shift under hypoxia in warm acclimated *E. marinus*. In order to investigate the link between responses at the physiological and molecular level, gene expression profiles were correlated with metabolic performance (Veilleux *et al.*, 2015) suggesting that the hypometabolic shift observed in warm acclimated individuals under hypoxia may be associated with the downregulation of mitochondrial, ribosomal and cuticle genes (Section 4.3.4). The majority of these genes represent significantly differentially expressed between acclimation temperatures (T_a = 10 vs T_a = 20 °C under normoxic conditions) and not as a direct result of hypoxic exposure. Therefore, they may represent preparative changes occurring during the acclimation period which has been termed “transcriptional/constitutive frontloading” (Barshis *et al.*, 2013).

The notion that thermal acclimation can induce mechanisms which prepare for hypoxia and vice versa has gained interest over recent years (Anttila *et al.*, 2015; McBryan *et al.*, 2016) perhaps stemming from the OCLTT hypothesis which suggests that warm acclimation may induce mechanisms to relieve internal oxygen limitation and thus potentially buffer against both stressors (Pörtner, 2012). At the physiological level, warm acclimation has been demonstrated to enhance hypoxic performance by mechanisms such as gill plasticity and cardiac function to increase oxygen supply (Anttila *et al.*, 2015; McBryan *et al.*, 2016). Surprisingly, mechanisms to increase oxygen supply were not implicated in the frontloaded transcripts of *E. marinus* and the direct interaction of temperature and hypoxia at the cellular level (Section 4.3.5). However, this could reflect mechanisms operating over different time scales as warm acclimation was chronic (1 week) but hypoxic exposure was acute

(several hours). *E. marinus* is a high intertidal species and may perhaps rely on a minimal transcriptional response to deal with short episodes of hypoxia similar to intertidal sculpins (Mandic *et al.*, 2014) rather than expending energy to produce gene products associated with enhancing oxygen supply. There was some overlap observed in the molecular response elicited by warm acclimation in *E. marinus* (Section 4.3.3) and exposure to chronic moderate hypoxia in *G. chevreuxi* (Section 2.3.5) where both species did display an upregulation of hemocyanin genes associated with increasing oxygen transport in crustaceans (Brouwer *et al.*, 2007; Johnson *et al.*, 2015). Aside from this effect, warm acclimation and moderate hypoxia had markedly different effects on gene expression profiles, particularly genes associated with oxygen and cellular energy demand. The responses may not be directly comparable given that they were identified from different species. However, moderate hypoxia induced an upregulation of genes involved in protein synthesis, aerobic enzymes and cellular defences (Section 2.3.5) whilst, in stark contrast, warm acclimation led to widespread downregulation of all of these groups of genes (Section 4.3.3).

It therefore seems that changes to metabolic performance under hypoxia in an increasingly warmer ocean may become more dependent upon frontloading of transcripts which reduce energetic demand rather than those that increase oxygen supply (Section 4.3.5). Hypometabolism has long been considered a key mechanism to deal with environmental stress (Storey and Storey, 2004; Gorr, 2016) and typically involves reductions in ATP demanding processes such as ion transport and protein synthesis (Storey and Storey, 2004). Downregulation of ribosomal pathways implicated reduced protein synthesis in the cellular response of *E. marinus* to chronic warming and hypoxia. Although

reductions in cellular processes such as protein synthesis may be essential to deal with environmental stress (Storey, 1988), they may not be optimal in the long term as it may come at a cost of longer term fitness traits such as growth and reproduction (Pörtner, 2010; Sheridan and Bickford, 2011). This is particularly the case as it coincided with downregulation of cuticle genes, associated with growth in crustacean species (Abehsera *et al.*, 2015) (Section 4.3.4).

5.5 Conclusions

It seems that at all levels of organisation, from genes to the whole organism, the responses of marine invertebrates to hypoxia can be modified by thermal acclimation. Acclimation may allow recovery of hypoxic performance in some species and the capacity of species to ameliorate the rise in MO_2 induced by an acute temperature increase may be a contributing factor. Within a species, thermal acclimation may drive widespread cellular reorganisation which alters the metabolic response to subsequent exposure to low oxygen. Future studies need to investigate how thermal acclimation affects hypoxic performance in a wider number of species using appropriate experimental designs in order to predict the consequences of rapidly spreading hypoxic regions under climate change in marine ecosystems (Keeling *et al.*, 2010; Breitburg *et al.*, 2018).

Appendices

Sections of Appendix 1 have been published in:

Collins, M., Tills, O., Spicer, J.I. and Truebano, M. (2017) *De novo* transcriptome assembly of the amphipod *Gammarus chevreuxi* exposed to chronic hypoxia, *Marine Genomics*, 33, 17-19 doi: 10.1016/j.margen.2017.01.006

Collins, M., Tills, O., Turner, L.M., Clark, M.S., Spicer, J.I. and Truebano, M. (2019) Moderate reductions in dissolved oxygen may compromise performance in an ecologically-important estuarine invertebrate, 693, 133444 doi: 10.1016/j.scitotenv.2019.07.250

Appendix 1 – to accompany Chapter 2

Table A1.1 MixS descriptors for the *Gammarus chevreuxi* transcriptome

Item	Description
Investigation_type	Eukaryote
Project_name	Adult transcriptome for *Gammarus chevreuxi*
Lat_lon	−50° 39′ 03″ N, 4° 08′ 56″W
Geo_loc_name	United Kingdom: Plymouth
Collected_by	Manuela Truebano
Collection_date	01-Jun-13
Environment	Brackish estuary
Biome	ENVO:00002137
Feature	ENVO:00000229
Material	ENVO:00002019
Depth	<0.5m
Alt-elev	0m
Temperature	15 °C
Salinity	15 PSU
Sequencing method	Illumina HiSeq
Assembly method	Trinity (v 2.2.0)
Assembly name	*Gammarus chevreuxi* adult transcriptome
Genome coverage	x10

Table A1.2 Assembly statistics for the *G. chevreuxi* transcriptome

Assembled bases	Number of contigs	Mean contig length	Median contig length	N50	GC content
117,494,825	144,501	813.11	350	1,618	43.94

Annotation statistics (transcripts)

Swissprot (blastx)	SignalP	GO	Eggnog	KEGG
23,498	2,902	24,030	17,852	18,833

Table A1.3 Top 40 gene ontology (GO) terms for biological processes (BP), cellular components (CC) and molecular function (MF) for genes significantly upregulated (P_{adj} < 0.01, log-2 fold change > 1) following exposure to 40% a.s. compared to 100% a.s.

40% a.s. upregulated

BP

GO term	Description	Padj
GO:0006518	peptide metabolic process	< 1e-30
GO:0043043	peptide biosynthetic process	< 1e-30
GO:0043604	amide biosynthetic process	< 1e-30
GO:0006412	translation	< 1e-30
GO:0043603	cellular amide metabolic process	< 1e-30
GO:1901566	organonitrogen compound biosynthetic pro...	1.30E-23
GO:1901564	organonitrogen compound metabolic proces...	3.30E-20
GO:0034641	cellular nitrogen compound metabolic pro...	2.60E-14
GO:0019538	protein metabolic process	1.20E-12
GO:0010467	gene expression	1.50E-12
GO:0042254	ribosome biogenesis	5.20E-12
GO:0043170	macromolecule metabolic process	6.90E-12
GO:0006807	nitrogen compound metabolic process	4.60E-11
GO:0044267	cellular protein metabolic process	6.00E-11
GO:0044238	primary metabolic process	8.70E-11
GO:0044260	cellular macromolecule metabolic process	4.30E-10
GO:0022613	ribonucleoprotein complex biogenesis	4.50E-09
GO:0034660	ncRNA metabolic process	5.00E-09
GO:0071704	organic substance metabolic process	5.20E-09
GO:0044271	cellular nitrogen compound biosynthetic ...	2.50E-08
GO:0034645	cellular macromolecule biosynthetic proc...	5.20E-08
GO:0016072	rRNA metabolic process	7.10E-08
GO:0006364	rRNA processing	2.30E-07
GO:0034470	ncRNA processing	2.40E-07
GO:0008152	metabolic process	2.50E-07
GO:0002181	cytoplasmic translation	3.30E-07
GO:0009059	macromolecule biosynthetic process	3.60E-07
GO:0032455	nerve growth factor processing	2.50E-06
GO:0006465	signal peptide processing	2.90E-06
GO:0044249	cellular biosynthetic process	3.00E-06
GO:0009058	biosynthetic process	3.10E-06
GO:0042255	ribosome assembly	3.60E-06
GO:0001991	regulation of systemic arterial blood pr...	3.90E-06
GO:0033260	nuclear DNA replication	5.60E-06
GO:1901576	organic substance biosynthetic process	6.80E-06
GO:0006281	DNA repair	7.40E-06
GO:0001999	renal response to blood flow involved in...	8.10E-06

GO:0002001	renin secretion into blood stream	8.10E-06
GO:0003081	regulation of systemic arterial blood pr...	8.60E-06
GO:0031135	negative regulation of conjugation	1.50E-05

CC

GO term	Description	Padj
GO:0005840	ribosome	< 1e-30
GO:0030529	intracellular ribonucleoprotein complex	1.40E-25
GO:1990904	ribonucleoprotein complex	1.40E-25
GO:0032991	macromolecular complex	1.50E-22
GO:0044391	ribosomal subunit	1.90E-21
GO:0022626	cytosolic ribosome	2.50E-19
GO:0044445	cytosolic part	1.70E-17
GO:0043232	intracellular non-membrane-bounded organ...	2.90E-16
GO:0043228	non-membrane-bounded organelle	6.50E-16
GO:0022625	cytosolic large ribosomal subunit	7.90E-15
GO:0044815	DNA packaging complex	8.30E-14
GO:0015934	large ribosomal subunit	1.70E-13
GO:0000786	nucleosome	3.60E-12
GO:0005829	cytosol	1.80E-10
GO:0032993	protein-DNA complex	2.30E-10
GO:0015935	small ribosomal subunit	7.80E-09
GO:0005816	spindle pole body	1.10E-08
GO:0044732	mitotic spindle pole body	1.80E-08
GO:0044424	intracellular part	4.40E-08
GO:0000785	chromatin	4.40E-08
GO:0044427	chromosomal part	8.80E-08
GO:0000329	fungal-type vacuole membrane	1.60E-07
GO:0005622	intracellular	7.70E-07
GO:0022627	cytosolic small ribosomal subunit	1.20E-06
GO:0000324	fungal-type vacuole	1.90E-06
GO:0005694	chromosome	4.50E-06
GO:0000790	nuclear chromatin	4.70E-06
GO:0000322	storage vacuole	5.80E-06
GO:0000228	nuclear chromosome	7.60E-06
GO:0044452	nucleolar part	8.60E-06
GO:0000502	proteasome complex	1.20E-05
GO:0043229	intracellular organelle	2.50E-05
GO:0072686	mitotic spindle	3.40E-05
GO:0031160	spore wall	7.10E-05
GO:0031310	intrinsic component of vacuolar membrane	7.10E-05
GO:0005796	Golgi lumen	8.30E-05
GO:0009506	plasmodesma	8.60E-05
GO:0055044	symplast	8.60E-05
GO:0044454	nuclear chromosome part	8.90E-05

GO:0005839	proteasome core complex	9.70E-05

MF

GO term	Description	Padj
GO:0003735	structural constituent of ribosome	< 1e-30
GO:0005198	structural molecule activity	1.60E-26
GO:0005344	oxygen transporter activity	7.50E-06
GO:0004386	helicase activity	1.20E-05
GO:0003678	DNA helicase activity	2.10E-05
GO:0019843	rRNA binding	2.40E-05
GO:0009378	four-way junction helicase activity	4.60E-05
GO:0047405	pyrimidine-5'-nucleotide nucleosidase ac...	7.70E-05
GO:0004298	threonine-type endopeptidase activity	0.00015
GO:0070003	threonine-type peptidase activity	0.00015
GO:0046961	proton-transporting ATPase activity, rot...	0.00017
GO:0003723	RNA binding	0.00018
GO:0036442	hydrogen-exporting ATPase activity	0.00032
GO:0031014	troponin T binding	0.00034
GO:0044769	ATPase activity, coupled to transmembran...	0.00059
GO:0008234	cysteine-type peptidase activity	0.00099
GO:0034062	RNA polymerase activity	0.00127
GO:0001872	(1->3)-beta-D-glucan binding	0.00129
GO:0003876	AMP deaminase activity	0.00129
GO:0043682	copper-transporting ATPase activity	0.00129
GO:0047623	adenosine-phosphate deaminase activity	0.00129
GO:0000991	transcription factor activity, core RNA ...	0.00193
GO:0003899	DNA-directed RNA polymerase activity	0.00237
GO:0008252	nucleotidase activity	0.00249
GO:0016787	hydrolase activity	0.00305
GO:0004197	cysteine-type endopeptidase activity	0.00353
GO:0019829	cation-transporting ATPase activity	0.00382
GO:0042625	ATPase coupled ion transmembrane transpo...	0.00422
GO:0004812	aminoacyl-tRNA ligase activity	0.00429
GO:0016875	ligase activity, forming carbon-oxygen b...	0.00429
GO:0016876	ligase activity, forming aminoacyl-tRNA ...	0.00429
GO:0008381	mechanically-gated ion channel activity	0.00523
GO:0022833	mechanically gated channel activity	0.00523
GO:0005302	L-tyrosine transmembrane transporter act...	0.00578
GO:0045309	protein phosphorylated amino acid bindin...	0.00654
GO:0015197	peptide transporter activity	0.00742
GO:0001871	pattern binding	0.00915
GO:0030247	polysaccharide binding	0.00915
GO:0016798	hydrolase activity, acting on glycosyl b...	0.00919
GO:0015181	arginine transmembrane transporter activ...	0.00959

40% a.s. down-regulated

BP

GO term	Description	Padj
GO:0032501	multicellular organismal process	1.90E-25
GO:0044707	single-multicellular organism process	2.80E-21
GO:0032502	developmental process	7.40E-20
GO:0044767	single-organism developmental process	7.70E-20
GO:0044699	single-organism process	9.50E-20
GO:0065007	biological regulation	2.20E-19
GO:0044700	single organism signaling	1.10E-18
GO:0023052	signaling	1.30E-18
GO:0030154	cell differentiation	1.10E-17
GO:0048856	anatomical structure development	2.20E-17
GO:0055001	muscle cell development	2.40E-17
GO:0007165	signal transduction	3.00E-17
GO:0048468	cell development	3.90E-17
GO:0007275	multicellular organism development	6.60E-17
GO:0055002	striated muscle cell development	1.80E-16
GO:0050789	regulation of biological process	2.00E-16
GO:0007154	cell communication	2.00E-16
GO:0007166	cell surface receptor signaling pathway	1.60E-15
GO:0048869	cellular developmental process	2.60E-15
GO:0009653	anatomical structure morphogenesis	5.50E-15
GO:0051146	striated muscle cell differentiation	1.30E-14
GO:0048731	system development	1.40E-14
GO:0009888	tissue development	2.00E-14
GO:0030239	myofibril assembly	3.40E-14
GO:0048513	animal organ development	1.50E-13
GO:0051239	regulation of multicellular organismal p...	1.70E-13
GO:0022610	biological adhesion	2.10E-13
GO:0060429	epithelium development	2.20E-13
GO:0048583	regulation of response to stimulus	2.50E-13
GO:0023051	regulation of signaling	2.90E-13
GO:0048519	negative regulation of biological proces...	6.20E-13
GO:0007155	cell adhesion	1.10E-12
GO:0050794	regulation of cellular process	1.90E-12
GO:0061061	muscle structure development	2.00E-12
GO:0042692	muscle cell differentiation	2.20E-12
GO:0031032	actomyosin structure organization	3.50E-12
GO:0010646	regulation of cell communication	4.70E-12
GO:0048523	negative regulation of cellular process	4.80E-12
GO:0003008	system process	1.30E-11
GO:0045214	sarcomere organization	1.60E-11

CC

GO term	Description	Padj
GO:0005886	plasma membrane	8.70E-26
GO:0071944	cell periphery	6.20E-25
GO:0031224	intrinsic component of membrane	1.20E-24
GO:0044425	membrane part	1.40E-24
GO:0016021	integral component of membrane	5.50E-24
GO:0016020	membrane	3.20E-23
GO:0043292	contractile fiber	4.20E-23
GO:0030016	myofibril	5.90E-23
GO:0044449	contractile fiber part	2.10E-22
GO:0030017	sarcomere	9.60E-20
GO:0044459	plasma membrane part	2.60E-17
GO:0030018	Z disc	9.70E-15
GO:0031674	I band	1.80E-14
GO:0015629	actin cytoskeleton	9.90E-13
GO:0005576	extracellular region	1.80E-12
GO:0031226	intrinsic component of plasma membrane	1.70E-11
GO:0098590	plasma membrane region	5.00E-11
GO:0005887	integral component of plasma membrane	6.20E-11
GO:0098589	membrane region	2.00E-10
GO:0032982	myosin filament	4.30E-10
GO:0016323	basolateral plasma membrane	1.20E-09
GO:0030315	T-tubule	2.50E-09
GO:0045177	apical part of cell	9.00E-09
GO:0042383	sarcolemma	3.10E-08
GO:0045202	synapse	3.30E-08
GO:0016328	lateral plasma membrane	4.30E-08
GO:0045178	basal part of cell	5.00E-08
GO:0030054	cell junction	5.30E-08
GO:0016459	myosin complex	6.00E-08
GO:0012505	endomembrane system	7.20E-08
GO:0044291	cell-cell contact zone	8.50E-08
GO:0031672	A band	1.40E-07
GO:0005615	extracellular space	1.50E-07
GO:0098805	whole membrane	2.50E-07
GO:0036379	myofilament	6.20E-07
GO:0097458	neuron part	1.20E-06
GO:0016324	apical plasma membrane	1.80E-06
GO:0030424	axon	2.00E-06
GO:0043296	apical junction complex	2.50E-06
GO:0044421	extracellular region part	2.80E-06

MF

GO term	Description	Padj
GO:0005515	protein binding	< 1e-30
GO:0008307	structural constituent of muscle	6.90E-12
GO:0005509	calcium ion binding	5.50E-09
GO:0004872	receptor activity	5.90E-09
GO:0060089	molecular transducer activity	5.90E-09
GO:0015291	secondary active transmembrane transport...	2.70E-08
GO:0043169	cation binding	5.70E-08
GO:0046914	transition metal ion binding	5.80E-08
GO:0046983	protein dimerization activity	1.10E-07
GO:0046872	metal ion binding	1.20E-07
GO:0038023	signaling receptor activity	2.30E-07
GO:0008270	zinc ion binding	2.60E-07
GO:0046943	carboxylic acid transmembrane transporte...	5.50E-07
GO:0042802	identical protein binding	6.60E-07
GO:0004871	signal transducer activity	1.00E-06
GO:0005342	organic acid transmembrane transporter a...	1.10E-06
GO:0016705	oxidoreductase activity, acting on paire...	1.70E-06
GO:0003779	actin binding	1.70E-06
GO:0004888	transmembrane signaling receptor activit...	2.50E-06
GO:0005343	organic acid:sodium symporter activity	6.20E-06
GO:0042803	protein homodimerization activity	6.40E-06
GO:0015296	anion:cation symporter activity	1.00E-05
GO:0008514	organic anion transmembrane transporter ...	1.10E-05
GO:0015171	amino acid transmembrane transporter act...	1.60E-05
GO:0015293	symporter activity	1.90E-05
GO:0008092	cytoskeletal protein binding	2.70E-05
GO:0044325	ion channel binding	2.90E-05
GO:0004630	phospholipase D activity	2.90E-05
GO:0070290	N-acylphosphatidylethanolamine-specific ...	2.90E-05
GO:0099600	transmembrane receptor activity	3.30E-05
GO:0015294	solute:cation symporter activity	3.80E-05
GO:0004497	monooxygenase activity	4.60E-05
GO:0046873	metal ion transmembrane transporter acti...	5.40E-05
GO:0019899	enzyme binding	6.10E-05
GO:0008509	anion transmembrane transporter activity	6.80E-05
GO:0005283	sodium:amino acid symporter activity	7.20E-05
GO:0050840	extracellular matrix binding	7.20E-05
GO:0015370	solute:sodium symporter activity	8.30E-05
GO:0015179	L-amino acid transmembrane transporter a...	0.00011
GO:0015081	sodium ion transmembrane transporter act...	0.00012

Table A1.4 Top 40 gene ontology (GO) terms for biological processes (BP), cellular components (CC) and molecular function (MF) for genes significantly upregulated (P_{adj} < 0.01, log-2 fold change > 1) following exposure to 20% a.s. compared to 100% a.s.

20% a.s. up-regulated

BP

GO term	Description	Padj
GO:0040003	chitin-based cuticle development	< 1e-30
GO:0042335	cuticle development	< 1e-30
GO:0006030	chitin metabolic process	1.00E-17
GO:1901071	glucosamine-containing compound metaboli...	2.30E-16
GO:0006040	amino sugar metabolic process	4.20E-15
GO:0006022	aminoglycan metabolic process	3.90E-14
GO:0032501	multicellular organismal process	1.10E-06
GO:0007275	multicellular organism development	1.40E-06
GO:0044707	single-multicellular organism process	1.80E-06
GO:0048856	anatomical structure development	4.90E-06
GO:0022617	extracellular matrix disassembly	3.40E-05
GO:0044767	single-organism developmental process	4.30E-05
GO:0050819	negative regulation of coagulation	4.50E-05
GO:0032502	developmental process	6.20E-05
GO:0007599	hemostasis	8.00E-05
GO:0050817	coagulation	9.90E-05
GO:0098869	cellular oxidant detoxification	0.00014
GO:0042730	fibrinolysis	0.00015
GO:0030198	extracellular matrix organization	0.00017
GO:0042744	hydrogen peroxide catabolic process	0.00018
GO:0002541	activation of plasma proteins involved i...	0.00019
GO:0002542	Factor XII activation	0.00019
GO:2000359	regulation of binding of sperm to zona p...	0.00019
GO:1990748	cellular detoxification	0.00022
GO:0031639	plasminogen activation	0.00022
GO:0042381	hemolymph coagulation	0.00022
GO:0098754	detoxification	0.00023
GO:0050818	regulation of coagulation	0.00024
GO:0043062	extracellular structure organization	0.00025
GO:0007306	eggshell chorion assembly	0.00027
GO:0002254	kinin cascade	0.00028
GO:0002353	plasma kallikrein-kinin cascade	0.00028
GO:0007597	blood coagulation, intrinsic pathway	0.00028
GO:0007566	embryo implantation	0.0003
GO:0030195	negative regulation of blood coagulation	0.00033
GO:1900047	negative regulation of hemostasis	0.00033
GO:0007304	chorion-containing eggshell formation	0.00035

GO:0030703	eggshell formation	0.00035
GO:0009636	response to toxic substance	0.00035
GO:0002675	positive regulation of acute inflammator...	0.00039

CC

GO term	Description	Padj
GO:0005576	extracellular region	< 1e-30
GO:0032992	protein-carbohydrate complex	< 1e-30
GO:0031012	extracellular matrix	1.40E-28
GO:0005615	extracellular space	2.40E-11
GO:0042600	chorion	7.20E-06
GO:0005796	Golgi lumen	0.00012
GO:0030312	external encapsulating structure	0.00145
GO:0044421	extracellular region part	0.00161
GO:0033645	host cell endomembrane system	0.00448
GO:0044165	host cell endoplasmic reticulum	0.00448
GO:0044167	host cell endoplasmic reticulum membrane	0.00448
GO:0097441	basilar dendrite	0.00448
GO:0097442	CA3 pyramidal cell dendrite	0.00448
GO:0071438	invadopodium membrane	0.00893
GO:0005578	proteinaceous extracellular matrix	0.01221
GO:0031258	lamellipodium membrane	0.01337
GO:0005874	microtubule	0.01406
GO:0002144	cytosolic tRNA wobble base thiouridylase...	0.01778
GO:0019031	viral envelope	0.01778
GO:0036338	viral membrane	0.01778
GO:0055036	virion membrane	0.02218
GO:0099512	supramolecular fiber	0.02458
GO:0099513	polymeric cytoskeletal fiber	0.02458
GO:0030141	secretory granule	0.02691
GO:0005766	primary lysosome	0.03092
GO:0042582	azurophil granule	0.03092
GO:0071437	invadopodium	0.03092
GO:0097440	apical dendrite	0.03092
GO:0019028	viral capsid	0.03838
GO:0044423	virion part	0.04042
GO:0005912	adherens junction	0.05254
GO:0070161	anchoring junction	0.05667
GO:0035003	subapical complex	0.06088
GO:0030313	cell envelope	0.0617
GO:0005604	basement membrane	0.08458
GO:0031231	intrinsic component of peroxisomal membr...	0.08584
GO:0016323	basolateral plasma membrane	0.08635
GO:0099503	secretory vesicle	0.09429
GO:0019012	virion	0.09683

GO:0030288	outer membrane-bounded periplasmic space	0.09807

MF

GO term	Description	Padj
GO:0042302	structural constituent of cuticle	< 1e-30
GO:0008061	chitin binding	< 1e-30
GO:0005214	structural constituent of chitin-based c...	< 1e-30
GO:0008010	structural constituent of chitin-based l...	< 1e-30
GO:0030023	extracellular matrix constituent conferr...	< 1e-30
GO:0097493	structural molecule activity conferring ...	< 1e-30
GO:0005201	extracellular matrix structural constitu...	< 1e-30
GO:0005198	structural molecule activity	7.50E-26
GO:0004252	serine-type endopeptidase activity	1.60E-08
GO:0008236	serine-type peptidase activity	2.20E-07
GO:0017171	serine hydrolase activity	2.30E-07
GO:0097367	carbohydrate derivative binding	0.00014
GO:0008011	structural constituent of pupal chitin-b...	0.00016
GO:0005200	structural constituent of cytoskeleton	0.00019
GO:0004806	triglyceride lipase activity	0.00098
GO:0004500	dopamine beta-monooxygenase activity	0.00121
GO:0004175	endopeptidase activity	0.00143
GO:0004666	prostaglandin-endoperoxide synthase acti...	0.00176
GO:0008201	heparin binding	0.00298
GO:0005539	glycosaminoglycan binding	0.00428
GO:0004601	peroxidase activity	0.00479
GO:0016684	oxidoreductase activity, acting on perox...	0.00493
GO:0070011	peptidase activity, acting on L-amino ac...	0.00531
GO:0004568	chitinase activity	0.00562
GO:0016209	antioxidant activity	0.00623
GO:0008233	peptidase activity	0.00757
GO:0016715	oxidoreductase activity, acting on paire...	0.00827
GO:0050840	extracellular matrix binding	0.00956
GO:0004447	iodide peroxidase activity	0.01052
GO:0033718	pyranose dehydrogenase (acceptor) activi...	0.01052
GO:0004181	metallocarboxypeptidase activity	0.01163
GO:0016705	oxidoreductase activity, acting on paire...	0.01268
GO:0099516	ion antiporter activity	0.01322
GO:0015019	heparan-alpha-glucosaminide N-acetyltran...	0.01574
GO:0042895	antibiotic transporter activity	0.01574
GO:0033218	amide binding	0.01632
GO:0003924	GTPase activity	0.01768
GO:0000827	inositol-1,3,4,5,6-pentakisphosphate kin...	0.02094
GO:0004483	mRNA (nucleoside-2'-O-)-methyltransferas...	0.02094
GO:0033857	diphosphoinositol-pentakisphosphate kina...	0.02094

20% a.s. down-regulated

BP

GO term	Description	Padj
GO:0006030	chitin metabolic process	1.00E-15
GO:0006026	aminoglycan catabolic process	2.00E-14
GO:1901071	glucosamine-containing compound metaboli...	2.50E-14
GO:0006032	chitin catabolic process	2.90E-13
GO:1901072	glucosamine-containing compound cataboli...	3.90E-13
GO:0006040	amino sugar metabolic process	5.10E-13
GO:0046348	amino sugar catabolic process	7.00E-13
GO:0006022	aminoglycan metabolic process	8.50E-13
GO:0000272	polysaccharide catabolic process	4.80E-11
GO:1901136	carbohydrate derivative catabolic proces...	5.20E-10
GO:1901565	organonitrogen compound catabolic proces...	3.30E-09
GO:0005976	polysaccharide metabolic process	4.00E-08
GO:0016052	carbohydrate catabolic process	3.40E-06
GO:0048514	blood vessel morphogenesis	1.00E-05
GO:0043171	peptide catabolic process	1.70E-05
GO:0050808	synapse organization	2.60E-05
GO:0001525	angiogenesis	5.50E-05
GO:0035814	negative regulation of renal sodium excr...	0.0001
GO:0050818	regulation of coagulation	0.0001
GO:0001568	blood vessel development	0.00015
GO:0055078	sodium ion homeostasis	0.00018
GO:0001944	vasculature development	0.0002
GO:0035812	renal sodium excretion	0.00023
GO:0035813	regulation of renal sodium excretion	0.00023
GO:0050819	negative regulation of coagulation	0.00024
GO:0002483	antigen processing and presentation of e...	0.00027
GO:0019883	antigen processing and presentation of e...	0.00027
GO:0019885	antigen processing and presentation of e...	0.00027
GO:0071205	protein localization to juxtaparanode re...	0.00027
GO:0050860	negative regulation of T cell receptor s...	0.00037
GO:0098801	regulation of renal system process	0.00037
GO:0007160	cell-matrix adhesion	0.00037
GO:0035335	peptidyl-tyrosine dephosphorylation	0.0004
GO:0035428	hexose transmembrane transport	0.00042
GO:1904659	glucose transmembrane transport	0.00042
GO:0072358	cardiovascular system development	0.00044
GO:0072359	circulatory system development	0.00044
GO:0050858	negative regulation of antigen receptor-...	0.00055
GO:0050856	regulation of T cell receptor signaling ...	0.00062
GO:0072661	protein targeting to plasma membrane	0.0007

CC

GO term	Description	Padj
GO:0005576	extracellular region	1.80E-14
GO:0044421	extracellular region part	3.50E-09
GO:0005615	extracellular space	7.00E-09
GO:0005886	plasma membrane	2.90E-08
GO:0044459	plasma membrane part	3.00E-08
GO:0071944	cell periphery	3.60E-07
GO:0031226	intrinsic component of plasma membrane	7.00E-07
GO:0005887	integral component of plasma membrane	9.20E-07
GO:0030054	cell junction	1.70E-06
GO:0005911	cell-cell junction	1.10E-05
GO:0043194	axon initial segment	3.80E-05
GO:0033267	axon part	4.50E-05
GO:0033268	node of Ranvier	5.20E-05
GO:0016021	integral component of membrane	0.00011
GO:0031224	intrinsic component of membrane	0.00011
GO:0033270	paranode region of axon	0.00017
GO:0070062	extracellular exosome	0.00017
GO:1903561	extracellular vesicle	0.00019
GO:0065010	extracellular membrane-bounded organelle	0.00021
GO:0043230	extracellular organelle	0.00023
GO:0016328	lateral plasma membrane	0.00027
GO:0044304	main axon	0.00029
GO:0001772	immunological synapse	0.0004
GO:0031983	vesicle lumen	0.00046
GO:0031256	leading edge membrane	0.00061
GO:0005921	gap junction	0.00084
GO:0043034	costamere	0.00094
GO:0044425	membrane part	0.00132
GO:0009986	cell surface	0.0017
GO:0005925	focal adhesion	0.00295
GO:0005924	cell-substrate adherens junction	0.00327
GO:0030055	cell-substrate junction	0.00362
GO:0016222	procollagen-proline 4-dioxygenase comple...	0.0041
GO:0005912	adherens junction	0.00436
GO:0070161	anchoring junction	0.00501
GO:0098590	plasma membrane region	0.00587
GO:0097454	Schwann cell microvillus	0.00692
GO:1904115	axon cytoplasm	0.00692
GO:0031253	cell projection membrane	0.0075
GO:0032587	ruffle membrane	0.00798

MF

GO term	Description	Padj
GO:0004568	chitinase activity	4.60E-11
GO:0008061	chitin binding	3.70E-10
GO:0004553	hydrolase activity, hydrolyzing O-glycos...	6.90E-09
GO:0016798	hydrolase activity, acting on glycosyl b...	1.20E-07
GO:0061134	peptidase regulator activity	3.00E-05
GO:0005138	interleukin-6 receptor binding	4.90E-05
GO:0070006	metalloaminopeptidase activity	8.70E-05
GO:0004867	serine-type endopeptidase inhibitor acti...	0.00012
GO:0070851	growth factor receptor binding	0.00021
GO:0004866	endopeptidase inhibitor activity	0.00022
GO:0004177	aminopeptidase activity	0.00023
GO:0015294	solute:cation symporter activity	0.00032
GO:0008235	metalloexopeptidase activity	0.00033
GO:0061135	endopeptidase regulator activity	0.00034
GO:0015370	solute:sodium symporter activity	0.00035
GO:0005343	organic acid:sodium symporter activity	0.00045
GO:0005243	gap junction channel activity	0.00047
GO:0030414	peptidase inhibitor activity	0.00052
GO:0005355	glucose transmembrane transporter activi...	0.00069
GO:0004725	protein tyrosine phosphatase activity	0.00072
GO:0015149	hexose transmembrane transporter activit...	0.00078
GO:0005344	oxygen transporter activity	0.00087
GO:0015145	monosaccharide transmembrane transporter...	0.00119
GO:0005001	transmembrane receptor protein tyrosine ...	0.00144
GO:0019198	transmembrane receptor protein phosphata...	0.00144
GO:0001618	virus receptor activity	0.00157
GO:0022829	wide pore channel activity	0.00157
GO:0008238	exopeptidase activity	0.00176
GO:0016755	transferase activity, transferring amino...	0.00203
GO:0015081	sodium ion transmembrane transporter act...	0.00203
GO:0005161	platelet-derived growth factor receptor ...	0.00211
GO:0045295	gamma-catenin binding	0.00257
GO:0070097	delta-catenin binding	0.00257
GO:0016848	carbon-halide lyase activity	0.00306
GO:0018833	DDT-dehydrochlorinase activity	0.00306
GO:0050839	cell adhesion molecule binding	0.00603
GO:0004563	beta-N-acetylhexosaminidase activity	0.00617
GO:0015296	anion:cation symporter activity	0.00642
GO:0008237	metallopeptidase activity	0.00657
GO:0015293	symporter activity	0.00688

Table A1.5 DEGs associated with physiological responses to hypoxia displayed in heatmaps (Fig. 3A-E). Log-2 fold changes (log-2 FC) for DEGs between 40 % a.s. or 20 % a.s. compared to the normoxic control (100 % a.s.) and the significance of this result (P_{adj}) are shown. Blank entries for a particular treatment indicate non-significant changes in gene expression ($P_{adj} > 0.05$) compared to 100 % a.s.

Hemocyanin

Gene	40 % a.s.		20 % a.s.	
	Log-2 FC	P_{adj}	Log-2 FC	P_{adj}
DN65240_c0_g2	3.77	8.30E-05		
DN69931_c0_g7	4.31	3.69E-11	-3.08	1.09E-04

Glycolytic enzymes

Enzyme family	Gene	40 % a.s.		20 % a.s.	
		Log-2 FC	P_{adj}	Log-2 FC	P_{adj}
Hexokinase	DN71565_c1_g1	-1.21	8.96E-04		
Phosphofructokinase	DN50894_c0_g1	7.5	3.22E-11		
Fructose -	DN59083_c0_g1	4.95	2.65E-04		
bisphosphate	DN149601_c0_g1	3.26	1.60E-03		
aldolase	DN70063_c0_g1	0.59	1.77E-03	0.68	5.40E-04
	DN48956_c0_g1	-1.95	9.30E-12	-1.02	3.20E-03
Triosephosphate isomerase	DN51533_c0_g1	5.74	7.96E-06		
	DN45705_c0_g1	3.85	0.02		
	DN103828_c0_g1	3.5	0.04		
Glyceraldehyde 3-	DN50141_c0_g1	8.07	3.49E-18		
phosphate	DN39408_c0_g1	4.68	9.33E-04		
dehydrogenase	DN95143_c0_g1	3.96	0.01		
	DN137445_c0_g1	3.95	7.87E-04		
	DN54835_c0_g1	3.49	0.04		
	DN39553_c0_g1	2.26	1.59E-04		
	DN138937_c0_g1	1.95	0.03		
	DN96995_c0_g1			-4.21	0.01
Phosphoglycerate kinase	DN55243_c0_g1	6.71	2.16E-09		
	DN43294_c0_g1	2.43	4.30E-04		
	DN59467_c1_g1	-0.86	1.64E-03		
Enolase	DN52128_c0_g1	7.37	4.86E-12		
	DN105798_c0_g1	-3.29	0.04		
	DN37445_c0_g2			-3.45	0.03
Pyruvate kinase	DN138537_c0_g1	4.21	1.60E-03		
	DN63898_c0_g2	-0.59	5.90E-03		

Table A1.5 (cont.)

TCA cycle enzymes

Enzyme family	Gene	40 % a.s.		20 % a.s.	
		Log-2 FC	P_{adj}	Log-2 FC	P_{adj}
Citrate synthase	DN43976_c1_g1	5.14	5.61E-05		
	DN43976_c0_g1	3.14	8.77E-03		
	DN52591_c0_g1	2.88	0.02		
Aconitase	DN45790_c0_g1	5.28	5.68E-05		
	DN41745_c0_g2	3.63	2.57E-04		
	DN70851_c1_g3	-1.02	1.04E-03	-0.90	0.01
Isocitrate dehydrogenase	DN52076_c0_g1	4.47	2.40E-03		
	DN43003_c0_g1	3.75	2.25E-03		
	DN89817_c0_g1	3.54	8.28E-04		
	DN48376_c0_g1	3.36	5.84E-08		
	DN68602_c0_g1	0.77	0.01		
Oxoglutarate	DN135927_c0_g1	2.90	9.72E-03		
dehydrogenase	DN71283_c2_g1	-0.76	1.44E-03		
	DN66886_c0_g2	-1.36	2.30E-07	-0.99	6.20E-04
Succinyl CoA ligase	DN104722_c0_g1	2.19	0.01		
	DN61677_c0_g1			-0.68	0.02
Succinate dehydrogenase	DN62370_c0_g1	-0.42	7.78E-03		
	DN67826_c0_g1	-0.63	8.55E-04		
	DN65942_c0_g1	-0.93	4.05E-03	-1.16	3.43E-04
Fumarase	DN67879_c2_g5	-0.91	3.15E-04		
Malate dehydrogenase	DN77596_c0_g1	1.93	0.05		
	DN58020_c0_g3	-0.91	3.32E-05		

Table A1.5 (cont.)

Electron transport chain

Complex	Gene	40 % a.s.		20 % a.s.	
		Log-2 FC	P_{adj}	Log-2 FC	P_{adj}
NADH dehydrogenase	DN104009_c0_g1	3.54	3.77E-03		
	DN73519_c0_g1	3.11	0.05		
	DN120507_c0_g1	2.75	0.04		
	DN108822_c0_g1	1.91	0.03		
	DN60497_c0_g1	0.65	9.48E-03		
	DN56853_c0_g2	-0.43	0.02		
	DN63788_c0_g1	-0.44	0.05		
	DN64682_c0_g1	-0.50	0.02		
	DN58321_c0_g2	-0.62	8.20E-03		
	DN65401_c1_g3	-0.62	0.02		
	DN71373_c2_g2	-0.80	4.95E-04	-0.62	0.02
	DN68136_c0_g1	-0.87	0.02		
	DN69542_c2_g1	-0.96	7.87E-05		
	DN61449_c2_g1	-0.99	6.04E-03		
	DN61449_c2_g5	-1.35	1.32E-03		
NADH ubiquinone	DN106294_c0_g1	3.44	8.60E-03		
oxidoreductase	DN70525_c0_g2	1.36	1.18E-04		
	DN70525_c0_g1	1.10	2.59E-04		
Succinate	DN62370_c0_g1	-0.42	7.78E-03		
dehydrogenase	DN67826_c0_g1	-0.63	8.55E-04		
	DN65942_c0_g1	-0.93	4.05E-03	-1.16	3.43E-04
Cytochrome bc1	DN136034_c0_g1	2.83	0.02		
	DN63331_c10_g9	-0.57	1.90E-03		
Cytchrome c1	DN152390_c0_g1	3.33	4.15E-04		
	DN88065_c0_g1	2.30	0.04		
Cytochrome c	DN40097_c0_g2	4.03	5.85E-05		
oxidase	DN70198_c1_g1	1.01	6.03E-04		
	DN73690_c0_g1	-3.17	0.04		
ATP synthase	DN50540_c0_g2	6.30	1.21E-07		
	DN56019_c0_g1	5.61	8.79E-07		
	DN28736_c0_g1	4.27	3.85E-03		
	DN25056_c0_g1	4.08	6.59E-03		
	DN32039_c0_g1	3.91	7.15E-04		
	DN90193_c0_g1	3.80	0.02		
	DN50540_c0_g1	3.61	1.52E-06		
	DN72939_c0_g1	3.58	0.03		
	DN50806_c0_g1	2.67	3.42E-05		
	DN88147_c0_g1	2.08	1.43E-04		
	DN14438_c0_g2	1.86	3.39E-03		

Table A1.5 (cont.)

Antioxidants and HSPs

Enzyme family	Gene	40 % a.s.		20 % a.s.	
		Log-2 FC	P_{adj}	Log2-FC	P_{adj}
Superoxide dismutase	DN135679_c0_g1	2.21	0.01		
	DN152180_c0_g1	3.26	0.03		
	DN27297_c0_g1	2.83	1.04E-03		
	DN31590_c0_g2	4.06	8.47E-03		
	DN34021_c0_g1			-3.06	0.03
	DN47936_c0_g1	6.83	3.81E-12		
	DN66796_c0_g4	-1.60	1.85E-03		
	DN71798_c2_g1	2.21	8.65E-05	1.99	1.48E-03
	DN8780_c0_g1	2.53	9.88E-03		
Catalase	DN37910_c0_g1	3.43	0.03		
	DN60804_c1_g1	3.27	5.01E-05		
Glutathione peroxidase	DN20010_c0_g1	3.46	0.04		
	DN50532_c0_g2	2.51	0.03		
	DN53657_c0_g1	3.51	4.98E-03		
	DN64248_c1_g1	-0.86	0.03		
	DN68685_c0_g1	1.03	0.03		
	DN71905_c2_g3	-1.39	3.67E-03		
Glutathione-S-transferase	DN15038_c0_g1	3.05	0.03		
	DN35228_c0_g1	3.74	0.02		
	DN39991_c0_g1	3.28	5.36E-03		
	DN40360_c0_g2	6.06	2.01E-07		
	DN40360_c0_g4	4.60	1.40E-12		
	DN42164_c0_g2	4.96	3.42E-04		
	DN42943_c0_g1	4.33	2.26E-07		
	DN46241_c0_g1	3.35	7.78E-09		
	DN48019_c0_g1	4.77	6.07E-04		
	DN50564_c0_g1	5.94	1.93E-06		
	DN51796_c0_g1			-1.07	0.02
	DN52652_c0_g1	2.98	1.29E-04		
	DN55192_c0_g1	7.90	1.03E-13		
	DN55718_c0_g1			-2.45	0.02
	DN56931_c1_g1			-1.65	8.70E-03
	DN58494_c0_g1			-2.47	5.02E-08
	DN59155_c2_g1	2.02	0.01		
	DN64645_c1_g1			-1.14	0.01
	DN65366_c0_g1			-0.86	0.05
	DN72953_c0_g1	4.51	1.29E-03		
	DN74596_c0_g1	3.44	0.02		

Table A1.5 (cont.)

Heat shock proteins	DN122421_c0_g1			-3.46	0.04
	DN13922_c0_g2	1.50	8.92E-03		
	DN20583_c0_g2	-3.45	8.78E-07	-3.58	4.48E-07
	DN38981_c0_g1	-1.92	0.01		
	DN46232_c0_g2	1.26	0.01		
	DN46232_c1_g2	1.56	5.12E-03		
	DN47317_c0_g1	6.04	2.73E-07		
	DN47667_c0_g1	4.76	7.10E-04		
	DN51923_c0_g1	2.82	2.18E-04		
	DN52592_c0_g1			-3.21	6.85E-04
	DN57597_c2_g1	2.86	1.42E-07		
	DN58276_c0_g1	-1.63	0.02		
	DN58462_c0_g2	2.83	1.63E-05		
	DN63773_c0_g1	-2.01	5.67E-03		
	DN64300_c2_g1	-1.37	5.96E-03		
	DN65773_c2_g2	-2.30	3.67E-03		
	DN67334_c6_g1	-3.72	5.81E-03	-3.48	0.02
	DN67794_c0_g1	-1.82	6.15E-03	-1.88	8.10E-03
	DN68961_c1_g1	-1.68	3.23E-04		
	DN69703_c0_g2	-1.50	0.01		
	DN71258_c1_g2	-0.98	0.02		
	DN71258_c1_g3	-1.63	0.04		
	DN71321_c0_g1	-1.10	1.65E-04		
	DN72485_c1_g1			-2.99	0.01
	DN72485_c3_g2	-2.23	2.35E-04		

Appendix 2 – to accompany Chapter 4

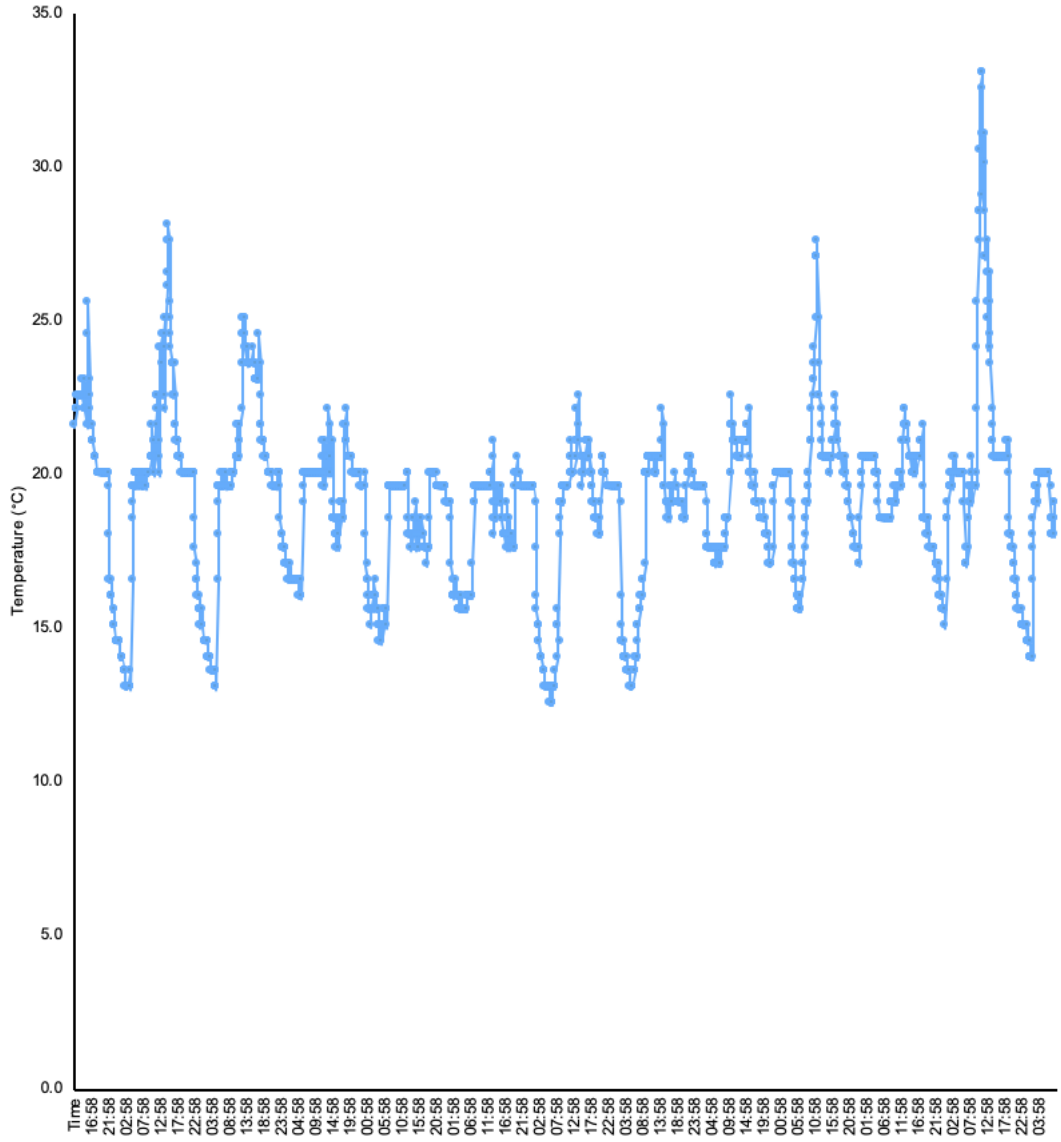

Fig. A2.1 Temperature profile of intertidal estuarine mudflat at Saltash, UK. Temperatures were logged every ten minutes for ~ 12 days in summer. Temperatures at high tide are fairly consistent at ~ 19 - 20°C, Temperatures were highly variable at low tide dependent upon whether low tide occurred during the day or at night (max: ~ 33 °C, min: ~ 12°C).

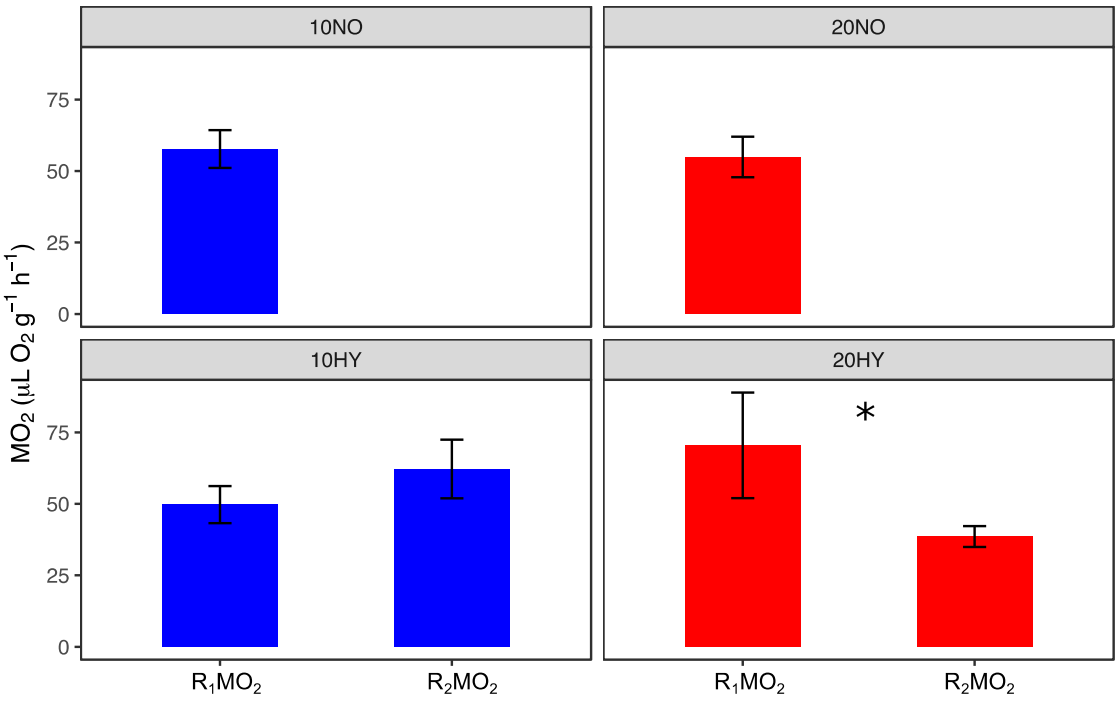

Fig. A2.2 MO_2 of samples utilised for transcriptomic analysis (n = 5 per treatment). Individuals from the normoxic treatments (10NO and 20NO) were allows to deplete oxygen within the respirometry chamber down to 80% a.s. Separate individuals had to be utilised for the hypoxia (HY) and normoxia (NO) treatments but R_1MO_2 did not significantly differ between any of the different treatment groups ($P > 0.05$). For the hypoxia treatments (10HY and 20HY), individuals were allowed to deplete oxygen within the respirometry chamber down to 30% a.s. Warm acclimated individuals (20HY) showed a significant hypometabolic shift ($P < 0.05$, indicated by asterisk) not present in cold acclimated individuals (10HY).

Table A2.1 Top 40 gene ontology (GO) terms for biological processes (BP), cellular components (CC) and molecular function (MF) for genes significantly upregulated (P_{adj} < 0.05, log-2 fold change > 1) following warm acclimation under normoxic conditions (80% a.s.) (20NO vs 10NO).

BP

GO term	Description	P_{adj}
GO:0005975	carbohydrate metabolic process	5.20E-11
GO:0000272	polysaccharide catabolic process	2.45E-10
GO:0044247	cellular polysaccharide catabolic process	1.15E-09
GO:0006026	aminoglycan catabolic process	4.27E-09
GO:0005976	polysaccharide metabolic process	6.45E-09
GO:0030245	cellulose catabolic process	6.94E-09
GO:0051275	beta-glucan catabolic process	6.94E-09
GO:0016052	carbohydrate catabolic process	7.82E-09
GO:0030243	cellulose metabolic process	9.25E-09
GO:0051273	beta-glucan metabolic process	1.42E-08
GO:1901136	carbohydrate derivative catabolic process	2.60E-08
GO:0044275	cellular carbohydrate catabolic process	2.60E-08
GO:0006022	aminoglycan metabolic process	3.05E-08
GO:0032501	multicellular organismal process	6.56E-08
GO:0061580	colon epithelial cell migration	1.31E-07
GO:0044264	cellular polysaccharide metabolic process	2.77E-07
GO:0051239	regulation of multicellular organismal process	1.29E-06
GO:0009251	glucan catabolic process	1.42E-06
GO:0023051	regulation of signaling	1.93E-06
GO:0071353	cellular response to interleukin-4	2.19E-06
GO:0043627	response to estrogen	2.56E-06
GO:0006073	cellular glucan metabolic process	3.27E-06
GO:0051240	positive regulation of multicellular organismal process	3.64E-06
GO:0061582	intestinal epithelial cell migration	4.54E-06
GO:0006030	chitin metabolic process	4.66E-06
GO:0010646	regulation of cell communication	8.09E-06
GO:0044042	glucan metabolic process	8.49E-06
GO:0044707	single-multicellular organism process	1.19E-05
GO:0044262	cellular carbohydrate metabolic process	1.72E-05
GO:0070670	response to interleukin-4	1.72E-05
GO:0009966	regulation of signal transduction	2.04E-05
GO:0007586	digestion	2.05E-05
GO:0006687	glycosphingolipid metabolic process	2.77E-05
GO:0006955	immune response	5.13E-05
GO:0002376	immune system process	6.31E-05
GO:0048583	regulation of response to stimulus	6.85E-05
GO:0006665	sphingolipid metabolic process	7.75E-05

Table A2.1 (cont.)

GO:0048646	anatomical structure formation involved in morphogenesis	9.09E-05
GO:1901071	glucosamine-containing compound metabolic process	0.00014673
GO:0051597	response to methylmercury	0.0001473

CC

GO term	Description	P_{adj}
GO:0005576	extracellular region	5.13E-25
GO:0005764	lysosome	1.10E-12
GO:0000323	lytic vacuole	3.00E-12
GO:0005615	extracellular space	4.54E-10
GO:0005887	integral component of plasma membrane	7.83E-10
GO:0031226	intrinsic component of plasma membrane	1.08E-09
GO:0044421	extracellular region part	1.82E-09
GO:0005773	vacuole	3.19E-09
GO:0044459	plasma membrane part	1.28E-08
GO:0009986	cell surface	3.20E-08
GO:0044449	contractile fiber part	1.47E-06
GO:0005886	plasma membrane	5.25E-06
GO:0043202	lysosomal lumen	6.52E-06
GO:0005775	vacuolar lumen	8.09E-06
GO:0030054	cell junction	6.61E-05
GO:0031224	intrinsic component of membrane	0.00014526
GO:0016021	integral component of membrane	0.00022565
GO:0032982	myosin filament	0.00023566
GO:0005859	muscle myosin complex	0.00042719
GO:1903561	extracellular vesicle	0.00051634
GO:0016460	myosin II complex	0.00059065
GO:0043230	extracellular organelle	0.00059757
GO:0070062	extracellular exosome	0.00062121
GO:0030018	Z disc	0.00068138
GO:0030017	sarcomere	0.00068138
GO:0005912	adherens junction	0.0062639
GO:0070161	anchoring junction	0.00946018
GO:0016459	myosin complex	0.01388179
GO:0042641	actomyosin	0.01472292
GO:0016942	insulin-like growth factor binding protein complex	0.01903398
GO:0036454	growth factor complex	0.01903398
GO:0042567	insulin-like growth factor ternary complex	0.01903398
GO:0044425	membrane part	0.02097749
GO:0005622	intracellular	0.02277918
GO:0005915	zonula adherens	0.02911471
GO:0005924	cell-substrate adherens junction	0.03103032

Table A2.1 (cont.)

GO:0001725	stress fiber	0.03496648
GO:0097517	contractile actin filament bundle	0.03496648
GO:0030055	cell-substrate junction	0.03549777
GO:0035579	specific granule membrane	0.04123063

MF

GO term	Description	P_{adj}
GO:0030246	carbohydrate binding	2.77E-25
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	7.49E-21
GO:0016798	hydrolase activity, acting on glycosyl bonds	7.96E-21
GO:0005537	mannose binding	3.12E-20
GO:0048029	monosaccharide binding	7.82E-14
GO:0030248	cellulose binding	1.37E-13
GO:0001871	pattern binding	1.01E-12
GO:0030247	polysaccharide binding	1.01E-12
GO:0008810	cellulase activity	4.39E-10
GO:0008484	sulfuric ester hydrolase activity	1.07E-08
GO:0001618	virus receptor activity	9.32E-08
GO:0003943	N-acetylgalactosamine-4-sulfatase activity	1.31E-07
GO:0004871	signal transducer activity	1.76E-07
GO:0004872	receptor activity	3.81E-07
GO:0060089	molecular transducer activity	3.81E-07
GO:0042806	fucose binding	4.19E-07
GO:0004065	arylsulfatase activity	4.24E-07
GO:0038023	signaling receptor activity	1.90E-06
GO:0004888	transmembrane signaling receptor activity	7.26E-06
GO:0004062	aryl sulfotransferase activity	8.09E-06
GO:0099600	transmembrane receptor activity	1.76E-05
GO:0019904	protein domain specific binding	8.87E-05
GO:0015020	glucuronosyltransferase activity	0.00014128
GO:0042802	identical protein binding	0.00022532
GO:0008061	chitin binding	0.00024531
GO:0004568	chitinase activity	0.00033624
GO:0004197	cysteine-type endopeptidase activity	0.00117844
GO:0042803	protein homodimerization activity	0.00119614
GO:0004348	glucosylceramidase activity	0.00124711
GO:0005344	oxygen transporter activity	0.00159504
GO:0008146	sulfotransferase activity	0.00205588
GO:0008426	protein kinase C inhibitor activity	0.00262419
GO:0016985	mannan endo-1,4-beta-mannosidase activity	0.00300222
GO:0051393	alpha-actinin binding	0.00302665
GO:0005515	protein binding	0.00313817

Table A2.1 (cont.)

GO:0005102	receptor binding	0.00337358
GO:0008509	anion transmembrane transporter activity	0.00498192
GO:0005501	retinoid binding	0.00540794
GO:0097153	cysteine-type endopeptidase activity involved in apoptotic process	0.00689963
GO:0097200	cysteine-type endopeptidase activity involved in execution phase of apoptosis	0.00689963

Table A2.2 Top 40 gene ontology (GO) terms for biological processes (BP), cellular components (CC) and molecular function (MF) for genes significantly downregulated (P_{adj} < 0.05, log-2 fold change < -1) following warm acclimation under normoxic conditions (80% a.s.) (20NO vs 10NO).

BP

GO term	Description	P_{adj}
GO:0006412	translation	2.34E-178
GO:0043043	peptide biosynthetic process	8.10E-176
GO:0006518	peptide metabolic process	4.25E-163
GO:0043604	amide biosynthetic process	5.80E-162
GO:0043603	cellular amide metabolic process	2.90E-143
GO:1901566	organonitrogen compound biosynthetic process	8.71E-120
GO:0002181	cytoplasmic translation	1.48E-54
GO:0019538	protein metabolic process	1.74E-35
GO:1901564	organonitrogen compound metabolic process	1.86E-35
GO:0044267	cellular protein metabolic process	7.78E-25
GO:0034645	cellular macromolecule biosynthetic process	4.78E-23
GO:0009059	macromolecule biosynthetic process	1.32E-20
GO:0044271	cellular nitrogen compound biosynthetic process	2.69E-20
GO:0000028	ribosomal small subunit assembly	4.89E-19
GO:0015992	proton transport	4.61E-18
GO:1902600	hydrogen ion transmembrane transport	4.61E-18
GO:0006818	hydrogen transport	5.85E-18
GO:0044249	cellular biosynthetic process	8.28E-16
GO:0009058	biosynthetic process	9.89E-16
GO:1901576	organic substance biosynthetic process	4.50E-15
GO:0000027	ribosomal large subunit assembly	4.68E-15
GO:0015988	energy coupled proton transmembrane transport, against electrochemical gradient	1.60E-12
GO:0015991	ATP hydrolysis coupled proton transport	3.09E-12
GO:0022618	ribonucleoprotein complex assembly	3.40E-12
GO:0090662	ATP hydrolysis coupled transmembrane transport	1.41E-11
GO:0099131	ATP hydrolysis coupled ion transmembrane transport	2.28E-11
GO:0099132	ATP hydrolysis coupled cation transmembrane transport	2.28E-11
GO:0071826	ribonucleoprotein complex subunit organization	4.82E-11
GO:0098655	cation transmembrane transport	1.82E-10
GO:0006457	protein folding	4.77E-10
GO:0098662	inorganic cation transmembrane transport	5.21E-10
GO:0022613	ribonucleoprotein complex biogenesis	7.28E-09
GO:0019752	carboxylic acid metabolic process	1.23E-08
GO:0016054	organic acid catabolic process	5.52E-08
GO:0046395	carboxylic acid catabolic process	5.52E-08
GO:0098660	inorganic ion transmembrane transport	1.50E-07
GO:0015672	monovalent inorganic cation transport	2.62E-07
GO:0006101	citrate metabolic process	9.16E-07

Table A2.2 (cont.)

GO:0006099	tricarboxylic acid cycle	1.46E-06
GO:0009063	cellular amino acid catabolic process	1.70E-06

CC

GO term	Description	P_{adj}
GO:0005840	ribosome	5.48E-148
GO:0044391	ribosomal subunit	2.64E-141
GO:0044445	cytosolic part	4.65E-129
GO:0022625	cytosolic large ribosomal subunit	1.14E-91
GO:0030529	intracellular ribonucleoprotein complex	2.59E-88
GO:1990904	ribonucleoprotein complex	3.52E-88
GO:0015934	large ribosomal subunit	3.10E-83
GO:0022627	cytosolic small ribosomal subunit	2.63E-54
GO:0015935	small ribosomal subunit	3.89E-53
GO:0005622	intracellular	2.06E-25
GO:0000786	nucleosome	3.10E-24
GO:0043228	non-membrane-bounded organelle	9.60E-24
GO:0043232	intracellular non-membrane-bounded organelle	9.60E-24
GO:0044444	cytoplasmic part	9.76E-24
GO:0032991	macromolecular complex	2.79E-22
GO:0044815	DNA packaging complex	7.15E-20
GO:0022626	cytosolic ribosome	1.22E-19
GO:0009506	plasmodesma	4.05E-19
GO:0005618	cell wall	7.49E-18
GO:0009507	chloroplast	1.77E-17
GO:0030312	external encapsulating structure	9.02E-17
GO:0009536	plastid	2.29E-16
GO:0032993	protein-DNA complex	1.03E-14
GO:0045335	phagocytic vesicle	1.32E-12
GO:0033178	proton-transporting two-sector ATPase complex, catalytic domain	2.23E-10
GO:0042788	polysomal ribosome	2.78E-09
GO:0030139	endocytic vesicle	8.37E-08
GO:0005773	vacuole	7.99E-07
GO:0005839	proteasome core complex	2.66E-06
GO:0042579	microbody	3.29E-06
GO:0005777	peroxisome	4.62E-06
GO:0033179	proton-transporting V-type ATPase, V0 domain	6.35E-05
GO:0098798	mitochondrial protein complex	6.69E-05
GO:0098800	inner mitochondrial membrane protein complex	0.00022303
GO:0005929	cilium	0.00023863
GO:0000788	nuclear nucleosome	0.00029277
GO:0044424	intracellular part	0.00032485
GO:0031514	motile cilium	0.00086096

Table A2.2 (cont.)

GO:0045261	proton-transporting ATP synthase complex, catalytic core F(1)	0.00104392
GO:0000502	proteasome complex	0.00137295

MF

GO term	Description	P_{adj}
GO:0003735	structural constituent of ribosome	6.28E-201
GO:0005198	structural molecule activity	7.75E-145
GO:0019843	rRNA binding	2.28E-30
GO:0044769	ATPase activity, coupled to transmembrane movement of ions, rotational mechanism	7.70E-13
GO:0015078	hydrogen ion transmembrane transporter activity	3.87E-12
GO:0019829	cation-transporting ATPase activity	8.84E-12
GO:0042625	ATPase coupled ion transmembrane transporter activity	2.39E-10
GO:0036442	hydrogen-exporting ATPase activity	1.23E-08
GO:0003723	RNA binding	1.71E-08
GO:0003674	molecular_function	3.68E-08
GO:0046961	proton-transporting ATPase activity, rotational mechanism	7.91E-08
GO:0004185	serine-type carboxypeptidase activity	1.42E-07
GO:0070008	serine-type exopeptidase activity	1.44E-07
GO:0015399	primary active transmembrane transporter activity	2.74E-07
GO:0015405	P-P-bond-hydrolysis-driven transmembrane transporter activity	2.74E-07
GO:0003729	mRNA binding	3.66E-07
GO:0008234	cysteine-type peptidase activity	8.72E-07
GO:0004298	threonine-type endopeptidase activity	1.01E-06
GO:0070003	threonine-type peptidase activity	1.01E-06
GO:0008233	peptidase activity	1.46E-06
GO:0070011	peptidase activity, acting on L-amino acid peptides	2.30E-06
GO:0022853	active ion transmembrane transporter activity	2.64E-06
GO:0004180	carboxypeptidase activity	6.86E-06
GO:0070181	small ribosomal subunit rRNA binding	7.20E-06
GO:0016491	oxidoreductase activity	1.16E-05
GO:0005509	calcium ion binding	1.35E-05
GO:0004364	glutathione transferase activity	1.88E-05
GO:0016820	hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement	0.00013166
GO:0042626	ATPase activity, coupled to transmembrane movement of substances	0.00013166
GO:0046933	proton-transporting ATP synthase activity, rotational mechanism	0.00016542
GO:0016859	cis-trans isomerase activity	0.0004511
GO:0003755	peptidyl-prolyl cis-trans isomerase activity	0.00218688
GO:0003924	GTPase activity	0.0022824
GO:0043492	ATPase activity, coupled to movement of substances	0.00302921
GO:0005525	GTP binding	0.00306571
GO:0004601	peroxidase activity	0.00320713
GO:0001883	purine nucleoside binding	0.00335229
GO:0032550	purine ribonucleoside binding	0.00335229

Table A2.2 (cont.)

GO:0051082	unfolded protein binding	0.00346347
GO:0016209	antioxidant activity	0.00431321

Table A2.3 Expression of selected genes (from Section 4.3.3) significantly upregulated ($P_{adj} < 0.05$) following warm acclimation under normoxic conditions (80% a.s.) (20NO vs 10NO).

Protein/Enzyme	Gene	Log-2 FC	P_{adj}
Lactate dehydrogenase	DN198236_c0_g1	1.73196563	0.00778345
Hemocyanin	DN196559_c4_g1	3.9030599	0.00177461
	DN197055_c1_g2	3.43002051	0.01391339
	DN197055_c1_g3	4.33470447	0.00023098
	DN198343_c2_g1	2.75233644	0.01151541
	DN198737_c0_g1	5.08021601	1.34E-05
	DN198737_c0_g2	3.1834551	0.00468498
	DN198737_c0_g3	7.09337449	9.98E-06

Table A2.4 Expression of selected genes (from Section 4.3.3) significantly downregulated (P_{adj} < 0.05) following warm acclimation under normoxic conditions (80% a.s.) (20NO vs 10NO).

Protein family	Gene	Log-2 FC	P_{adj}
Heat shock proteins	DN171758_c1_g1	-3.7880529	0.03984782
	DN172545_c0_g1	-4.360902	0.01482666
	DN176452_c0_g2	-3.5194351	0.02810268
	DN176586_c0_g2	-5.0015875	0.00361992
	DN178998_c0_g2	-4.2093082	0.00059305
	DN179756_c2_g2	-5.3561908	0.00100942
	DN188671_c0_g1	-4.5952748	0.01346073
	DN188872_c0_g1	-4.0482108	0.02082815
	DN189350_c3_g7	-4.1739126	0.03838879
	DN189350_c5_g1	-5.0939466	0.00246094
	DN189792_c2_g3	-6.7289947	1.06E-06
	DN189792_c2_g5	-5.2677567	0.0092429
	DN189792_c2_g15	-2.4912077	0.04721371
	DN190767_c0_g2	-3.9848924	0.03231365
	DN191498_c0_g1	-3.8857798	0.00257239
	DN191773_c0_g1	-3.0907045	0.00941485
	DN191896_c4_g2	-3.2937842	0.01226099
	DN192577_c7_g2	-4.2964556	1.51E-05
	DN192626_c0_g1	-5.2272935	0.00438789
	DN192887_c4_g1	-3.938765	0.00099485
	DN192887_c4_g4	-4.5315317	6.55E-05
	DN193172_c3_g3	-4.215329	0.00066256
	DN194056_c1_g1	-6.4566492	3.97E-05
	DN194437_c3_g1	-5.6072908	0.00044705
	DN194437_c7_g2	-4.2260919	5.09E-05
	DN194437_c8_g4	-4.1897772	0.01708024
	DN194437_c8_g8	-4.1634671	0.03485447
	DN194733_c1_g1	-5.4891676	5.37E-05
	DN194733_c1_g2	-5.1256341	1.09E-05
	DN195273_c3_g2	-3.465194	0.04313875
	DN195273_c4_g1	-4.4979876	0.00065956
	DN195273_c4_g4	-4.1071199	0.00480593
	DN195273_c4_g6	-2.8988622	0.03941767
	DN195521_c1_g1	-2.9277629	0.01465535
	DN195521_c1_g2	-3.7536263	0.00258324
	DN195521_c1_g4	-4.4807023	0.00025812
	DN196402_c3_g2	-4.262897	0.008206
	DN197344_c3_g1	-2.8964815	0.03916402
	DN197344_c3_g3	-5.511041	1.63E-05
	DN198189_c3_g2	-3.0300369	0.01355854

Table A2.4 (cont.)

	DN199684_c0_g1	-3.7574434	0.00215155
	DN199684_c0_g7	-6.4499672	9.90E-07
	DN199684_c0_g10	-5.9034232	0.0002457
	DN199684_c0_g11	-5.2041588	1.22E-05
	DN199684_c0_g12	-4.6223821	0.0055419
	DN199779_c6_g1	-6.3383071	3.92E-06
	DN199779_c6_g2	-7.0402226	5.45E-06
	DN199779_c6_g6	-4.4749363	0.00014608
Citrate synthase	DN116832_c0_g1	-5.2961161	2.36E-05
	DN128900_c0_g1	-6.2241408	2.11E-07
	DN131645_c0_g1	-4.3071892	0.01407816
	DN143503_c1_g1	-3.8691711	0.02487942
	DN143867_c0_g2	-5.4064473	7.36E-05
	DN151908_c0_g1	-3.2247502	0.01755492
	DN165653_c0_g1	-6.960159	8.92E-07
	DN177534_c0_g1	-6.1012817	1.23E-05
	DN177534_c1_g1	-5.2766517	8.13E-05
	DN184558_c0_g1	-3.1958171	0.00587183
	DN185119_c2_g2	-4.8483678	0.00457863
	DN185119_c2_g4	-7.1464687	1.37E-08
	DN185119_c2_g5	-5.5908648	3.67E-06
	DN187378_c1_g1	-4.9096218	0.00045187
	DN188289_c0_g1	-6.4941131	1.14E-06
	DN193262_c0_g1	-3.2407921	0.00570846
	DN195128_c1_g1	-4.8867252	0.00111163
	DN195128_c1_g5	-3.8826669	0.00375676
	DN198681_c1_g1	0.71417627	0.01894579
	DN79526_c0_g1	-4.7798334	0.00262202
Cytochrome c oxidase	DN134343_c0_g1	-4.5543722	0.00427085
	DN150528_c0_g1	-3.2706424	0.03446215
	DN167944_c0_g1	-3.9717506	0.04597658
	DN178630_c0_g1	-0.9579108	0.0394591
	DN197284_c1_g1	-0.9994484	0.02830977
	DN24853_c0_g1	-4.0734412	0.03972237

Table A2.5 All significantly enriched gene ontology (GO) terms for genes significantly downregulated (P_{adj} < 0.05, log-2 fold change < -1) under hypoxia (30% a.s.) compared to normoxia (80% a.s.) in cold acclimated animals (10HY vs 10NO). Data not shown for upregulated genes (P_{adj} < 0.05, log-2 fold change > 1) in cold acclimated animals (10HY vs 10NO) or for both up or downregulated genes in warm acclimated animals (20HY vs 20NO) as no significant GO enrichment was observed.

Ontology	GO term	Description	P_{adj}
BP	GO:0043043	peptide biosynthetic process	7.03E-06
BP	GO:0006412	translation	8.90E-06
BP	GO:0043604	amide biosynthetic process	8.90E-06
BP	GO:0006518	peptide metabolic process	2.08E-05
BP	GO:0043603	cellular amide metabolic process	7.88E-05
BP	GO:1901566	organonitrogen compound biosynthetic process	0.00499138
BP	GO:0034645	cellular macromolecule biosynthetic process	0.01553867
BP	GO:0009059	macromolecule biosynthetic process	0.0196086
CC	GO:0005840	ribosome	1.30E-05
CC	GO:0030529	intracellular ribonucleoprotein complex	7.88E-05
CC	GO:1990904	ribonucleoprotein complex	7.88E-05
CC	GO:0044391	ribosomal subunit	0.0028929
CC	GO:0043228	non-membrane-bounded organelle	0.01247005
CC	GO:0043232	intracellular non-membrane-bounded organelle	0.01247005
CC	GO:0044445	cytosolic part	0.02406129
CC	GO:0005622	intracellular	0.03132207
CC	GO:0015934	large ribosomal subunit	0.04260791
MF	GO:0003735	structural constituent of ribosome	7.03E-06
MF	GO:0005198	structural molecule activity	2.69E-05
MF	GO:0005509	calcium ion binding	0.03132207

Table A2.6 All significantly enriched gene ontology (GO) terms for Cluster 1 genes which were significantly correlated with changes to metabolic function (P < 0.05). Data not shown for upregulated genes as no significant functional enrichment was observed (P_{adj} < 0.05).

Ontology	GO term	Description	P_{adj}
BP	GO:0034767	positive regulation of ion transmembrane transport	0.01482289
BP	GO:1902578	single-organism localization	0.01956669
BP	GO:0034764	positive regulation of transmembrane transport	0.0235078
BP	GO:0051453	regulation of intracellular pH	0.02547464
BP	GO:0060372	regulation of atrial cardiac muscle cell membrane repolarization	0.02547464
BP	GO:0015701	bicarbonate transport	0.02562511
BP	GO:0032417	positive regulation of sodium:proton antiporter activity	0.02562511
BP	GO:0030497	fatty acid elongation	0.03996493
BP	GO:0030641	regulation of cellular pH	0.03999941
BP	GO:0044765	single-organism transport	0.04161613
BP	GO:0030003	cellular cation homeostasis	0.04161613
BP	GO:1904064	positive regulation of cation transmembrane transport	0.0416969
BP	GO:0006633	fatty acid biosynthetic process	0.04656015
CC	GO:0031012	extracellular matrix	0.00019367
CC	GO:0005578	proteinaceous extracellular matrix	0.00061149
CC	GO:0044421	extracellular region part	0.01482289
CC	GO:0031226	intrinsic component of plasma membrane	0.02547464
CC	GO:0005887	integral component of plasma membrane	0.02562511
MF	GO:0042302	structural constituent of cuticle	2.57E-06
MF	GO:0015294	solute:cation symporter activity	6.36E-05
MF	GO:0015293	symporter activity	0.00014798
MF	GO:0015081	sodium ion transmembrane transporter activity	0.00033222
MF	GO:0015291	secondary active transmembrane transporter activity	0.00046833
MF	GO:0008324	cation transmembrane transporter activity	0.00046833
MF	GO:0022891	substrate-specific transmembrane transporter activity	0.00061149
MF	GO:0015075	ion transmembrane transporter activity	0.0011729
MF	GO:0046873	metal ion transmembrane transporter activity	0.00213697
MF	GO:0022890	inorganic cation transmembrane transporter activity	0.00388455
MF	GO:0022892	substrate-specific transporter activity	0.00467336
MF	GO:0015077	monovalent inorganic cation transmembrane transporter activity	0.0047917
MF	GO:0004312	fatty acid synthase activity	0.00494205
MF	GO:0015370	solute:sodium symporter activity	0.00494205
MF	GO:0005215	transporter activity	0.00494205
MF	GO:0008514	organic anion transmembrane transporter activity	0.00494205
MF	GO:0004316	3-oxoacyl-[acyl-carrier-protein] reductase (NADPH) activity	0.00494205
MF	GO:0102131	3-oxo-glutaryl-[acp] methyl ester reductase activity	0.00494205
MF	GO:0102132	3-oxo-pimeloyl-[acp] methyl ester reductase activity	0.00494205
MF	GO:0015106	bicarbonate transmembrane transporter activity	0.00663696
MF	GO:0022857	transmembrane transporter activity	0.00683235

Table A2.6 (cont.)

MF	GO:0005198	structural molecule activity	0.01278762
MF	GO:0005328	neurotransmitter:sodium symporter activity	0.01278762
MF	GO:0005326	neurotransmitter transporter activity	0.02547464
MF	GO:0046943	carboxylic acid transmembrane transporter activity	0.04469821

Table A2.7 All significantly enriched gene ontology (GO) terms for significantly downregulated genes ($P_{adj} < 0.05$) identified as frontloaded genes during warm acclimation. Data not shown for frontloaded upregulated genes as no significant functional enrichment was observed ($P_{adj} = 1$)

Ontology	GO term	Description	P_{adj}
BP	GO:0043043	peptide biosynthetic process	9.88E-06
BP	GO:0043604	amide biosynthetic process	9.88E-06
BP	GO:0006412	translation	1.06E-05
BP	GO:0006518	peptide metabolic process	1.41E-05
BP	GO:0043603	cellular amide metabolic process	4.95E-05
BP	GO:1901566	organonitrogen compound biosynthetic process	0.00192453
BP	GO:0034645	cellular macromolecule biosynthetic process	0.04454594
BP	GO:0009059	macromolecule biosynthetic process	0.04454594
CC	GO:0005840	ribosome	1.08E-05
CC	GO:0030529	intracellular ribonucleoprotein complex	7.63E-05
CC	GO:1990904	ribonucleoprotein complex	7.63E-05
CC	GO:0043228	non-membrane-bounded organelle	0.00192453
CC	GO:0043232	intracellular non-membrane-bounded organelle	0.00192453
CC	GO:0044391	ribosomal subunit	0.00298311
CC	GO:0005622	intracellular	0.00984928
CC	GO:0044445	cytosolic part	0.02373793
MF	GO:0003735	structural constituent of ribosome	9.88E-06
MF	GO:0005198	structural molecule activity	1.51E-05
MF	GO:0005509	calcium ion binding	0.00984928

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