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The role of hydrostatic pressure in constraining the bathymetric distribution of marine ectotherms

by

Alastair Edward Brown

Thesis for the degree of Doctor of Philosophy

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“Nothing in biology makes sense except in the light of evolution”

Dobzhansky, 1973

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ABSTRACT

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THE ROLE OF HYDROSTATIC PRESSURE IN CONSTRAINING THE BATHYMETRIC DISTRIBUTION OF MARINE ECTOTHERMS

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A hyperbaric and thermal physiological bottleneck at bathyal depths is thought to contribute to bathymetric zonation of marine benthic invertebrates and demersal fishes on deep continental margins. The focus of this thesis was to investigate hyperbaric tolerance in the lithodid crab *Lithodes maja* as a case study for the effects of hydrostatic pressure on upper bathyal marine ectotherms. Experimental hyperbaric exposures revealed that hyperbaric tolerance is oxygen- and capacity-limited. Hyperbaric tolerance appears proximately oxygen-limited, but ultimately limited by cardiac capacity: adverse hyperbaric impacts on cardiac capacity appear mediated by the effects of pressure on membranes and membrane related functions. However, bathymetric range appears constrained by increased metabolic cost at elevated hydrostatic pressure. Hyperbaric limitation of bathymetric range supports a role for hydrostatic pressure in structuring bathymetric zonation in the deep sea, and lineage-specific physiological tolerances appear to contribute to global phylogenetic bottlenecks. Further, physiological effects of high hydrostatic pressure and low temperature at bathyal depths, acting on shallow-water taxa at the lower limits of their distribution, may invoke a stress–evolution mechanism. The resulting bathymetric variation in speciation rates could drive a unimodal diversity–depth pattern, typically peaking at bathyal depths, over time.

Marine ectotherms' thermal tolerance is also oxygen- and capacity-limited, and functionally associated with hypoxia tolerance. Comparing hypoxia thresholds and hyperbaric thresholds of taxonomic groups of shallow-water fauna revealed significant correlation, supporting the proposition that hydrostatic pressure tolerance is oxygen-limited. Consequently, it appears that the combined effects of temperature, pressure, and oxygen concentration constrain the fundamental ecological niches of marine invertebrates and fishes. Including depth in a conceptual model of oxygen- and capacity-limited fundamental ecological niches' responses to ocean warming and deoxygenation confirms that polar taxa are most vulnerable to the effects of climate change, but reveals for the first time that temperate fauna as well as tropical fauna may experience substantial fundamental ecological niche expansion with ocean warming and deoxygenation.

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DECLARATION OF AUTHORSHIP

I, Alastair Edward Brown, declare that the thesis entitled

'The role of hydrostatic pressure in constraining the bathymetric distribution of marine ectotherms'

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
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- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
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1. Introduction

1.1 Bathymetric biodiversity patterns*

Many macroevolutionary patterns display both ecological and biogeographical components. Clear bathymetric (depth-related) patterns have been identified in the extant biodiversity of the deep continental margins, a region covering approximately 40% of the total ocean surface area (Fig. 1.1) (reviewed by Merrett & Haedrich, 1997;

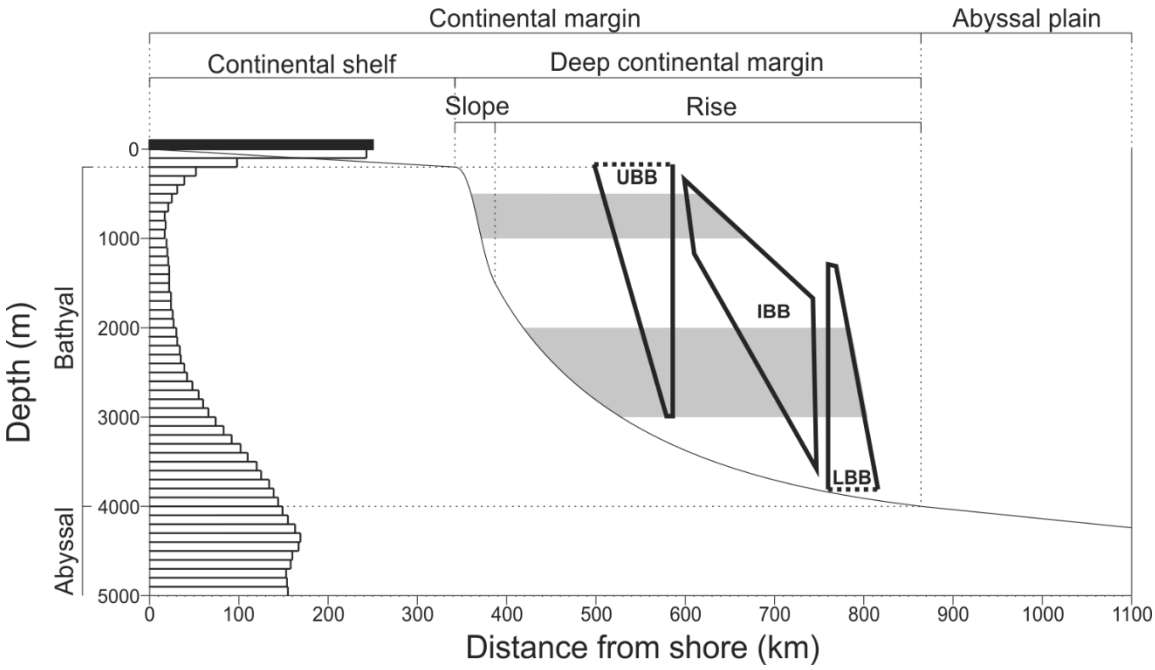

Figure 1.1. Conceptual profile of a passive aseismic continental margin (adapted from Gage & Tyler, 1991). Horizontal bars on the left indicate percentage of total ocean surface area of each 100 m depth interval (note that this is not restricted exclusively to continental margins) estimated from figure 9.2 in Mackenzie & Lerman (2006) (black scale bar = 5%; depths greater than 5000 m not shown). Conceptual components of bathymetric patterns of diversity (adapted from Carney, 2005; height = bathymetric range, width = species richness) are included, representing three groups of species: upper boundary biota (UBB) species extend downwards from or above the upper boundary of the deep continental margin but do not reach the lower boundary; lower boundary biota (LBB) species extend upwards from or below the lower boundary of the deep continental margin but do not reach the upper boundary; inter-boundary biota (IBB) species reach neither boundary. Shaded areas indicate depths of high species turnover consistently identified in studies of bathymetric diversity.

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Constraint of bathymetric distribution by hydrostatic pressure

Levin *et al*., 2001; Stuart *et al*., 2003; Carney, 2005; Menot *et al*., 2010; Rex & Etter, 2010). The deep continental margin comprises continental slope and rise. In topographic terms, the continental slope is characterised by increasing topographic gradient at the continental shelf break. The topographic gradient reduces onto the continental rise, formed by a thick slope-derived sediment wedge, and reduces further onto the relatively flat abyssal plain. Prevailing deep-sea biodiversity concepts define the deep continental margin ecological zones as bathyal (200–4000 m), with a transitional fauna comprising both shallow and deep water species, and abyssal (4000–6000 m), with an exclusively deep-sea fauna (Gage & Tyler, 1991).

A unimodal diversity–depth pattern has been indicated by qualitative (Rex, 1981) and quantitative (Etter & Grassle, 1992) sampling studies in the western North Atlantic, the most intensively sampled region of the deep sea. Diversity appears depressed at upper bathyal depths and at abyssal depths, with a peak in diversity at intermediate depths (typically between 1000 and 3000 m; Rex & Etter, 2010), despite the relatively low area represented by these depths (1000–3000 m represents approximately 13% of the total ocean surface area; Fig. 1.1), at a level comparable to the most diverse ecosystems known (Grassle & Maciolek, 1992; Levin & Dayton, 2009). Almost all organisms are distributed between a high and a low depth limit (Pradillon & Gaill, 2007) and geometric constraints models, which stochastically place bathymetric ranges between boundaries, have yielded unimodal patterns of diversity similar to bathymetric gradients observed in the deep sea (Pineda & Caswell, 1998). However, such models cannot explain most characteristics of the parabolic bathymetric diversity pattern, i.e. curvature, or the magnitude and position of the peak (Pineda & Caswell, 1998; McClain & Etter, 2005). This unimodal diversity pattern has been attributed to varied environmental gradients, particularly in productivity and disturbance (Paterson & Lambshead, 1995; Cosson-Sarradin *et al*., 1998; Rex *et al*., 2005*a*; Tittensor *et al*., 2011), and a source–sink hypothesis has been suggested for abyssal biodiversity where abyssal populations are regulated by a balance between immigration from bathyal sources and chronic extinction arising from vulnerabilities to Allee effects (Rex *et al*., 2005*b*).

Although the first test of the source–sink hypothesis strongly suggests that source–sink dynamics contribute to the unimodal diversity pattern, it is also clear that species turnover is more important at bathyal depths (Brault *et al*., 2013*b*), and shallowing of a unimodal peak in diversity in low-temperature environments (e.g. the Arctic; Yasuhara *et al*., 2012) clearly suggests a role for temperature and physiology (see Yasuhara and Danovaro, 2014). However, the mechanisms proposed to drive the unimodal diversity–depth relationship do not consider the evolutionary history of the deep-sea fauna. Speciation rates appear to drive other biodiversity patterns (Allen & Gillooly, 2006); consequently the unimodal bathymetric diversity pattern may be influenced by discordance in the environmental pattern of evolutionary origin (Stuart & Rex, 2009). Whilst ecological processes tend to dominate over short time periods and local scales, evolutionary processes are more important over long time periods and regional or global scales (Lambshead & Boucher, 2003), and it appears that species diversity is driven largely by abiotic factors (for review see Benton, 2009). The importance of considering evolutionary processes is apparent in analysis of bathymetric zonation in the deep sea.

Although the unimodal diversity pattern seems typical of the western North Atlantic and also occurs widely in other locations, data from some geographical regions suggest that it may not be ubiquitous, being interrupted by oceanographic conditions at specific depths such as oxygen minimum zones (see Levin *et al*., 2001; Stuart *et al*., 2003; Menot *et al*., 2010; Rex & Etter, 2010). The geological history of regions may also contribute to the absence of unimodal diversity patterns with depth. For example, following the Mediterranean Sea desiccation event ~5.5 million years ago (MYA) (Krijgsman *et al*., 1999) recolonisation by marine fauna may have been limited to shallower species by the depth of the Mediterranean sill, resulting in an impoverished Mediterranean deep-sea fauna (see Tyler, 2003) without a clear unimodal bathymetric diversity pattern (Danovaro *et al*., 2010). Similarly, the Madeira archipelago evolved volcanically and relatively recently (<14.3 MYA) from a hotspot approximately 700 km off the African coastline (Geldmacher *et al*., 2000), which explain may the contrasting bathymetric diversity patterns reported in crustaceans: the unimodal bathymetric diversity pattern is present in pelagic taxa but absent in benthic taxa (Rosa *et al*.,

2012), likely as a result of dispersal limitation (see e.g. McClain *et al*., 2011). Regardless of the absolute global validity of the unimodal bathymetric diversity model, bathymetric patterns of species turnover and zonation in the deep sea are widespread (see Merrett & Haedrich, 1997; Carney, 2005; Menot *et al*., 2010; Rex & Etter, 2010). Rapid depth-correlated turnover in species composition is consistently indicated at the shelf-slope transition between the shelf break and 1000 m and at the slope-abyss transition between 2000 and 3000 m. Consequently, the shelf, continental slope, and abyssal plain faunas are clearly distinct, suggesting that these transitions are biodiversity bottlenecks (Fig. 1.1) (Carney, 2005; Menot *et al*., 2010). It remains uncertain which factors play a dominant role in these distributional barriers but it appears that thermal effects may contribute to bathymetric zonation patterns since temperature-related shifts in the upper transition zone have been identified, and zonal boundaries appear to become less distinct with increasing latitude (see Carney, 2005). However, bathymetric zonation persists in isothermal water columns, such as those at high latitudes or in the Mediterranean Sea (see Carney, 2005; Gale *et al*., 2014). These diversity patterns suggest that the deep-sea margin ecosystem may offer novel contributions to ecological theory (Levin & Dayton, 2009).

1.2 Origin of the deep-sea fauna and the colonisation of the deep sea*

The evolutionary origin and antiquity of the extant deep-sea fauna remains uncertain, with the contemporary fauna appearing to comprise clades which originated throughout the ~541 million years (myr) of the Phanerozoic (Jablonski *et al*., 1983; Jablonski & Bottjer, 1991). Over these geological timescales there have been at least five (Raup & Sepkoski, 1982; see also Harnik *et al*., 2012) relatively sudden (*c*. 1–10 myr; Briggs, 1995) major marine extinctions, estimated to have eradicated at least half of marine species (Briggs, 2003; but see Bambach, 2006). Although some fauna appear to have survived these events (see e.g. Raupach *et al.*, 2009; Thuy *et al*., 2012, 2014;

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Thuy, 2013), in the most severe case approximately half of all marine families (Sepkoski, 1986) and more than 95% of marine species (Raup, 1979; Benton & Twitchett, 2003) disappeared. Consequently, it has been suggested that climate-driven dysoxic extinction events in the deep sea and subsequent recolonisations have occurred on multiple occasions (Wignall & Twitchett, 1996; Horne, 1999; Wilson, 1999; Rogers, 2000; Kiehl & Shields, 2005; Wignall *et al*., 2005). General onshore–offshore patterns of evolution have been reported from extensive analyses of the fossil record of shelf communities of the Phanerozoic: higher taxonomic level innovation occurred predominantly in nearshore settings before expanding into offshore environments, while rates of genera-level evolution appear to be diversity dependent, shaped by clade-specific bathymetric gradients (Jablonski, 2005, and references cited therein; but see Tomasových *et al*., 2014). For example, scleractinian corals appear to have originated ~237 MYA in shallow water (see Jablonski & Bottjer, 1991) before invading the deep sea (Kitahara *et al*., 2010) perhaps on several occasions during the last 65.5 myr (Os'kina *et al*., 2010). Similarly, molluscs appear to have made invasions of the deep sea from multiple shallow-water regions, although no families or higher groups of mollusc appear to have originated in the deep sea (Clarke, 1962; Allen, 1978). Deep-sea fishes appear to have originated in shallow water too, before colonising the deep sea during the last ~70 myr (see Merrett & Haedrich, 1997, and references cited therein; Priede & Froese, 2013). For example, the fossil record of gadoid and macrouroid fishes suggests origination in a shallow continental shelf environment, but with adaptation to deep-water settings early in their evolution prior to radiation (see Merrett & Haedrich, 1997; Kriwet & Hecht, 2008). Chondrichthyian fishes also appear to have originated in shallow-water, with holocephalian fishes diverging from elasmobranchian fishes after deep-sea colonisation ~400 MYA, before diversifying *in situ* (Priede & Froese, 2013).

Phylogenetic analyses have supported the relatedness of extant deep-sea and shallow-water species, predominantly consistent with diversification and invasion of deep-sea environments from shallow water, albeit over differing timescales. Plumulariid hydroids appear to have invaded deep water ~8 MYA (Moura *et al*., 2011). Bresiliid shrimp from deep-sea vents and seeps are reported to have radiated less than 20 MYA

and from shallow ancestry (Shank *et al*., 1999; Tokuda *et al*., 2006). Similarly, vesicomyid clams appear to have invaded the deep sea from coastal habitats between 22 and 44 MYA, occupying cold seep habitats before colonising hydrothermal vent environments (Little & Vrijenhoek, 2003; Decker *et al*., 2012). Other vent and seep fauna predominantly originated relatively recently in geological terms too, within the last 100 myr (see Little & Vrijenhoek, 2003; Roterman *et al*., 2013; Vrijenhoek, 2013; Yang *et al*., 2013; Herrera *et al*., 2015). Although the deep-sea abyssochrysoid gastropod snails diverged from shallow-water caenogastropods ~154 MYA (Osca *et al*., 2014), the divergence of vent abyssochrysoid species from non-vent abyssochrysoid species has not been explored, consequently dating abyssochrysoid vent colonisation is not possible.

Pagodulid snails appear to have radiated even more recently, from a shallow-water Antarctic lineage, and colonised the deep sea approximately 3 MYA (Barco *et al*., 2012). However, colonisation of the deep sea by shallow-water Antarctic fauna is not exclusively recent. The notothenioid fish species flock also appears to have radiated in the last ~21 myr (Bargelloni *et al*., 2000), evolving in Antarctic shallow water before invading the deep sea (see Clarke & Johnston, 1996, and references therein). Molecular phylogenetic evidence indicates that a deep-sea octopus lineage invaded from shallow-water Antarctic origin, diverging around 33 MYA and subsequently radiating ~15 MYA (Strugnell *et al*., 2008). Similarly, nudipleuran evolution is proposed to have taken place around the cooling of Antarctica about 40 or 30 MYA, prior to invasion of temperate and tropical seas along northward flowing currents of Antarctic origin (see Göbbeler & Klussmann-Kolb, 2010). Fossil evidence also suggests submergence of shallow-water Antarctic bivalves, gastropods, asteroids, crinoids, and decapods into the deep sea during this period (Zinsmeister & Feldmann, 1984). Although the timing remains unclear, molecular evidence indicates that palinurid spiny lobsters originated around Antarctica, invading deep-sea habitats from shallower rocky reefs and then radiating (Tsang *et al*., 2009).

Molecular evidence suggests that lysianassoid amphipods colonised the deep-sea in a single event approximately 70 MYA (Corrigan *et al.*, 2014). However, palaeontological

and molecular data indicate that echinoids have made migrations to the deep sea over multiple timescales: generalist omnivores migrated to the deep sea in low numbers over the last 200 myr in contrast to the majority of specialist detritivore clades, which made independent off-shelf migrations between approximately 75 and 55 MYA (Smith & Stockley, 2005). Similarly, deep-sea asellote isopods originate from at least four major and independent migrations from shallow water, but these isopods are proposed to have invaded the deep sea and radiated prior to the dysoxic events during the end-Permian extinction event *ca.* 250 MYA (see Raupach *et al*., 2009; Lins *et al*., 2012; Riehl *et al*., 2014). Torquaratorid acorn worms also appear to have colonised the deep sea prior to ~250 MYA, invading from shallow water before demonstrating an extensive radiation *in situ* (Osborn *et al*., 2012). Bivalve molluscs were well represented in the Ordovician (485–443 MYA) but also show evidence of more recent radiations (Allen, 1978). For example, protobranch bivalves appear to have colonised the deep Atlantic during its formation approximately 130 MYA (Allen and Sanders, 1996; Etter *et al*., 2005; Etter *et al*., 2011), and deep-sea bathymodiolinid mussels found at hydrothermal vents and cold seeps represent a recent evolutionary radiation from modern shallow-water mytilid taxa as organic fall specialists (Distel *et al*., 2000; Lorion *et al*., 2010; Thubaut *et al*., 2013; but see Kiel & Amano, 2013), within the last 30 myr (Miyazaki *et al*., 2010; Lorion *et al*., 2013; but see Kiel & Amano, 2013). The deep-water lithodid crabs also appear to have originated recently, with at least three radiations from North Pacific shallow-water ancestors since the evolution of the lithodids (Hall & Thatje, 2009) approximately 29 MYA (Bracken-Grissom *et al*., 2013). The potential for depth-range extension and colonisation of the deep sea by shallow-water species persists: there is evidence that the echinoid sea urchin *Echinus acutus* is extending its bathymetric range, indicating that migrations to the deep sea are still occurring (Tyler & Young, 1998; Minin, 2012).

Re-emergence from the deep sea has also been reported, for example for lithodid crabs (Hall & Thatje, 2009) and possibly for cylindroleberidid ostracods (Syme & Oakley, 2012; see their discussion for contrasting conclusions from different analytical methods). Further, some taxa originated in the deep sea and ascended to shallow water. Following origination ~62 MYA (Bernecker & Weidlich, 1990) stylasterid corals

diversified extensively in the deep sea before making three distinct invasions of the shallow-water tropics and a single invasion of temperate shallow water (Lindner *et al*., 2008). Similarly, chrysogorgiid soft corals and pennatulid sea pens appear to have originated in the deep sea before radiating globally and into shallow water (Dolan, 2008; Pante *et al*., 2012; Dolan *et al*., 2013), and shallow-water isthanid anemones may have been seeded by ancestors living in the deep sea (Lauretta *et al*., 2014). Molecular phylogeny indicates that freshwater eels also originated in the deep ocean following invasion from shallow water, reflecting their evolutionary origin in their catadromous life cycle (Inoue *et al*., 2010). Carcharinid sharks invaded shelf habitats following origination in the deep sea, too (Sorenson *et al*., 2014). Fossil evidence suggests that the ophiacanthid brittle star family originated in the deep sea before invading shallow-water (Thuy, 2013), and morphological and molecular evidence suggests that the macrostylid isopod family also colonised shallow environments following origination in the deep sea (Riehl, 2014). However, examples of deep-sea origination of higher taxonomic levels are relatively few (but see Thuy *et al*., 2014), and the extant deep-sea fauna is considered to result predominantly from both ancient and more recent radiations of shallow-water lineages into deep water (Horne, 1999; Wilson, 1999).

Since shallow-water fauna are adapted to relatively warm conditions currently dominating the upper oceans except at high latitudes, the low temperatures prevalent in the deep sea are considered to limit invasion by such fauna (deep-sea temperature is typically between 4 and -1°C; Gage & Tyler, 1991). Consequently, it is believed that the colonisation of the deep sea may have been predominantly limited to periods and regions with an isothermal water column (Tyler *et al*., 2000). Warm water columns are currently restricted to isolated seas, e.g. water temperature is 21.5°C at 2 km depth in the Red Sea and is 13°C at 4 km depth in the Mediterranean Sea (Gage & Tyler, 1991), but were widespread in some earlier geological periods. For example, the vertically homogenous warm ocean of the late Mesozoic and early Cenozoic (with deep-sea bottom temperatures up to 16°C; Lear *et al*., 2000; Zachos *et al*., 2001; Cramer *et al*., 2011) could have permitted invasion of deep water, later requiring adaptation to cold temperatures as the oceans gradually evolved to the current psycrospheric state

(Young *et al*., 1997; Thatje *et al.*, 2005*b*). Invasion of the deep sea by the majority of specialist detritivore echinoids occurred during this period (Smith & Stockley, 2005). At other geological times near-isothermal cold water columns in regions of deep-water formation at high latitudes have presented an opportunity for deep-sea invasion. Molecular phylogeny has indicated Antarctic shallow water as the origin of both deep-sea asellote isopods (>250 MYA; Raupach *et al*., 2009) and deep-sea octopus (~33 MYA; Strugnell *et al*., 2008) during periods with low-temperature deep-water formation at high latitudes (Horne, 1999), prior to deep-sea radiation.

In both warm and cold isothermal water columns the major limiting factor for range extension into the deep sea is predicted to be tolerance of high hydrostatic pressure (pressure exerted by the overlying mass of water) (Young *et al*., 1997; Thatje *et al*., 2005*b*). Phylogenetic and physiological studies have certainly emphasised thermal and hyperbaric bottlenecks in an evolutionary context, with passage to deeper water requiring adaptation to low temperatures and high hydrostatic pressures (Macdonald, 1972; Menzies & George, 1972; Macdonald & Teal, 1975; George, 1979; Hall & Thatje, 2009; Mestre *et al*., 2009; Thatje *et al*., 2010; Brown & Thatje, 2011; Oliphant *et al*., 2011; Smith & Thatje, 2012). Evidence of critical biological effects of hydrostatic pressure and temperature could support the imposition of limits on bathymetric distribution by these factors.

1.3 Physiological limitation by low temperature and high hydrostatic pressure*

Thermal tolerance is proposed to relate directly to the physiological ability of an organism to avoid the transition from aerobic to anaerobic metabolism, with a systemic to molecular hierarchy of limitation (see Pörtner, 2001, 2002; but see e.g. Ern *et al*., 2014). Under environmental conditions beyond optimum, the homeostatic effort required to maintain internal conditions within physiological tolerance boundaries increases. Low temperatures have been shown to interrupt protein

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structure for numerous proteins (for review see Privalov, 1990; Kunugi & Tanaka, 2002; Marqués *et al*., 2003). Molecular and physiological studies of cold stress suggest that this can result in elevated protein chaperoning in response to cold denaturation (Place & Hofmann, 2005; Schmid *et al*., 2009). These protein chaperones counteract the stabilisation of the secondary structures of RNA and DNA and the consequent reduction in the efficiency of translation, transcription, and DNA replication (Phadtare *et al*., 1999), and may be required for ribosome assembly at suboptimal temperatures (Gualerzi *et al*., 2003). Low temperature also decreases the fluidity of biological membranes, significantly reducing membrane function (Hazel, 1995). Mitochondrial activity increases to facilitate the increased homeostatic effort, however increased mitochondrial oxygen demand is not directly matched by increased respiratory capacity delivered through ventilation and circulation (e.g. Frederich & Pörtner, 2000). Subsequently, a transition from aerobic to anaerobic mitochondrial respiration occurs (oxygen limitation) at the critical threshold where mitochondrial oxygen demand exceeds the respiratory capacity of the animal (capacity limitation); survival under such conditions is time limited. The effects of oxygen limitation on the cardiac muscle are amplified as mitochondrial oxygen demand increases ultimately forming a positive feedback loop (Somero, 2005). This model is commonly known as the oxygen- and capacity-limitation hypothesis (see Pörtner, 2010, and references cited therein).

Mitochondrial densities and their functional properties appear to be critical in defining thermal tolerance windows. For example, at low temperatures the aerobic capacity of mitochondria may become limiting for ventilation and circulation. Adjustments in mitochondrial densities and functional properties can shift temperature envelopes tolerated by organisms (Sommer & Pörtner, 2002). However, integrated molecular modifications in lipid saturation, kinetic properties of metabolic enzymes, contractile proteins, and transmembrane transporters are also essential for maintaining higher functions (Pörtner, 2002). Outside the optimal range basic metabolic processes can be maintained before the critical threshold, but non-essential processes such as growth, reproduction, feeding, and voluntary movement are reduced (Cossins & Bowler, 1987; Peck, 1998; Peck *et al*., 2004, 2007, 2008; Pörtner, 2004; Young *et al*., 2006). At a species level diminished performance induced by environmental factors may have

significant impacts; reductions in growth and reproductive output will affect the survival of species (Pörtner, 2002). Complex animals rely on ventilation and circulatory systems to supply their cells with oxygen and, consistent with the oxygen- and capacity-limitation hypothesis, inter- and intraspecific analyses have indicated that smaller individuals survive to higher temperatures than larger ones in marine species and that more active species survive higher elevated temperatures (Peck *et al*., 2002, 2004, 2007, 2009; Pörtner, 2002; Pörtner *et al*., 2006, 2007). This may explain the apparent preferential survival of small species during extinction events (Cardillo, 2003).

There are significant physical effects of hydrostatic pressure on proteins and lipoprotein membranes (reviewed by Pradillon, 2012). Relatively moderate pressure increase may deform proteins resulting in functional changes (e.g. Barstow *et al*., 2008, and references cited therein), or induce protein subunit dissociation and consequently denature enzymes (for review see Gross & Jaenicke, 1994; Mozhaev *et al*., 1996; Boonyaratanakornkit *et al*., 2002; Winter & Dzwolak, 2005). For example, macromolecular protein assemblages such as cytoskeleton tubulin and actin are dissociated by pressure in the range of a few tens of MPa in shallow-water organisms, affecting basic cell morphology and organisation (Kennedy & Zimmerman, 1970; Salmon, 1975*a*,*b*; Begg *et al*., 1983; Swezey & Somero, 1985; Bourns *et al*., 1988). Synthesis of proteins is also susceptible to elevated pressure (Gross & Jaenicke, 1994).

Lipid bilayers of biological membranes appear one of the most pressure-sensitive molecular assemblages (Wann & Macdonald, 1980; DeLong & Yayanos, 1985; Somero, 1992; Macdonald, 1997; Winter & Dzwolak, 2005; Winter, 2010): pressure increase orders structures and reduces flexibility in lipids, nucleic acids and carbohydrates (Behan *et al*., 1992; Balny *et al*., 2002). An increase in pressure of 100 MPa (1 MPa ≈ 100 m water depth) is equivalent to a decrease in temperature of approximately 13–21°C depending on membrane composition (Somero, 1992); a temperature increase of 2.8°C has been reported to reverse the reduction in membrane fluidity imposed by a hydrostatic pressure of 10 MPa (De Smedt *et al*., 1979). The effects of reduced membrane functionality on action potential transmission in nervous cells (Wann & Macdonald, 1980; Siebenaller & Garrett, 2002) are clearly visible in a high-hydrostatic-

pressure neurological syndrome in organisms exposed to pressures radically different from those within their natural distribution; signs are motor coordination impairment, spasm and even paralysis (Menzies & George, 1972; Macdonald & Teal, 1975; Wilcock *et al*., 1978; Yayanos, 1981; Avrova, 1984; Heinemann *et al*., 1987; Treude *et al*., 2002; Oliphant *et al*., 2011). The interference can affect cardiac function (Mickel & Childress, 1982*b*; Airriess & Childress, 1994), with clear implications for aerobic scope. Observed respiratory and cardiac responses to pressure change appear to support the application of the oxygen- and capacity-limitation hypothesis to hydrostatic pressure tolerance (George, 1979; Mickel & Childress, 1982*a*,*b*,*c*; Robinson *et al*., 2009; Brown & Thatje, 2011; Thatje & Robinson, 2011), with further consistent indications that voluntary movement and feeding are affected by hyperbaric conditions beyond optimum (Thatje *et al*., 2010; Thatje & Robinson, 2011). Aerobic scope certainly appears the crucial factor setting tolerance limits (Peck *et al*., 2002, 2004, 2009; Pörtner, 2002; Pörtner *et al*., 2006, 2007; Brown & Thatje, 2011; Thatje & Robinson, 2011).

Cellular responses to thermal and hyperbaric environmental challenges can contribute directly to biogeographic limitation (e.g. see Tomanek, 2010). The effects of increases in pressure and temperature on proteins and lipid bilayers of biomembranes are largely antagonistic within ecologically relevant ranges (Balny *et al*., 1997; Winter & Dzwolak, 2005), suggesting that low temperatures and high hydrostatic pressures may act together in limiting bathymetric distribution of species (e.g. Brown & Thatje, 2011). Cells of atmospheric-pressure-adapted organisms respond to pressure changes by altering synthetic capacity (Parkkinen *et al*., 1993; Lammi *et al*., 1994; Smith *et al*., 1996). Analysis of transcription in articular cartilage cells indicates hyperbaric up-regulation of mRNA of several genes mediating growth arrest (Sironen *et al*., 2002), and exposure to high hydrostatic pressure causes cellular growth arrest (Abe & Horikoshi, 2000; Koyama *et al*., 2005) and decreased levels of mRNA of genes involved in cell-cycle progression (Fernandes *et al*., 2004). Reduced transcription has also been reported for genes involved in protein synthesis (Fernandes *et al*., 2004; Elo *et al*., 2005). However, cells exposed to continuous high hydrostatic pressure regimes have also responded by up-regulating several heat shock proteins (Kaarniranta *et al*., 1998,

2000; Elo *et al*., 2000, 2005). This response protects proteins from acute and chronic stress by stabilising and refolding protein-folding intermediates or by facilitating protein degradation (Morimoto *et al*., 1997), and has recently been reported in atmospheric-pressure-adapted shrimp exposed to high hydrostatic pressure (10 MPa) without onset of systemic failure (Cottin *et al*., 2012). In contrast, exposure to 10 MPa did not stimulate a heat-shock response in shallow-water-acclimated deep-sea brachyuran crabs (Mestre *et al*., 2015). Cold stress can result in elevated protein chaperoning too, in response to cold denaturation (Place & Hofmann, 2005; Schmid *et al*., 2009). Expression of cold shock proteins can also be induced in organisms exposed to increased hydrostatic pressure (e.g. Welch *et al*., 1993; Wemekamp-Kamphuis *et al*., 2002). Considering the analogous effects of high hydrostatic pressure and low temperature, such responses may be critical to colonising the deep sea: the cold shock response of the microorganism *Listeria monocytogenes* after exposure to 10°C for 4 hours following culture at 37°C has been reported to result in a 100-fold increase in survival of exposure to 300 MPa for 20 minutes (Wemekamp-Kamphuis *et al*., 2002).

Evidently, hydrostatic pressure and temperature both have significant biological effects perturbing every level of biological organisation sufficiently to limit biogeographic range (Table 1.1). It appears likely that an organism's capacity for survival at any given depth is determined by the sum of hydrostatic pressure and temperature interactions (Sébert, 2002) in advance of other ecological considerations. Experimental evidence assessing hyperbaric limitation across a range of shallow-water benthic invertebrate taxa at *in situ* temperatures at bathyal depths could support the contribution of hydrostatic pressure to bathymetric zonation.

1.4 Tolerance of high hydrostatic pressure and low temperature*

Recently, attempts to determine potential for invasion of the deep sea have focused on mollusc and echinoderm propagule tolerance of hydrostatic pressure and low temperature in shallow-water species, with and without close phylogenetic links to

* Updated from 'Brown, A. & Thatje, S. (2014). Explaining bathymetric diversity patterns in marine benthic invertebrates and demersal fishes: physiological contributions to adaptation of life at depth. *Biological Reviews* **89**, 406-426'.

Table 1.1. Proposed timescales and known physiological effects of high hydrostatic pressure and low temperature, and responses across hierarchical levels of organisation.

Process	Time scale	Level	Effects and responses
	Seconds to minutes	Molecular	Macromolecular damage is sustained (e.g. cytoskeletal tubulin and actin are dissociated) and functionality of membranes is reduced (e.g. decreased flexibility of lipids, nucleic acids and carbohydrates) during exposure to high hydrostatic pressure and low temperature.
Acclimatisation	Seconds to months	Cellular	In the short term, cellular growth and cell-cycle progression cease and stress proteins (e.g. heat shock and cold shock proteins) are expressed. Longer term, the cellular homeostatic response is induced promoting acclimatisation (e.g. through increasing mitochondrial density and enzyme concentrations, and adjusting functional properties; cell membranes accumulate higher levels of lipid, the proportion of unsaturated fatty acids increases, and sterol and protein concentrations also increase). Hidden variation or mutagenic activity is released by the cellular stress response, or transposable elements are activated or released in larvae or adults, initiating the process of adaptation; the timescale of acclimatisation and adaptation processes therefore overlap.
	Minutes to decades	Individual	Cellular stress response and increased homeostatic effort elevates metabolic demand and individual cardiac and ventilatory activity rise in response. Mutation is increased by stress-induced DNA damage in germ cells in the absence of a stress response during embryogenesis, or canalisation is inactivated during embryonic or larval development.
	Years to centuries	Population	Organismal survival and reproduction permits propagation of variation through a population.
Adaptation	Decades to millenia	Species	Differentiation of populations ultimately results in speciation.

deep-sea species, in order to test the validity of theories of deep-sea colonisation (Young *et al*., 1995, 1996, 1997; Tyler & Young, 1998; Tyler *et al*., 2000; Benitez Villalobos *et al*., 2006; Aquino-Souza *et al*., 2008; Mestre *et al*., 2009, 2013; Smith & Thatje, 2012). These studies suggest that hydrostatic pressure tolerance is influenced by species' thermal adaptive history (e.g. Smith & Thatje, 2012), but in all cases have indicated impressive pressure tolerances considerably beyond those experienced in the known adult distributions of the study species. However, although juveniles of several echinoderm species are known to settle outside of the adult bathymetric range these animals do not normally survive; growth in these individuals is reported to be slower suggesting that temperature and/or pressure may be important contributing factors (Gage & Tyler, 1981*a*,*b*; Sumida *et al*., 2000, 2001; Howell *et al*., 2002). Experimental evidence assessing tolerance to hydrostatic pressure indicates that organismal tolerance to the effects of high hydrostatic pressure can vary through ontogeny (George, 1984; Young *et al*., 1997; Tyler & Young, 1998; Tyler *et al*., 2000; Aquino-Souza, 2006; Benitez Villalobos *et al*., 2006; Yoshiki *et al*., 2006, 2008, 2011; Aquino-Souza *et al*., 2008; Smith & Thatje, 2012; Mestre *et al*., 2013). It appears that tolerance increases following early cleavages and subsequently decreases through further life-history stages (e.g. Tyler *et al*., 2000). A similar pattern through ontogeny has been observed for thermal tolerance, attributed to increasing functional and/or structural complexity through development (Pörtner and Farrell, 2008). Aerobic thermal tolerance windows of fish initially increase as diffusion across the cell membrane in early embryonic and larval stages is replaced by ventilation and circulation approaching the juvenile stage, before narrowing again as increasing size increases the difficulty of maintaining oxygen supply (Pörtner & Farrell, 2008). However, variation in environmental tolerance may also reflect changes in cellular responses to environmental extremes (Hamdoun & Epel, 2007), and therefore variation in hyperbaric tolerance through development has been proposed to reflect changes in the cellular stress response as well as in functional and/or structural complexity (Mestre *et al*., 2013). Early hyperbaric intolerance putatively results from the absence of stress response during embryogenesis, with consequent inability to counteract the effects of high hydrostatic pressure resulting in developmental failure. Subsequent increases in hyperbaric tolerance may reflect the increasing ability of

larvae to express a stress response as well as ventilatory and circulatory development (Mestre *et al*., 2013). Later decreases in tolerance are proposed to result from increasing difficulty in maintaining oxygen supply with increasing structural and/or functional complexity and size in the absence of adaptations to high hydrostatic pressure (Mestre *et al*., 2013). This may explain known settlement of several echinoderm species outside the adult bathymetric range (Gage & Tyler, 1981*a*,*b*; Sumida *et al.*, 2000, 2001; Howell *et al*., 2002), although it has been conjectured that these adult distributions may be limited by post-settlement selective forces other than pressure tolerance (e.g. suitability of habitat or food availability; Howell *et al*., 2002). The only investigation of hyperbaric pressure tolerance through embryonic, larval, juvenile and adult life-history stages suggests that such differential tolerances can drive ontogenetic bathymetric migrations in the Antarctic krill *Euphausia superba* (George, 1984). Whilst knowledge of larval tolerance to hydrostatic pressure and/or temperature may be critical to understanding dispersal pathways and may contribute, for example, to theories regarding hydrothermal vent and cold seep colonisation (Tyler & Dixon, 2000; Brooke & Young, 2009; Arellano & Young, 2011), it is clear that studies involving adult organisms are also essential to understanding bathymetric patterns of biodiversity and evolution. Indeed, adult-specific genes have experienced greater positive selection than those expressed in larvae in the urchin *Allocentrotus fragilis* during adaptation to the deep-sea environment (Oliver *et al*., 2010). Hyperbaric experimental studies have focused predominantly on acute exposures using closed isolated systems. However, recent technological development of flow-through pressure vessels has permitted investigation of the effects of sustained pressure over longer periods using both behavioural and molecular approaches (e.g. Cottin *et al*., 2012; New *et al*., 2014). Recent studies have highlighted the importance of holistic investigations evaluating the physiological effects of pressure in a variety of routine behaviours. For example, the metabolic requirements of feeding in the shallow-water crab *Maja brachydactyla* appear to be greater under hyperbaric conditions, potentially critical in restricting bathymetric distributions (Thatje & Robinson, 2011).

Thorough investigation of both temperature and hydrostatic pressure tolerances of adult specimens of shallow-water species are few and focus on crustaceans, but have

also demonstrated tolerance of pressures outside known natural distributions (Naroska, 1968; Menzies & George, 1972; Macdonald & Teal, 1975; George, 1979; Thatje *et al*., 2010; Oliphant *et al*., 2011; Thatje & Robinson, 2011; Cottin *et al*., 2012). For example, the shallow-water shrimp *Palaemonetes varians* tolerates pressures equivalent to 1000 m depth for at least a month and retains the ability to feed and successfully moult at this pressure, despite naturally inhabiting depths of less than 10 m (Cottin *et al*., 2012). Interaction of temperature and pressure effects has also been identified in behavioural and molecular responses of this species, with lower temperature reducing critical pressure tolerance and stimulating a significant molecular stress response at pressure equivalent to 1000 m depth (Oliphant *et al*., 2011; Cottin *et al*., 2012; New *et al*., 2014). Only a single organism-level study has extensively examined the interaction of hydrostatic pressure and temperature effects on a deep-sea species. Respiratory measurements suggest that the 2000 m lower bathymetric limit of the bathyal lysianassoid amphipod *Stephonyx biscayensis* is determined by the combination of low-temperature and high-hydrostatic-pressure effects (Brown & Thatje, 2011). Beyond this maximum depth limit oxygen consumption is significantly reduced, indicating that oxygen supply is functionally limited and suggesting that this restricts the bathymetric range of this species. Seasonal acclimatisation to low temperature appears to increase tolerance to hydrostatic pressure (Naroska, 1968), implying that the requirements for hypothermal and hyperbaric acclimatisation may be congruent, and similar variation in hydrostatic pressure tolerance has been reported for latitudinally distinct populations of species, although genetic variation may contribute to this pattern (Aquino-Souza, 2006). Experimental study of pressure tolerance in the cold-adapted whelk *Buccinum undatum* indicates that long-term acclimation (phenotypic plasticity; Ohlberger, 2013) to low temperature through development can increase hyperbaric resistance (Smith & Thatje, 2012). However, whilst low-temperature acclimation rapidly (<8 days) increases the critical hyperbaric maximum of the shallow-water shrimp *Palaemonetes varians* by ~1.4 MPa, there is no further change over time in contrast to a continuing decrease in critical thermal maximum for at least 78-80 days (New *et al*., 2014). Differing responses to low-temperature acclimation in critical temperature and critical pressure thresholds in the short term (i.e. decreasing temperature tolerance and

increasing pressure tolerance after 8-10 days acclimation) indicate that physiological adjustments during acclimation to low temperature increase pressure tolerance. However, continuing shifts in critical temperature threshold during longer term low-temperature acclimation contrast with constant critical pressure threshold, suggesting that multiple acclimation processes may occur over different timescales during cold acclimation, and that pressure tolerance may only be sensitive to those occurring in the short term (New *et al*., 2014). Although homeoviscous modifications do not occur in the very short term during cold acclimation (e.g. 24 hours; Ronges *et al*., 2012) they can occur over slightly longer timescales (e.g. unsaturation of phospholipid-derived fatty acids over 72 hours; Waagner *et al*., 2013). Elevated hydrostatic pressure reduces membrane fluidity (Hazel & Williams, 1990), impacting transmembrane signalling (Siebenaller & Garrett, 2002) and affecting action potential transmission in nervous cells (Campenot, 1975*a*,*b*), causing loss of equilibrium for example (Oliphant *et al*., 2011). Consequently, homeoviscous acclimation will affect pressure tolerance, and may cause the 1.4 MPa increase in pressure tolerance observed after cold acclimation (New *et al*., 2014). High hydrostatic pressure exposure (10 MPa; 7 days) of low-temperature acclimated shrimp increases critical hyperbaric tolerance of *P*. *varians* by a further 2.2 MPa (New *et al*., 2014). Surprisingly, however, limited research has so far indicated that critical pressure tolerance in bathyal taxa is unaffected by hydrostatic pressure acclimation (Mickel & Childress, 1982*a*; Brown & Thatje, 2011).

Existing studies of hyperbaric pressure tolerance of shallow-water benthic invertebrates consistently indicate limitation at bathyal depths (Fig. 1.2; Table 1.2), coinciding with regions of high species turnover. This pattern is constrained by studies on larval molluscs and echinoderms and adult crustaceans, demanding caution in adopting this as a model for other taxa. However, this model suggests that a physiological bottleneck for deep-sea-colonising shallow-water organisms may contribute to establishing bathymetric zonation. Since the onset of hyperbaric effects can occur at lower pressure at low temperature (Thatje *et al*., 2010; Brown & Thatje, 2011; Oliphant *et al*., 2011; New *et al*., 2014) this may explain decreasingly distinct and blurring bathymetric zonal boundaries with increasing latitude, despite the persistence of such boundaries even in the Antarctic (e.g. Kaiser *et al*., 2011). It is recognised that

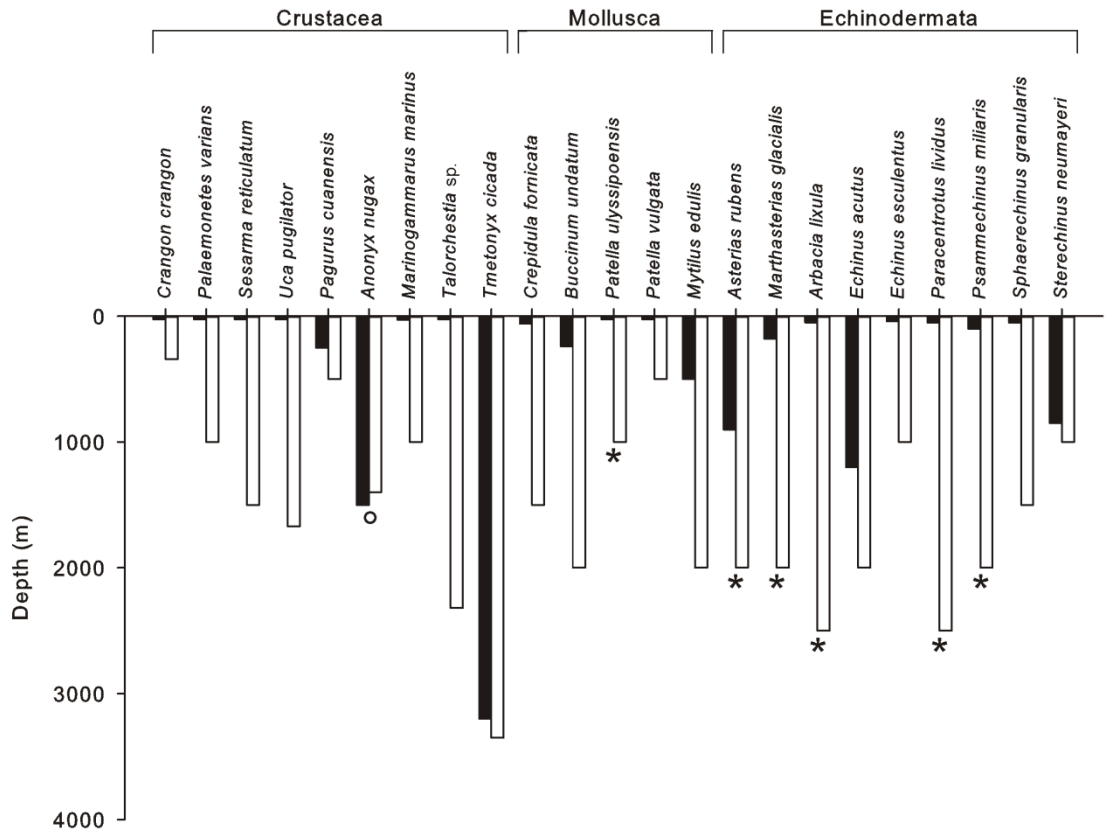

Figure 1.2. Experimentally determined hydrostatic pressure tolerances (white bars) and reported adult bathymetric distributions of shallow-water benthic invertebrate species (black bars). Tolerances presented are for the most developmentally advanced stage examined at ecologically relevant temperatures and are determined by a variety of measures (see Table 1.2 for details). Studies using coarse measures or temperatures not ecologically relevant are excluded. Asterisks indicate tolerance of the highest hydrostatic pressure assessed. °Note that the slight discrepancy in hydrostatic pressure tolerance and adult bathymetric distribution of *Anonyx nugax* is likely to result from the resolution of pressure treatments used to assess hydrostatic pressure tolerance. Maximum tolerance is consistently identified at bathyal pressures, indicating that temperature and hydrostatic pressure equating to these depths may impose a physiological bottleneck at bathyal depths on shallow-water fauna colonising the deep sea following mass extinctions. This coincides with high bathymetric turnover of species, suggesting that the hyperbaric and thermal physiological bottleneck contributes to bathymetric zonation.

challenges across biological scales can drive evolution over variable timescales (e.g. Peck, 2011), and adaptive traits appear to define the limits of species distributions and to affect demographic dynamics significantly (Carnicer *et al*., 2012). It therefore appears likely that invasion of the deep sea by shallow-water taxa must promote adaptation to the effects of the high hydrostatic pressure and low temperature

Table 1.2. Shallow-water benthic invertebrate species assessed for elevated hydrostatic pressure tolerance, indicating the most developmentally advanced ontogenetic stage assessed (A = adult, L = larva, J = juvenile), pressure treatment (pressures assessed, rate of pressurisation and duration of exposure), tolerance measure (B = behaviour, R = respiration, D = development), and maximum pressure treatment tolerated (P_{tol}) at an ecologically relevant temperature (T) determined from bathymetric profiles presented by Locarini *et al*. (2010). Studies using coarse measures or temperatures not ecologically relevant are excluded.

Taxon	Stage	Pressure treatment (MPa)	Measure	P_{tol} (MPa)	T (°C)	Reference
CRUSTACEA						
Crangon crangon	A	0.1 - 20 stepwise (1 6m^{-1})	B	3.4	8	Wilcock *et al*. (1978)
Palaemonetes varians	A	0.1 - 30 stepwise (1 5m^{-1})	B	10	5	Oliphant *et al*. (2011)
Sesarma reticulatum	A	0.1 - 207 ramped (0.98 s^{-1})	B	15	10	Menzies & George (1972)
Uca pugilator	A	0.1 - 207 ramped (0.98 s^{-1})	B	16.7	10	Menzies & George (1972)
Pagurus cuanensis	A	0.1, 2, 5, 10 acute; 1 h	R	5	10	Thatje *et al*. (2010)
Anonyx nugax	A	0.1, 6, 14, 20, 25 ramped (0.4 m^{-1}), 4 h	B, R	14	-1	George (1979)
Marinogammarus marinus	A	0.1 - 40 stepwise (5 5m^{-1})	B	10	3	Macdonald (1972)
Talorchestia sp.	A	0.1 - 207 ramped (0.98 s^{-1})	B	23.2	10	Menzies & George (1972)
Tmetonyx cicada	A	0.1 - 50 stepwise (5 5m^{-1})	B	33.5	6	Macdonald & Gilchrist (1978)
MOLLUSCA						
Crepidula fornicata	L	0.1, 5, 10, 15, 20, 25, 30, 35, 40	B	15	10	Mestre *et al*. (2013)
Buccinum undatum	J	0.1, 10, 20, 30, 40 acute; 4 h	R	20	6	Smith & Thatje (2012)
Patella ulyssipoensis	L	0.1, 5, 10 acute; 24 h	B	10	10	Aquino-Souza (2006)
Patella vulgata	L	0.1, 5, 10 acute; 24 h	B	5	10	Aquino-Souza (2006)
Mytilus edulis	E	0.1, 10, 20, 30, 40, 50 acute; 24 h	D	20	10	Mestre *et al*. (2009)
ECHINODERMATA						
Asterias rubens	L	0.1, 5, 10, 15, 20 acute; 24 h	B	20	5	Benitez Villalobos *et al*. (2006)
Marthasterias glacialis	L	0.1, 5, 10, 15, 20 acute; 24 h	B	20	5	Benitez Villalobos *et al*. (2006)
Arbacia lixula	L	0.1, 5, 15, 25 acute; 20 h	B	25	10	Young *et al*. (1997)
Echinus acutus	L	0.1, 10, 20, 25 acute; 24 h	B	20	4	Tyler & Young (1998)
Echinus esculentus	L	0.1, 10, 20, 25 acute; 24 h	B	10	4	Tyler & Young (1998)
Paracentrotus lividus	L	0.1, 5, 15, 25 acute; 20 h	B	25	10	Young *et al*. (1997)
Psammechinus miliaris	L	0.1, 5, 10, 15, 20 acute; 24 h	B	20	5	Aquino-Souza *et al*. (2008)
Sphaerechinus granularis	L	0.1, 5, 15, 25 acute; 20 h	B	15	10	Young *et al*. (1997)
Sterechinus neumayeri	L	0.1, 5, 10, 15, 20, 25 acute; 24 h	B	10	0.9	Tyler *et al*. (2000)

environmental conditions. Evidence of such adaptation would provide further support for the role of these environmental factors in establishing bathymetric zonation.

1.5 Adaptations to high hydrostatic pressure and low temperature*

The oxygen requirements of cold and deep-living species do not appear to be elevated (Childress *et al*., 1990; Peck & Conway, 2000; Drazen & Seibel, 2007; Seibel & Drazen, 2007) suggesting they are functionally adapted to high hydrostatic pressure and low temperature (Childress, 1995). Adaptation to environmental conditions can be demonstrated through comparison of natural populations of related taxa (see Franks & Hoffman, 2012). Increased mitochondrial concentration, enzyme concentration, adoption of enzymes with greater efficacy at low temperatures, and inclusion of modulator compounds that facilitate enzyme reactions, are all strategies identified in successful adaptation to cold habitats (Hazel, 1995; Clarke, 1998). Enzyme adaptation and modulation has increased in importance in updated cold-adaptation models (Clarke, 1998; Hochachka & Somero, 2002; Somero, 2003). *In vitro* evidence indicates that critical enzyme functionality can be maintained under different pressure and temperature regimes by changes of relatively few amino acids at critical positions in a protein chain, or by the inclusion of stabilizing compounds in the intracellular matrix (Carney, 2005). Indeed, positive selection of genes involved in metabolism is reported in the deep-sea urchin *Allocentrotus fragilis*, in contrast to selection in the shallow-water urchin *Stronglyocentrotus purpuratus* (Oliver *et al*., 2010). Three categories of dehydrogenases have shown functional depth adaptation (Somero, 1998) and the importance of enzyme-stabilising compounds has been confirmed for the osmolyte trimethylamine N-oxide (TMAO), which counteracts the effects of pressure by increasing cell volume (Yancey & Siebenaller, 1999; Samerotte *et al*., 2007). TMAO elevation may be limited by approaching isosmoticity at ~8200 m depth, biochemically constraining marine fish from inhabiting the deepest ocean depths (Yancey *et al*., 2014). Low molecular weight compounds mediating pressure effects have been

* Updated from 'Brown, A. & Thatje, S. (2014). Explaining bathymetric diversity patterns in marine benthic invertebrates and demersal fishes: physiological contributions to adaptation of life at depth. *Biological Reviews* **89**, 406-426'.

reported from a variety of deep-sea fish, invertebrates and microbes (Kelly & Yancey, 1999; Yancey & Siebenaller, 1999; Yin *et al*., 1999, 2000; Yin & Yancey, 2000; Yancey *et al*., 2000, 2002, 2004; Fiess *et al*., 2001, 2002; Martin *et al*., 2002; Siebenaller& Garrett, 2002; Yancey, 2005). Similarly, functional depth adaptation has been identified in cytoskeletal actin and tubulin filaments (Morita, 2003, 2004; Koyama *et al*., 2005) amongst other proteins, with associated increase in thermal stability (Swezey & Somero, 1982). Positive selection of genes involved in skeletal development in the deep-sea urchin *Allocentrotus fragilis* contrasts with selection in the shallow-water urchin *Stronglyocentrotus purpuratus* and may reflect adaptation to environmental effects of the deep-sea (Oliver *et al*., 2010). Adaptive protein sequences appear consistent with selection for high-hydrostatic-pressure and low-temperature structural stability, for example in deep-sea fishes (see Stefanni *et al*., 2014).

The reduced fluidity of bio-membranes under increased hydrostatic pressure and decreased temperature necessitates homeoviscous adaptations in membrane structure and composition (Hazel & Williams, 1990; Balny *et al*., 2002). Accumulation of higher levels of lipid and an increased proportion of unsaturated fatty acids have been observed, counteracting pressure- and temperature-induced decrease in membrane fluidity (White & Somero, 1982; Avrova, 1984; Cossins & Macdonald, 1984; Macdonald, 1984; DeLong & Yayanos, 1985, 1986; Phleger & Laub, 1989; Hazel, 1995; Sébert *et al*., 2004; Pond *et al*., 2014). Membrane fluidity may also be maintained by adjusting concentrations of sterols or proteins (Winter & Dzwolak, 2005). Protein adaptations similar to those proposed in Somero's (2003) descriptive model have been identified in membranes, where shifts in pressure induce changes in transmembrane signalling (Siebenaller & Garrett, 2002; Campanaro *et al*., 2008). Positive selection of genes involved in endo- and exocytosis in the deep-sea urchin *Allocentrotus fragilis* is in contrast to selection in the shallow-water urchin *Stronglyocentrotus purpuratus* and may indicate that membranes or membrane-related functions have undergone environmental selection during deep-sea colonisation (Smith & Stockley, 2005; Oliver *et al*., 2010). Linear relationships between such adaptations and the depth of capture in marine fish, from shallow to >4500 m, have been interpreted as causal evidence for pressure adaptations (Cossins & Macdonald, 1986; Samerotte *et al*., 2007). Theoretical

calculations of homeoviscous adaptation in hadal organisms indicate that this is not simply temperature compensation (Somero, 1992).

Adaptations to high hydrostatic pressure can result in near pressure insensitivity, for example in malate dehydrogenases (see Somero, 1992), but it is not necessarily the case that all adaptations confer this effect. Although it is clear that some bathyal fauna can tolerate recovery from ~2000 m and even flourish at surface pressures for up to several years (e.g. Brown & Thatje, 2011; Smith *et al*., 2013*a*), low-pressure intolerance has been reported for deeper bathyal and abyssal fauna (e.g. Yayanos, 1981; Treude *et al*., 2002; Shillito *et al*. 2006). This suggests that deeper living fauna may display upper bathymetric limits imposed by reduced hydrostatic pressure. These limited data appear to offer further support for a physiological bottleneck at bathyal depths.

Hyperbaric and thermal effects do appear to have made significant adaptive demands on shallow-water organisms colonising the deep sea, further supporting the influence of these factors in establishing bathymetric zonation patterns. However, the physiological mechanism of hyperbaric limitation remains unknown, and consequently there has not been a sufficiently convincing demonstration of constraint of bathymetric distribution by hydrostatic pressure. Successful demonstration of hyperbaric limitation of bathymetric distribution requires experimental investigation of hyperbaric tolerance in a species otherwise adapted to deep-sea conditions.

1.6 The Lithodidae: a model family for hyperbaric investigation

All lithodids share preference for cold-water environments and their shallow-water latitudinal distribution appears to be constrained by the detrimental effects of temperature extremes (Hall & Thatje, 2009). However, phylogenetic evidence supports the hypothesis that lineage-specific physiological thresholds may have differentiated lithodid king crab genera in successful colonisation of the deep-sea (Hall & Thatje, 2009). Recent phylogenetic investigations have revealed the evolutionary history of the anomuran king crab family Lithodidae (Cunningham *et al*., 1992; Hall, 2010; Tsang *et al*., 2011; Bracken-Grissom *et al*., 2013, but see McLaughlin *et al*., 2007). Lithodids appear to have diverged from their most recent hermit crab ancestor approximately 29

Constraint of bathymetric distribution by hydrostatic pressure

MYA (Bracken-Grissom *et al*., 2013), but lithodid phylogeny fossil calibration is low resolution making dating divergences challenging. The only reported lithodid fossils are *Paralomis debodeorum* from ~10 MYA in New Zealand (Feldmann, 1998; Feldmann *et al*., 2006), and an undetermined putative lithodid species from ~16 MYA in Japan (Karasawa & Ohara, 2012). Decapods are underrepresented in the fossil record, particularly deep-water forms (Feldmann, 2003): disarticulation following rapid decomposition of the arthroidal membrane, and mechanical disintegration and dissolution of extremely brittle cuticular elements may cause poor taphonomy (Plotnick *et al*., 1988).

The North Pacific endemic lithodid subfamily Hapalogastrinae are considered morphologically primitive, and 89% of hapalogastrine species occur in shallow water in the eastern part of this region, in contrast to 45% in shallow water in the western part (Zaklan, 2002*a*). Consequently, the north-eastern Pacific coastline is regarded as the evolutionary environment of the incipient lithodid subfamily Lithodinae (Makarov, 1962; Zaklan 2002*a*). All five hapalogastrine genera and six of the 10 genera in the subfamily Lithodinae, which diverged from the hapalogastrines approximately 19 MYA (Bracken-Grissom *et al*., 2013), are restricted to depths shallower than 300 m in the North Pacific (Hall, 2010). The genera occuring deeper than 300 m (*Glyptolithodes*, *Lithodes*, *Neolithodes* and *Paralomis*) appear to result from at least three distinct and relatively rapid radiations (Hall & Thatje, 2009) beginning approximately 15 MYA (inferred from Hall & Thatje, 2009; Hall, 2010; Bracken-Grissom *et al*., 2013) and giving rise to the majority of lithodid diversity (108 of 129 described species; McLaughlin *et al*., 2010). Isothermal submergence into the deep-sea and subsequent dispersal out of the North Pacific and into other water bodies globally is considered to result from ecological adaptation (Makarov, 1962; Zaklan, 2002*a*). Reemergence from deep waters has occurred in the genera *Lithodes* and *Paralomis* (Hall, 2010).

Although the limited bathymetric distribution of the shallow-water lithodid genera hints at a role for hydrostatic pressure in constraint of bathymetric distribution, other factors may contribute to depth restriction of these taxa. Morphological adaptation may be necessary for colonisation of deep continental margins (Morrison *et al*., 2002).

Hapalogastrines are amongst the only anomurans to have undergone full carcinisation whilst retaining a fully uncalcified abdomen (Zaklan, 2002*a*). These crabs inhabit intertidal and shallow subtidal rocky shores, residing in a variety of habitats that afford protection: rocks, crevices, and kelp. The deep sea affords considerably less shelter, and calcification of the abdomen may be a necessary defensive adaptation to avoid predation (Zaklan, 2002*b*). Similarly, the locomotory mechanics of walking legs have adaptive significance: the long legs of deep-sea taxa confer greater efficiency in covering long distances of relatively homogenous seafloor than the short and compact legs of the hapalogastrines, which are more suited to their cryptic habits (Hall, 2010). However, species of the genus *Paralithodes* have similar morphology to deep-water lithodids (calcified abdomen and long legs capable of migrating 13.1 km day^{-1}; Stone *et al*., 1992) yet remain shallow-water taxa (Hall, 2010), suggesting that these adaptations may not be the only requirements for deep-sea living.

The typically differing developmental modes of deep- and shallow-water lineages of king crab may be more important in explaining differences in distribution. Shallow-water lithodids predominantly follow a planktotrophic mode of larval development (Zaklan, 2002*b*), which may restrict these taxa to depths of high planktonic productivity and food availability (Thatje *et al*., 2005*a*). In contrast, deep-water species follow a lecithotrophic developmental mode (Zaklan, 2002*b*), facilitated by enhanced maternal energy investment per offspring and putative energy-saving mechanisms such as abbreviated development, low exuvial losses, and reduced locomotory activity (e.g. Calcagno *et al*., 2003). Lecithotrophy allows independence from seasonal planktonic productivity during development and enables tolerance of protracted development times associated with the low temperatures of the deep-sea environment (Thatje *et al*., 2005*a*). Although development in deep- and shallow-water lithodids appears to offer a parsimonious explanation for lithodid distribution patterns, lecithotrophy appears to have evolved in two *Paralithodes* spp. (inferred from egg size >1.2 mm; Hall 2010). Persisting restriction to shallow water in these species (Hall, 2010) suggests that lecithotrophy may have evolved prior to bathymetric range extension, but also that larval developmental mode may not have been the ultimate bathymetric range limiting factor.

Constraint of bathymetric distribution by hydrostatic pressure

The uncertain cause of bathymetric limitation in shallow-water lithodids precludes these taxa from an unequivocal demonstration of hyperbaric limitation of bathymetric distribution. In contrast, lineages of deep-sea lithodids remain bathymetrically constrained to bathyal depths despite morphological adaptation and lecithotrophic development capable of maintaining reproductive populations at temperatures representative of abyssal depths (Hall and Thatje, 2009). The distribution of deep-water lithodids may, therefore, be limited by hydrostatic pressure.

1.7 Rationale, aim, objectives and hypotheses

The well-constrained evolutionary history and ecology of deep-sea lithodids make these taxa ideal model species for resolving uncertainty surrounding the mechanism limiting hydrostatic pressure tolerance and hyperbaric limitation of bathymetric distribution. However, it is essential that the study species occurs naturally at, or close to, pressures of shallow surface waters to minimise any potential confounding from maintaining specimens in aquaria, outside their natural depth distribution. Although several species of the deep-water lithodid lineage occur in the North Atlantic, only one occurs in the shallow sublittoral (Macpherson, 1988): the bathymetric distribution of the northern stone crab *Lithodes maja* is reported as 4-790 m (Williams, 1984; Macpherson, 1988; see also Zaklan, 2002*b*). *Lithodes maja* is distributed from 40°N along the North American coast to Newfoundland, across to the west and east coasts of Greenland, the coasts of Iceland, and down the Norwegian coast to the British Isles and the Netherlands (Hansen, 1908; Williams, 1984). Single specimens have also been reported further north, off Bear Island and West Spitzbergen (Hansen, 1908). Available metadata support the reported distribution (38-68°N, 20-739 m; OBIS, 2013). Consequently, the initial aim of this study is:

Aim 1: to assess whether *L*. *maja*'s bathymetric distribution is limited by hydrostatic pressure, by

Objective 1A: determining the mechanisms limiting hyperbaric tolerance in *L*. *maja*, and

Objective 1B: determining whether *L*. *maja*'s depth limit coincides with the species' sublethal hyperbaric threshold.

Subsequently, the aim of this study is:

Aim 2: to assess whether the mechanism of hyperbaric limitation is consistent across taxonomic groups, by

Objective 2A: examining variation in hyperbaric tolerance among taxonomic groups of shallow-water fauna, and

Objective 2B: examining correlation between hyperbaric and in hypoxic tolerance thresholds of taxonomic groups of shallow-water fauna.

Specific hypotheses relating to aims 1 and 2 are presented within individual chapters. The final aims of this study are addressed in the Synthesis:

Aim 3: to explore potential mechanisms through which hydrostatic pressure may influence evolution and contribute to bathymetric biodiversity patterns, by

Objective 3: reviewing existing literature examining environmental impacts on evolution and developing a scenario for colonisation of the deep sea;

Aim 4: to examine the implications of oxygen- and capacity-limited hyperbaric tolerance for shifts in species distributions with changing ocean climate, by

Objective 4: constructing a simple conceptual model of oxygen- and capacity-limited species ranges' responses to ocean warming and deoxygenation.

2. Evidence for oxygen- and capacity-limited hyperbaric tolerance and support for bathymetric range limitation by metabolic cost

2.1 Introduction

The role of hydrostatic pressure in constraining the bathymetric distribution of marine organisms remains uncertain. Bathymetric biodiversity patterns on deep continental margins appear driven by a bathyal hyperbaric and hypothermal physiological bottleneck imposed on shallow-water organisms colonising the deep sea following multiple mass extinction events during the Phanerozoic (the last 542 million years) (Brown & Thatje, 2014). However, the mechanism limiting hydrostatic pressure tolerance remains hypothetical, and there has been no unequivocal demonstration of hyperbaric limitation of bathymetric distribution. Recent evidence suggests that the physiological model of oxygen- and capacity-limited thermal tolerance (see Pörtner, 2010, and references cited therein) may be applicable to hyperbaric limitation (see Brown and Thatje, 2014, and references cited therein). This physiological model has been integrated with fundamental principles of energy allocation and trade-offs developed in dynamic energy budget models (Kooijman, 2010) to deliver a conceptual framework of environmental stress tolerance (Sokolova *et al*., 2012; Sokolova, 2013). Such a bioenergetic framework facilitates integration of multiple environmental stressor effects, highly relevant in a changing world (see Todgham & Stillman, 2013, and references cited therein). Critically, the bioenergetic framework links the physiological effects of stressors to population-level consequences in the long term, differentiating moderate environmental stress compatible with sustainable populations from high and extreme stress limiting species distributions (Sokolova *et al*., 2012; Sokolova, 2013), thus resolving potential discrepancies between optimal environmental conditions for physiological performance and optimal environmental conditions for fitness (see Clark *et al*., 2013*b*; Gräns *et al*., 2014). Examining hyperbaric tolerance in the context of this framework may reveal hydrostatic pressure's

contribution to bathymetric range limitation in marine organisms. Based on existing evidence it is proposed that:

Hypothesis 1: hyperbaric tolerance in *Lithodes maja* is proximately oxygen-limited, but ultimately limited by cardiac capacity;

Hypothesis 2: hyperbaric cardiac capacity is limited through hydrostatic pressure effects on membrane effects;

Hypothesis 3: *L. maja*'s depth limit coincides with the species' sublethal hyperbaric threshold.

2.2 Materials and Methods

2.2.1 Sample collection, transfer, and maintenance

Adult specimens of the king crab species *Lithodes maja* were collected from depths of approximately 60 m in Gullmarsfjord, Sweden, by local fishermen using baited traps. Individuals were maintained in an open aquarium system at the Sven Lovén Centre for Marine Sciences – Kristineberg (seawater from a 30 m deep intake: salinity 35, temperature 6-10°C; natural light cycle) for several weeks prior to relocation to the National Oceanography Centre Southampton (NOCS). Only active specimens without extensive necrosis or externally visible parasites were retained and transferred to the NOCS. Animals were starved for 3 days prior to relocation to reduce the potential for adverse impact of specific dynamic action during transport. Each specimen was isolated in a polystyrene box lined with a towel wetted with seawater. Although air exposure may induce oxidative stress (Romero *et al.*, 2007; 2011) and water loss (Urbina *et al.*, 2013), recovery can be relatively rapid in lithodids: antioxidant enzyme activities recovered to control values within 24 hours in *Paralomis granulosa* after 6 hour exposure to dry air at 7°C (Romero *et al.*, 2011). Specimens were transported by temperature-controlled vehicle (6°C; <24 hours) and animals were transferred to a recirculating aquarium at atmospheric pressure (1 µm-filtered seawater: salinity 32.7, temperature 6°C; 24-hour darkness) immediately upon arrival at the NOCS. The 6°C maintenance temperature was selected to match the temperature in the field at the

time of sampling. Animals were fed squid mantle weekly (*Nototodarus sloanii*); excess unconsumed food was removed after 24 hours. These animals continued to flourish under maintenance conditions: feeding, moulting, competition for mates, mating, and larval release and development to juvenile were all observed post-transfer. However, animals for experimental treatments were sampled and transported on several occasions (October, 2011; April, 2012; June, 2013) to reduce the potential for experimental confounding by any adverse effects of long-term aquarium maintenance. In each case transportation survival was >90%. Animals were used in experimental treatments within 18 months of capture.

2.2.2 The IPOCAMP pressurised incubator

The pressurised incubator IPOCAMP (Incubateur Pressurisé pour l'Observation et la Culture d'Animaux Marins Profonds; previously described by Shillito *et al.*, 2001; Ravaux *et al.*, 2003; Shillito *et al.*, 2014) is a 19 l stainless steel flow-through pressure circuit. The IPOCAMP operates as a recirculating system with an external head tank, permitting continuous monitoring of water parameters and maintenance of water quality. The system is filled with 1 µm-filtered seawater (salinity 32.7), and the head tank is bubbled with air continuously to ensure constant oxygen saturation of water supplied to the pressure chamber. Constant experimental temperature is achieved using a temperature regulation unit (CC240, Huber) to circulate ethylene-glycol around the seawater inlet line and through steel jackets surrounding the pressure vessel. Connecting plugs in the wall of the IPOCAMP permit use of sensors within the pressure chamber, for example a photoplethysmographic heartbeat sensor.

2.2.3 Physiological measurements

Metabolic rate is the fundamental biological rate: the rate of energy uptake, transformation, and allocation (Brown *et al.*, 2004). Since energy is obtained by oxidising carbon compounds, aerobic metabolic rate is equivalent to the rate of respiration (Brown *et al.*, 2004). Cardiac activity in malacostracan crustaceans is regulated by extrinsic neuronal and hormonal factors, and has been used previously as a proxy for respiratory capacity in crustaceans and a sensitive indicator of physiological

impairment (e.g. Frederich & Pörtner, 2000). However, respiration is defined by complex dynamics: cardiac activity is only one aspect. Cardiac output is also affected by the amplitude of each beat, which can vary independently of cardiac activity (see McMahon, 1999; Wilkens, 1999). Systemic haemolymph distribution may be altered by selective allocation of cardiac output (see Wilkens, 1999) and the partial pressure gradient driving oxygen diffusion across gaseous exchange sites may be adjusted by modification of haemocyanin oxygen binding properties (Bridges, 2001). Consequently, a direct metabolic measure is often employed as an alternative indicator of environmental tolerance (e.g. Frederich & Pörtner, 2000). Maintenance costs are measured as standard or basal metabolic rate (Guderley & Pörtner, 2010; Kooijman, 2010). Measuring metabolic rate accurately and consistently is challenging (Brown *et al.*, 2004), but the fixed stochiometry of respiratory gas exchange means that it is nearly as accurate and more practical to measure the rate of oxygen consumption (Withers 1992). Further, the ratio of oxygen consumption to cardiac activity may reveal variation in cardiac stroke volume and/or oxygen utilisation coefficient (metabolic rate = cardiac rate × cardiac stroke volume × oxygen utilisation coefficient) (Maynard, 1960; Ansell, 1973). Consequently, both cardiac rate and oxygen consumption were measured during experimental treatments.

L-lactate accumulation in crustacean haemolymph represents increasing reliance on anaerobic metabolism (e.g. Booth *et al*., 1982). Consequently, haemolymph L-lactate accumulation during sustained exposure to moderate stress at hydrostatic pressure approximately equivalent to *L*. *maja*'s depth limit was also measured. Shifts in haemolymph ion concentration reflect adjustments in homeostatic activity (e.g. Wittmann *et al*., 2012) and may therefore indicate changes in homeostatic effort, but may also reveal homeostatic mechanisms. Therefore, haemolymph ion concentration during these treatments was measured too. Vertical integration of physiological processes across organisational levels, a current challenge in comparative physiology (Mykles *et al.*, 2010), may deliver insight into the mechanisms underlying responses to stressors (e.g. Morris *et al*., 2015). Consequently, transcription was examined to reveal both cellular challenges and responses during hyperbaric exposures, and to identify potential critical hyperbaric effects modulating systemic responses. Transcription

analysis was conducted by J. P. Morris and is therefore presented in Appendix 1:the interpretation of transcription data was developed entirely independently.

2.2.4 Measuring cardiac activity and oxygen consumption

Movements of the cardiac muscle were recorded using the photoplethysmographic technique (as precise and accurate as the impedance method and noninvasive; Burnett *et al.*, 2013) adapted for hyperbaric use (Robinson *et al.*, 2009). The heartbeat sensor was attached to the carapace over the cardiac region. Lithodids have an incompletely closed circulatory system (see Reiber & McGaw, 2009, for definition) with a large single chambered heart located in the centre of the body (McGaw & Duff, 2008; Keiler *et al*., 2013). The photoplethysmographic sensor emits infrared light, which penetrates the carapace and is backscattered by the underlying heart tissue. The direction and intensity of the backscatter depend on the shape of the tissue, and the sensor detects variation in backscatter. Any change in backscatter reflects the contraction and relaxation of the cardiac muscle. The raw signal was recorded at a sampling rate of 1000 min^{-1}. The signal was converted into voltage and passed to a voltage amplifier (Electrode Amplifier, Vernier), before the analogue signal was digitalised by the *LabPro* system (Vernier), and recorded and interpreted using *LoggerPro* software (version 3.4.2, Vernier).

Oxygen consumption was measured using the depletion of oxygen from water flowing through the pressure vessel. Oxygen saturation of the water was recorded as it passed out of the pressure vessel using an oxygen microoptode connected to a Microx TX3 array (PreSens) at a sampling rate of 1 s^{-1} (Thatje & Robinson, 2011). The rate of oxygen consumption was obtained by comparison with the oxygen saturation of the water entering the pressure vessel, which remains stable over time (Thatje & Robinson, 2011). Molar oxygen consumption was calculated using Benson & Krause's (1984) formula for determining air-saturated seawater's oxygen concentration.

Cardiac activity and oxygen consumption were recorded over the duration of the treatment, but the large quantity of data collected made it necessary to interrupt recording briefly (<10 min for cardiac activity; <1 min for oxygen consumption) every

12 hours (09:00 and 21:00) to save data and reset software. Oxygen saturation did not fall below 75% (= 236.9 μmol O_2 l^{-1}) during any treatment, reducing the potential for any hypoxic exposure effect (McMahon, 2001; Vaquer-Sunyer & Duarte, 2008).

2.2.5 Experimental protocols

2.2.5.1 Hyperbaric tolerance

Stepped hydrostatic pressure exposures were conducted across and beyond the range equivalent to *L*. *maja*'s bathymetric distribution to determine the mechanism of hyperbaric limitation and assess tolerance to hydrostatic pressure. The experimental temperature (6°C) was selected to approximate the temperature of *L*. *maja*'s deepest recorded occurrence (6.6°C at 739 m depth; OBIS, 2013). The experimental temperature also represents the deep-sea temperature at the proposed time of lithodid deep-sea radiation ~15 MYA (inferred from Hall & Thatje, 2009; Hall, 2010; Cramer *et al.*, 2011; Bracken-Grissom *et al.*, 2013) and is therefore within the thermal adaptive history of the genus, which may be key to understanding adaptive trajectories from shallow- to deep-living (New *et al.*, 2014).

Experimental treatments were conducted following a minimum of 2 months exposure to experimental temperature to reduce potential impact of pre-capture temperature conditions. Five animals were exposed to control treatments and five to pressure treatments; animals were not reused. *L*. *maja*'s reported distribution is likely informed by adult occurences, and sex and reproductive state may influence temperature and/or hydrostatic pressure tolerance (George, 1984; Madeira *et al*., 2012). Putatively lower gametogenic energetic cost incurred in males suggests that physiologically-defined distributional limits may therefore represent adult male tolerances. Consequently, for each treatment a single mature male was selected at random. Maturity was assessed as >55 mm carapace length (CL; measured from the baseline of the orbit to the posterior edge of the carapace excluding any protruding crests or spines; Macpherson, 1988; Hall, 2010) based on the 0.87 male to female CL ratio for gonadal maturity established in the congeneric species *Lithodes santolla* (Vinuesa, 1984), and on personal observation of gonadal maturity in 100% of female *L*. *maja* at

≤65 mm CL through dissection or post-moult oviposition (*n* = 5): female CL at first maturity may be smaller than 37 mm in *L*. *maja* (Squires, 1990). The animal was cleaned of encrusting detritus and epibiota to minimise potential inaccuracies in oxygen consumption measurement. The animal was starved for 7 days prior to experimental treatment to minimise the potential for reduced hydrostatic pressure tolerance resulting from specific dynamic action effects (see Thatje & Robinson, 2011); production of faecal matter ceased by 4 days post-feeding. Carapace length was measured to the nearest 0.05 mm using vernier calipers. Total wet mass was determined to the nearest 0.1 g using an SG-5001 balance (Fisher Scientific): external body cavities (i.e. gill chambers) were drained by holding the animal perpendicular to the ground anterior downward for 1 minute, and the animal was blotted dry. A heartbeat sensor adapted for use in the IPOCAMP system under hydrostatic pressure (Robinson *et al.*, 2009) was attached to the carapace over the cardiac region (Fig. 2.1) using dental wax and cyanoacrylate glue (*Loctite*, Henkel). The animal was isolated in the IPOCAMP pressure chamber at 10:30; the IPOCAMP was run for at least 24 hours prior to treatments to ensure the system was fully temperature acclimated and oxygen saturation was stable. For control treatments, 0.1 MPa was maintained for 240 hours following introduction of the animal to the pressure vessel (Fig. 2.2). In pressure treatments 0.1 MPa was maintained for 24 hours (Fig. 2.2), to allow acclimation and recovery from potential handling stress. Pressure was then increased stepwise to 2.5 MPa at a rate of 0.5 MPa 10 min^{-1}. Further stepwise 2.5 MPa increases were made at the same rate daily at 10:30, to a maximum pressure of 20.0 MPa. Hydrostatic pressure levels were selected to represent *L*. *maja*'s bathymetric distribution (0.1 MPa – 7.5 MPa) and beyond (≥10.0 MPa). In both control and pressure treatments 24-hour darkness was maintained to simulate deep-sea light conditions and minimise light-dependent circadian physiological cyclicity. After a further 24 hours, pressure was decreased to 0.1 MPa at a rate of 1.0 MPa 5 min^{-1}, and maintained to the end of the treatment (total treatment duration = 240 hours). Both temperature and pressure within the system were recorded using a temperature/pressure logger (SP2T4000, NKE Instrumentation). Individuals from both treatments were removed from the IPOCAMP system, returned directly to maintenance conditions, and observed daily for any subsequent behavioural changes or mortality.

Constraint of bathymetric distribution by hydrostatic pressure

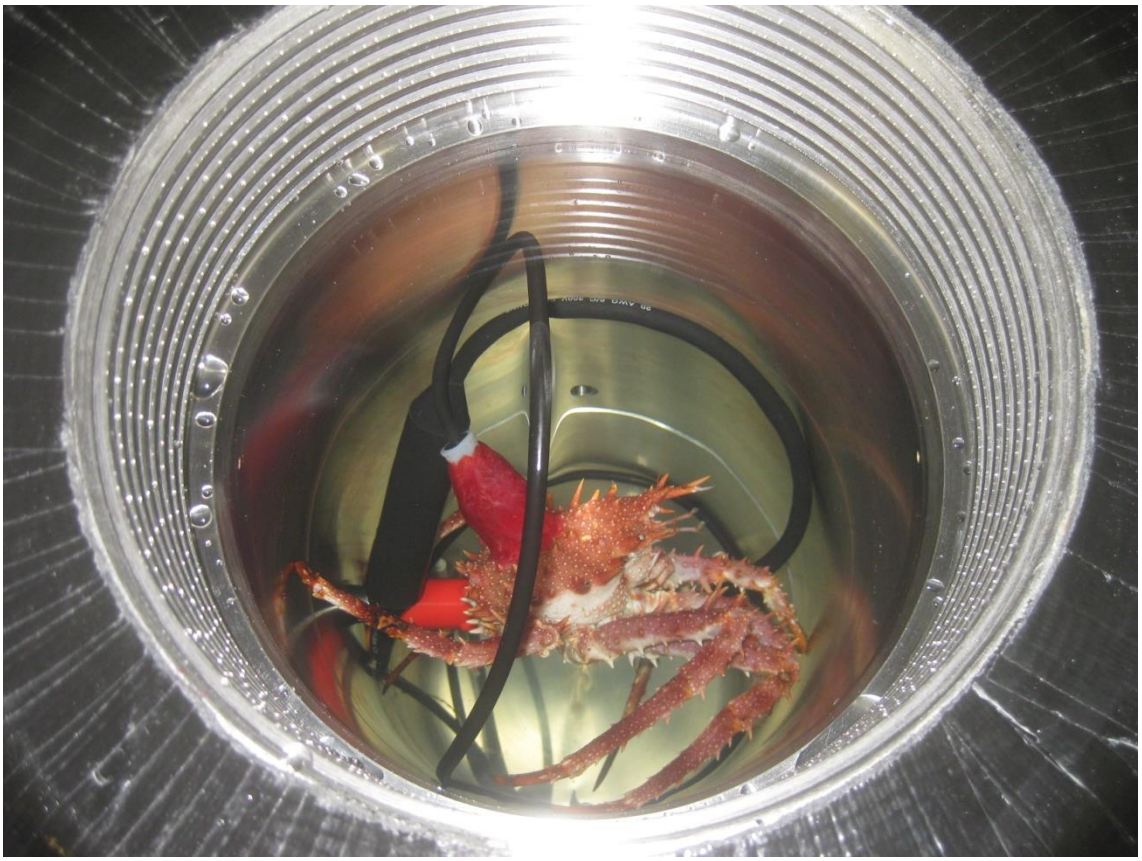

Figure 2.1. *Lithodes maja* inside the IPOCAMP hyperbaric aquaria (internal diameter 20 cm; internal depth ~60 cm), with a photoplethysmographic heartbeat sensor attached over the cardiac region.

2.2.5.2 Sustained hyperbaric tolerance

Analysis of hyperbaric tolerance data indicated transition to moderate stress above 5.0 MPa and transition to extreme stress above 12.5 MPa (see Discussion). Consequently, further hyperbaric tolerance exposures were conducted at 12.5 MPa, 10.0 MPa, and 7.5 MPa to assess sustained hyperbaric tolerance, and to identify the contribution of hydrostatic pressure to bathymetric range limitation. Five animals were exposed to each pressure treatment; animals were not reused. The sustained hyperbaric tolerance protocol was a modification of the hyperbaric tolerance protocol to permit reuse of control treatment data from the hyperbaric tolerance experiment (Fig. 2.3). 0.1 MPa was maintained for 24 hours following introduction of the animal to the pressure vessel, to allow acclimation and recovery from potential handling stress. Pressure was then increased stepwise to experimental pressure (12.5 MPa, 10.0 MPa, or 7.5 MPa) at

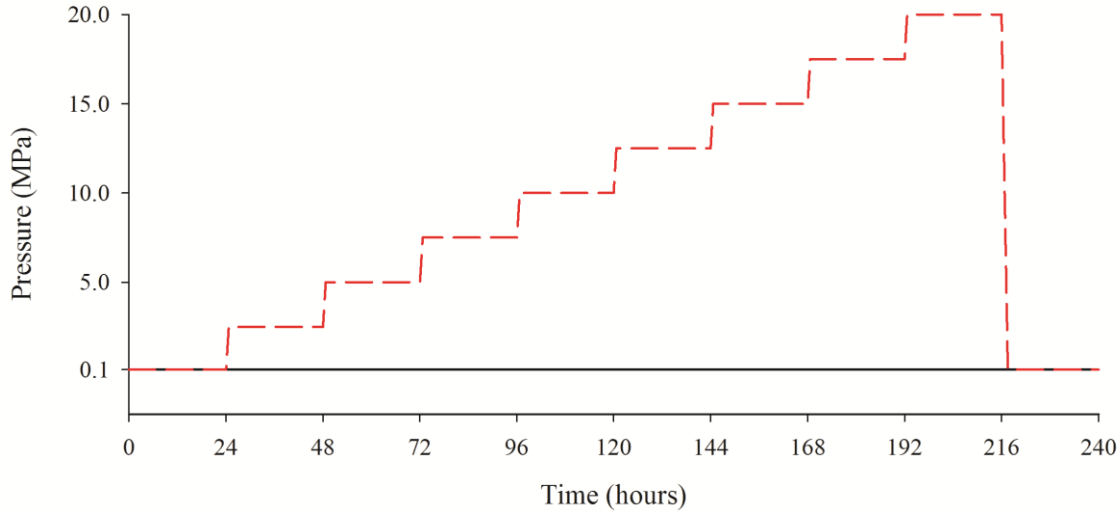

Figure 2.2. Schematic overview of 240-hour pressure regimes used to assess hyperbaric tolerance in adult male *Lithodes maja*; 0.1 MPa control treatment in black, pressure treatment in red. Incremental steps in pressure change are indicated as a straight line due to scale.

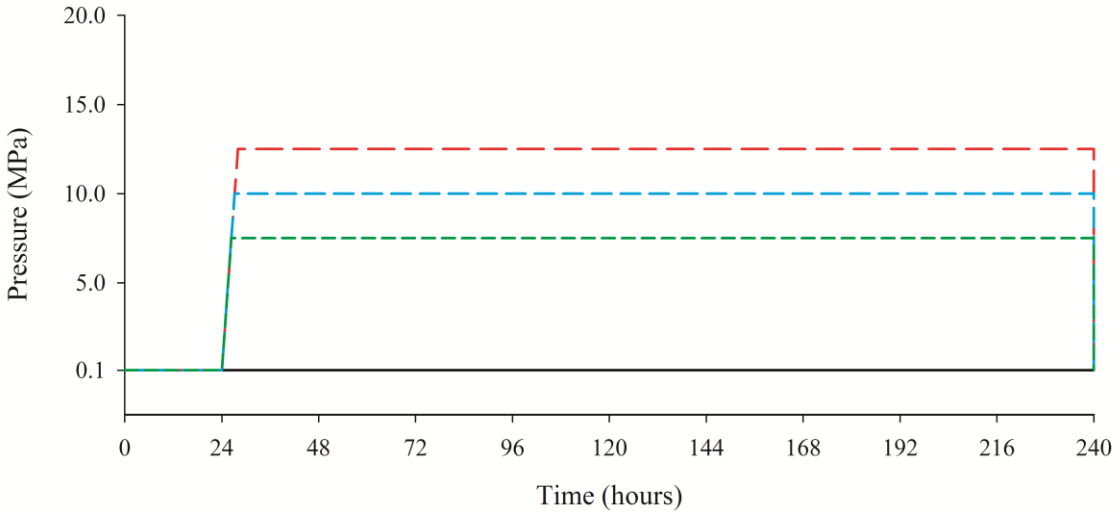

Figure 2.3. Schematic overview of 240-hour pressure regimes used to assess sustained hyperbaric tolerance in adult male *Lithodes maja*; 0.1 MPa control treatment in black, 7.5 MPa treatment in green, 10.0 MPa treatment in blue; 12.5 MPa treatment in red. Incremental steps in pressure change are indicated as a straight line due to scale.

a rate of 0.5 MPa 10 min^{-1}, and maintained to the end of the treatment (total treatment duration = 240 hours). Pressure was returned to ambient over a period of one minute. Individuals were removed from the IPOCAMP system and tissue was

sampled for subsequent analysis of gene expression: the 3rd right pereopod was severed at the coxal end of the merus, tissue was transferred to a 1.5 ml centrifuge tube, and snap frozen in liquid nitrogen within 10 minutes of departure from experimental pressure. Short depressurisation period and rapid preservation of samples were employed to minimise introduction of depressurisation artefacts and to minimise transcriptional recovery (Morris *et al.*, 2015). Once frozen, tissue was preserved at -80°C until analysed (see Appendix 1). Cardiac activity and oxygen consumption data from the hyperbaric tolerance 0.1 MPa control treatment were available for direct comparison. Consequently, tissue samples for sustained hyperbaric tolerance 0.1 MPa control treatments were generated following the sustained hyperabaric tolerance protocol in an identical alternative IPOCAMP system. Although cardiac activity and oxygen consumption were not monitored in the sustained hyperbaric tolerance control treatment due to limited equipment availability (i.e. only one Vernier *LabPro* system was available), a heartbeat sensor was attached to the carapace as previously described to minimise the potential for treatment artefacts. Accurate temperature and hydrostatic pressure calibration of the alternative IPOCAMP system were confirmed using a temperature/pressure logger (SP2T4000, NKE Instrumentation).

Haemolymph was also sampled from sustained hyperbaric tolerance treated animals for L-lactate and ion analyses: a sterile ice-cooled 23g hypodermic needle (*Neolus*, Terumo) was inserted though the 3rd left coxal arthroidal membrane and ~0.5 ml haemolymph was withdrawn using a sterile 2 ml syringe (*Plastipak*, BD). Haemolymph samples were transferred to sterile 0.3 ml centrifuge vials and frozen at -20°C until analysed. Haemolymph was successfully sampled from 4 individuals following 0.1 MPa control treatments and 3 individuals following 7.5 MPa treatments. Individuals from all treatments were returned to maintenance conditions and observed daily for any subsequent behavioural changes or mortality.

2.2.5.3 Hyperbaric acclimation

Analysis of sustained hyperbaric tolerance cardiac activity and oxygen consumption data indicated a transient decrease in cardiac activity and sustained moderate stress at

7.5 MPa (see Results and Discussion). Consequently, shorter duration hyperbaric exposures were conducted at 7.5 MPa to assess potential shifts in gene expression and haemolymph composition during hyperbaric acclimation. Hyperbaric acclimation treatments were conducted in the alternative IPOCAMP system. Five animals were exposed to control treatments and five to pressure treatments; animals were not reused. The hyperbaric acclimation protocol was a modification of the sustained hyperbaric tolerance protocol to permit comparison of tissue and haemolymph samples. A heartbeat sensor was attached to the carapace despite no cardiac activity or oxygen consumption data being recorded, to minimise the potential for treatment artefacts. As in earlier treatments, 0.1 MPa was maintained for 24 hours following introduction of the animal to the pressure vessel, to allow acclimation and recovery from potential handling stress. Pressure was then increased stepwise to experimental pressure (7.5 MPa) at a rate of 0.5 MPa 10 min^{-1} and maintained for 4 or 24 hours, or maintained at 0.1 MPa for 6 hours 20 minutes or 26 hours 20 minutes (control treatments), achieving consistent sampling time and eliminating potential confounding from diel cyclic variability (Fig. 2.4). Treatments were terminated, and tissue and haemolymph sampled for subsequent analysis following the sustained hyperbaric tolerance protocol; haemolymph was successfully sampled from all individuals.

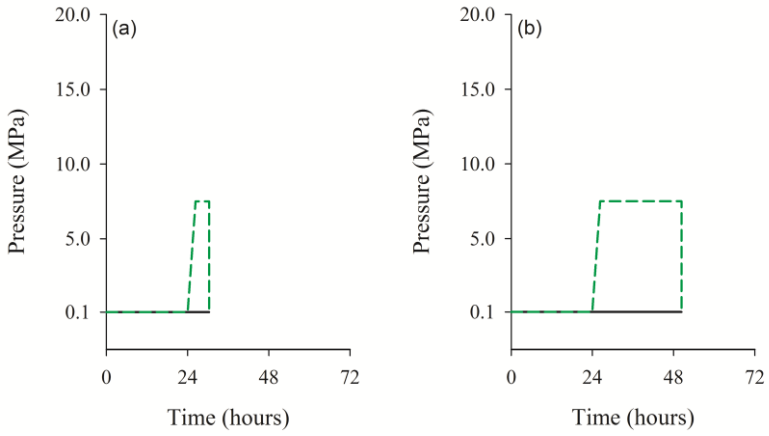

Figure 2.4. Schematic overview of (a) 4-hour and (b) 24-hour pressure regimes used to assess hyperbaric acclimation in adult male *Lithodes maja*; 0.1 MPa control treatments in black, 7.5 MPa treatments in green. Incremental steps in pressure change are indicated as a straight line due to scale.

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Individuals from all treatments were returned to maintenance conditions and observed daily for any subsequent behavioural changes or mortality.

2.2.6 Cardiac activity and oxygen consumption data normalisation and statistical analysis

Means were calculated for cardiac activity and mass-specific molar oxygen consumption for each 1 hour period of the treatment. Data were normalised to the mean mass of experimental subjects (464.5 g) to compensate for significant mass-dependent variation in cardiac activity and mass-specific molar oxygen consumption (non-linear regression using a power function: $F_{1,23} = 4.362$, $p = 0.048$ and $F_{1,23} = 9.676$, $p = 0.049$ respectively). Mass-scaling functions were derived using data from all specimens at 0.1 MPa over the initial 24 hours of treatments ($n = 25$), assuming intraspecific mass scaling follows a power function (see Glazier 2005). Both cardiac activity and mass-specific molar oxygen consumption mass-scaling exponents (-0.232 and -0.595 respectively) were within the range reported elsewhere (see West & Brown, 2005; Glazier, 2005). Linear regression of mass-normalised cardiac activity (CA) and mass-normalised mass-specific molar oxygen consumption (MO_2) indicated mass independence ($F_{1,23} = 0.000$, $p = 0.987$ and $F_{1,23} = 0.029$, $p = 0.867$ respectively).

Clear daily rhythms were evident in CA and MO_2 in control treatments, limiting comparisons between treatments to corresponding periods. To integrate diel variation into the statistical analysis, means were calculated for the period at which all treatments were at experimental pressure, i.e. the last 22 hours of each 24-hour period for hyperbaric tolerance treatments, and the last 18 hours of each 24-hour period for sustained hyperbaric tolerance treatments. CA means for hyperbaric tolerance and sustained hyperbaric tolerance were normally distributed and homoscedastic (Shapiro-Wilk test, $p = 0.187$ and $p = 0.201$; Levene's test, $p = 0.900$ and $p = 0.272$). MO_2 means for hyperbaric tolerance and sustained hyperbaric tolerance were square-root transformed to achieve normality and homoscedasticity (Shapiro-Wilk test, $p = 0.053$ and $p = 0.179$; Levene's test, $p = 0.250$ and $p = 0.598$). MO_2:CA means for hyperbaric tolerance and sustained hyperbaric tolerance were fourth-root transformed to achieve normality and homoscedasticity (Shapiro-Wilk test, $p = 0.097$

and $p = 0.148$; Levene's test, $p = 0.222$ and $p = 0.231$). The effect of hydrostatic pressure on CA, MO_2, and MO_2:CA was assessed using two-way repeated measures analysis of variance (ANOVA), with treatment (control or hydrostatic pressure) and time (proxy for pressure in pressure treatments) as fixed factors ($\alpha = 0.05$). The post-hoc Holm-Sidak multiple comparisons test was used to determine significant differences from the initial 0.1 MPa period within both control and pressure treatments, and significant differences between control and pressure treatments during corresponding periods.

2.2.7 Haemolymph L-lactate concentration measurement and statistical analysis

10 µl haemolymph subsamples were deproteinized by addition of 10 µl 0.6 M perchloric acid. Subsamples were subsequently neutralised by addition of 2.5 M potassium carbonate. The denatured sample was centrifuged at 10,000 g for 20 minutes at 4°C and the supernatant removed for use in the L-lactate assay. Haemolymph L-lactate concentration was determined in a microplate reader at 552 nm absorbance (Lactate assay kit no. 735, Trinity Biotech; FLUOstar OPTIMA, BMG Labtech).

L-lactate concentrations were normal and homoscedastic (Shapiro-Wilk test, $p > 0.05$; Levene's test, $p > 0.05$). The effect of hydrostatic pressure on L-lactate concentration was assessed using two-way ANOVA, with treatment (control or hydrostatic pressure) and exposure duration as fixed factors.

2.2.8 Haemolymph ion concentration determination and statistical analysis

100 µl haemolymph subsamples were deproteinised in concentrated sub-boiled nitric acid to break down metal-protein bonds, dried, and then dissolved in 0.45 M sub-boiled nitric acid. Subsamples were filtered through 0.2 µm syringe filters, then analysed using an ICP-OES (Optima 4300DV, Perkin Elmer), calibrated using synthetic standards. Instrumental drift was assessed every 10 samples and corrections were applied subsequently.

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Haemolymph ion concentrations were normal and homoscedastic (Na^+, K^+, Ca^{2+}, Cu^{2+}, Shapiro-Wilk test, $p > 0.05$; Levene's test, $p > 0.05$) or were square-root transformed to achieve normality and homoscedasticity (Mg^{2+}, Mn^{2+}, B^{3+}; Shapiro-Wilk test, $p > 0.05$; Levene's test, $p > 0.05$). The effect of hydrostatic pressure on ion concentration was assessed using two-way ANOVA, with treatment (control or hydrostatic pressure) and exposure duration as fixed factors. The post-hoc Holm-Sidak multiple comparisons test was used to identify which treatments differed significantly.

2.3 Results

Experimental individuals' carapace length ranged from 56.0 mm to 102.5 mm and total wet mass ranged from 231.5 g to 841.7 g. The mean mass of individuals used in each experimental treatment ($n = 5$) did not vary significantly (one way ANOVA: $F_{4,20} = 0.668$, $p = 0.622$; data were normally distributed and homoscedastic: Shapiro-Wilk test, $p = 0.994$; Levene's test, $p = 0.141$).

2.3.1 Survival

All individuals survived the duration of the hyperbaric tolerance exposures and survived ≥3 months post-treatment. Individuals exposed to the control treatment resumed normal behaviour (e.g. feeding) immediately upon return to the aquarium. In contrast, individuals exposed to the pressure treatment were quiescent for several days following treatment before resuming normal activity. Individuals from both treatments later moulted and engaged in mating. All individuals survived the duration of the sustained hyperbaric tolerance treatments too, but post-treatment survival following tissue sampling varied: all individuals exposed to 0.1 MPa control and 7.5 MPa pressure treatments survived; 3 individuals exposed to the 10.0 MPa pressure treatment survived; no individuals exposed to the 12.5 MPa pressure treatment survived. All individuals survived the duration of the hyperbaric acclimation treatments as well, and survival following tissue sampling again varied: all individuals exposed to 4-hour and 24-hour 0.1 MPa treatments survived; no individuals exposed to 4-hour and 24-hour 7.5 MPa treatments survived.

2.3.2 Hyperbaric tolerance

The effect of time (proxy for pressure in pressure treatments) on CA, MO_2, and MO_2:CA depended on treatment (Figs. 2.5, 2.6; $F_{9,72} = 9.374$, $p < 0.001$; $F_{9,72} = 5.669$, $p < 0.001$; $F_{9,72} = 8.883$, $p < 0.001$). In the control treatment, mean CA, MO_2, and MO_2:CA remained approximately constant over time. In contrast, in the pressure treatment mean CA, MO_2, and MO_2:CA differed among periods.

Mean CA in the pressure treatment was significantly lower at pressures ≥10.0 MPa than during corresponding periods in the control treatment; mean CA decreased with each pressure increase. Rhythms evident in control treatment CA appeared reduced in the pressure treatment at 5.0 and 7.5 MPa, and were not apparent at pressures ≥10.0 MPa. Variability in CA appeared reduced at pressures of ≥12.5 MPa. Rhythms did not recommence after depressurisation to 0.1 MPa. CA increased rapidly after depressurisation and continued to increase over time; mean CA was significantly greater than during the corresponding period in the control treatment.

Mean MO_2 in the pressure treatment was significantly greater at pressures of 7.5 MPa to 17.5 MPa than during corresponding periods in the control treatment; mean MO_2 increased with each pressure increase to 12.5 MPa, before decreasing with each pressure increase to 20.0 MPa. Rhythms evident in control treatment MO_2 appeared reduced in the pressure treatment at 5.0 MPa, and were not apparent at pressures ≥7.5 MPa. At 17.5 MPa MO_2 appeared to decline initially before rising again, and at 20.0 MPa MO_2 appeared to decline over time. Rhythms did not recommence after depressurisation to 0.1 MPa. MO_2 rose rapidly after decompression but declined over time; mean MO_2 was not significantly different from the corresponding period in the control treatment.

Mean MO_2:CA in the pressure treatment increased was significantly greater at pressures ≥7.5 MPa than during corresponding periods in the control treatment; mean MO_2:CA increased with each pressure increase to 12.5 MPa, before decreasing with each pressure increase to 20.0 MPa. Variability in MO_2:CA appeared increased at pressures ≥5.0 MPa. MO_2:CA rose rapidly after decompression to 0.1 MPa but declined

Constraint of bathymetric distribution by hydrostatic pressure

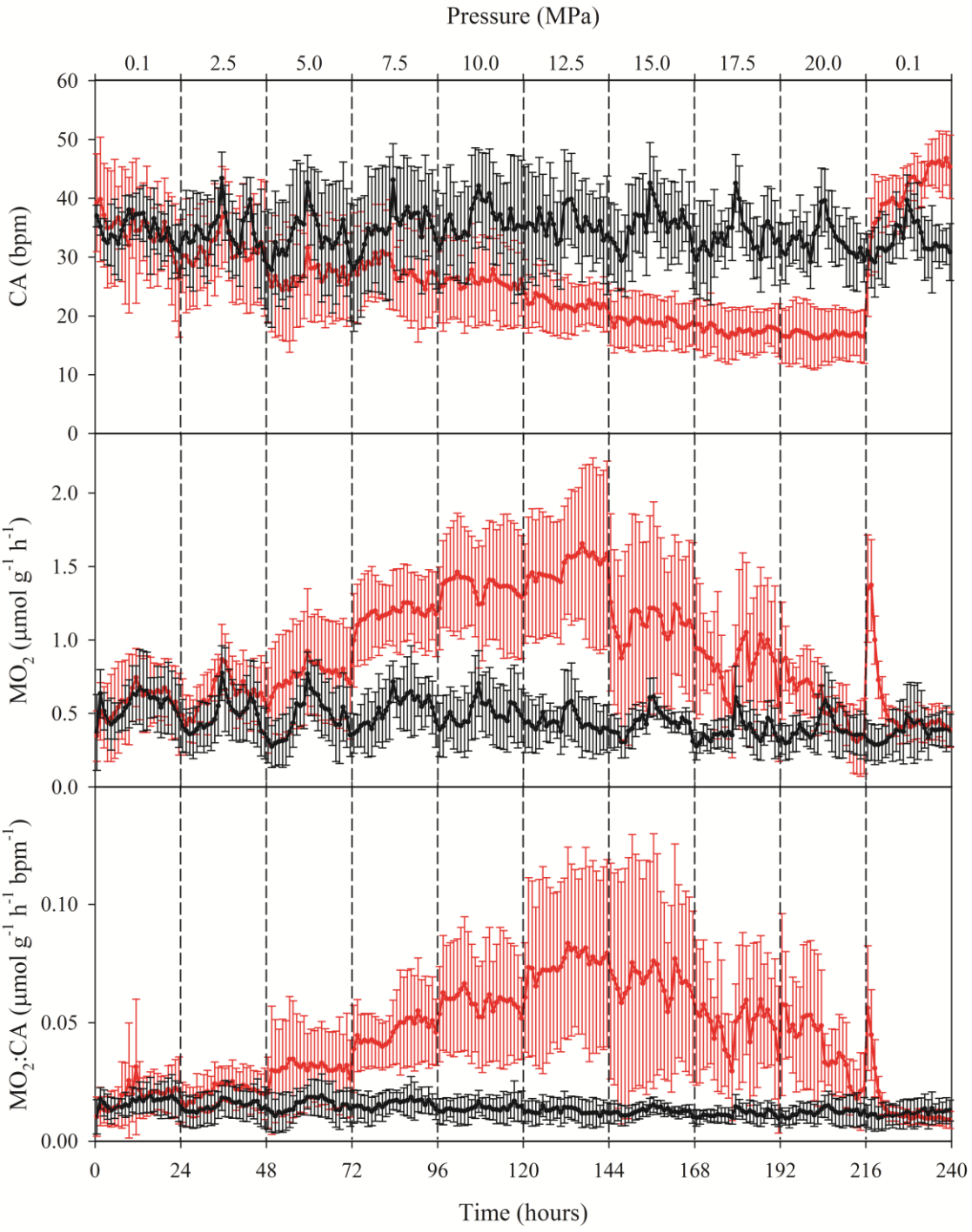

Figure 2.5. Effect of hydrostatic pressure on mean mass-normalised cardiac activity (CA), mean mass-normalised mass-specific molar oxygen consumption (MO_2), and mean MO_2:CA of adult male *Lithodes maja* at 6°C; 0.1 MPa control treatment in black, pressure treatment in red (n = 5). Error bars represent 95% confidence intervals.

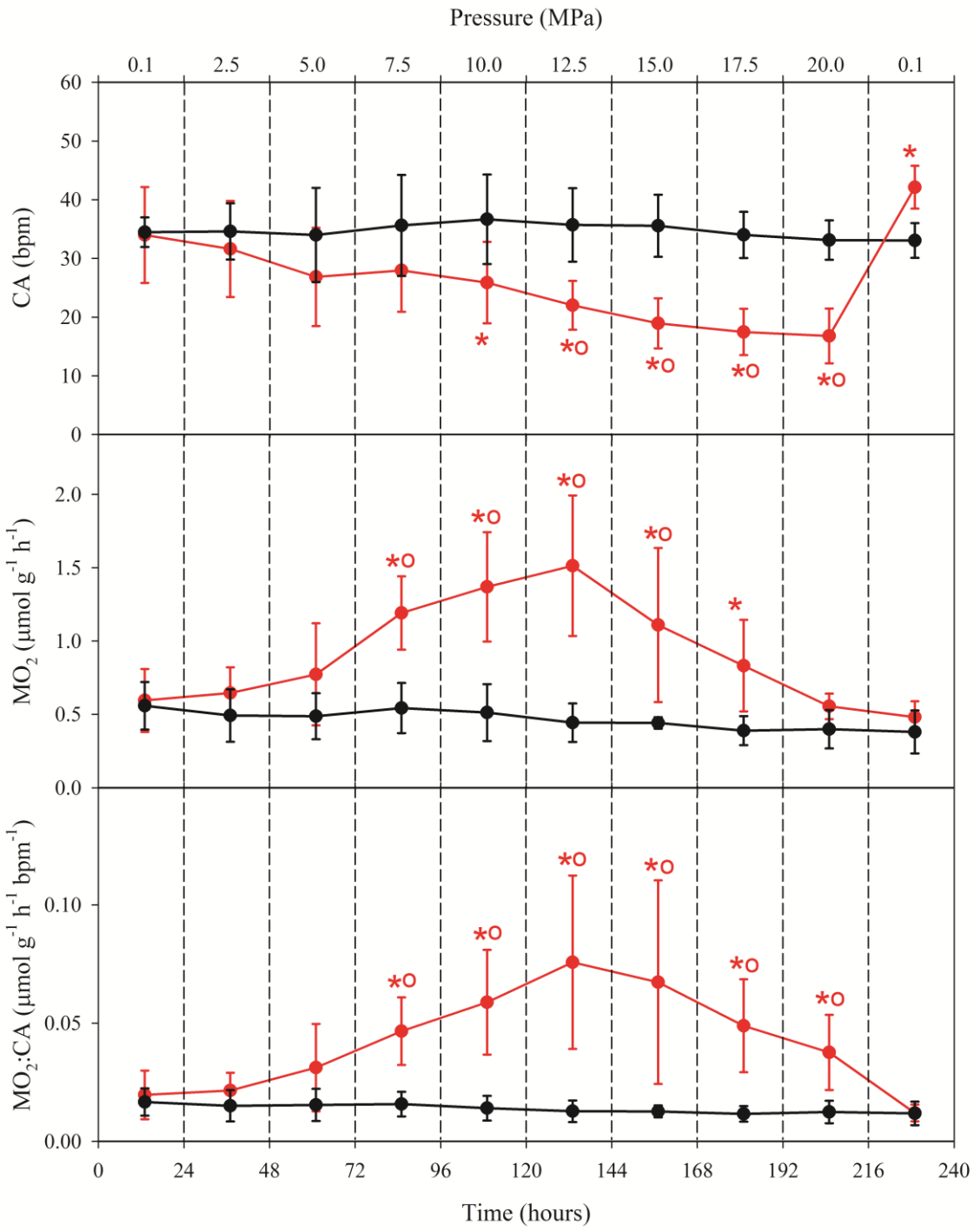

Figure 2.6. Effect of hydrostatic pressure on time-period mean mass-normalised cardiac activity (CA), mean mass-normalised mass-specific molar oxygen consumption (MO_2), and mean MO_2:CA of adult male *Lithodes maja* at 6°C; 0.1 MPa control treatment in black, pressure treatment in red (n = 5). Error bars represent 95% confidence intervals. Significant difference from the same period in the control treatment is indicated by an asterisk. Significant difference from the initial 24-hour period of a treatment (= 0.1 MPa in pressure treatment) is indicated by an open circle; symbol colour reflects treatment.

over time; mean MO_2:CA was not significantly different from the corresponding period in the control treatment.

2.3.3 Sustained hyperbaric tolerance

The effect of time (proxy for pressure in the pressure treatment) on CA, MO_2, and MO_2:CA depended on treatment (Figs. 2.7, 2.8; $F_{27,144} = 3.557$, $p < 0.001$; $F_{27,144} = 4.647$, $p < 0.001$; $F_{27,144} = 7.327$, $p < 0.001$). In the control treatment, mean CA, mean MO_2, and mean MO_2:CA remained approximately constant over time. In contrast, mean CA demonstrated a sustained significant decrease at 12.5 MPa, and a transient significant decrease at 10.0 MPa and at 7.5 MPa. Mean MO_2 and mean MO_2:CA demonstrated a sustained significant increase in all pressure treatments.

2.3.4 Hyperbaric acclimation

The effects of sustained hydrostatic pressure and exposure duration varied among haemolymph components. Haemolymph L-lactate concentration was not significantly affected by hydrostatic pressure or exposure duration (Fig. 2.9; $F_{2,23} = 0.018$, $p = 0.982$). Similarly, haemolymph $[Na^+]$, $[K^+]$, $[Mg^{2+}]$, $[Ca^{2+}]$, and $[Cu^{2+}]$ were not significantly affected by hydrostatic pressure or duration (Fig. 2.10; $p > 0.05$), despite the appearance of lower haemolymph concentrations of these ions after 4 hours exposure to 7.5 MPa. In contrast, haemolymph $[Mn^{2+}]$ was significantly lower at 7.5 MPa ($F_{1,21} = 12.084$, $p = 0.002$), and haemolymph $[B^{3+}]$ was significantly greater at 7.5 MPa ($F_{1,21} = 4.913$, $p = 0.038$). Although statistical analysis indicated that the effect of pressure on haemolymph $[B^{3+}]$ did not depend on exposure duration ($F_{2,21} = 3.437$, $p = 0.051$) the statistic was marginal, suggesting that haemolymph $[B^{3+}]$ may increase with 7.5 MPa exposure duration.

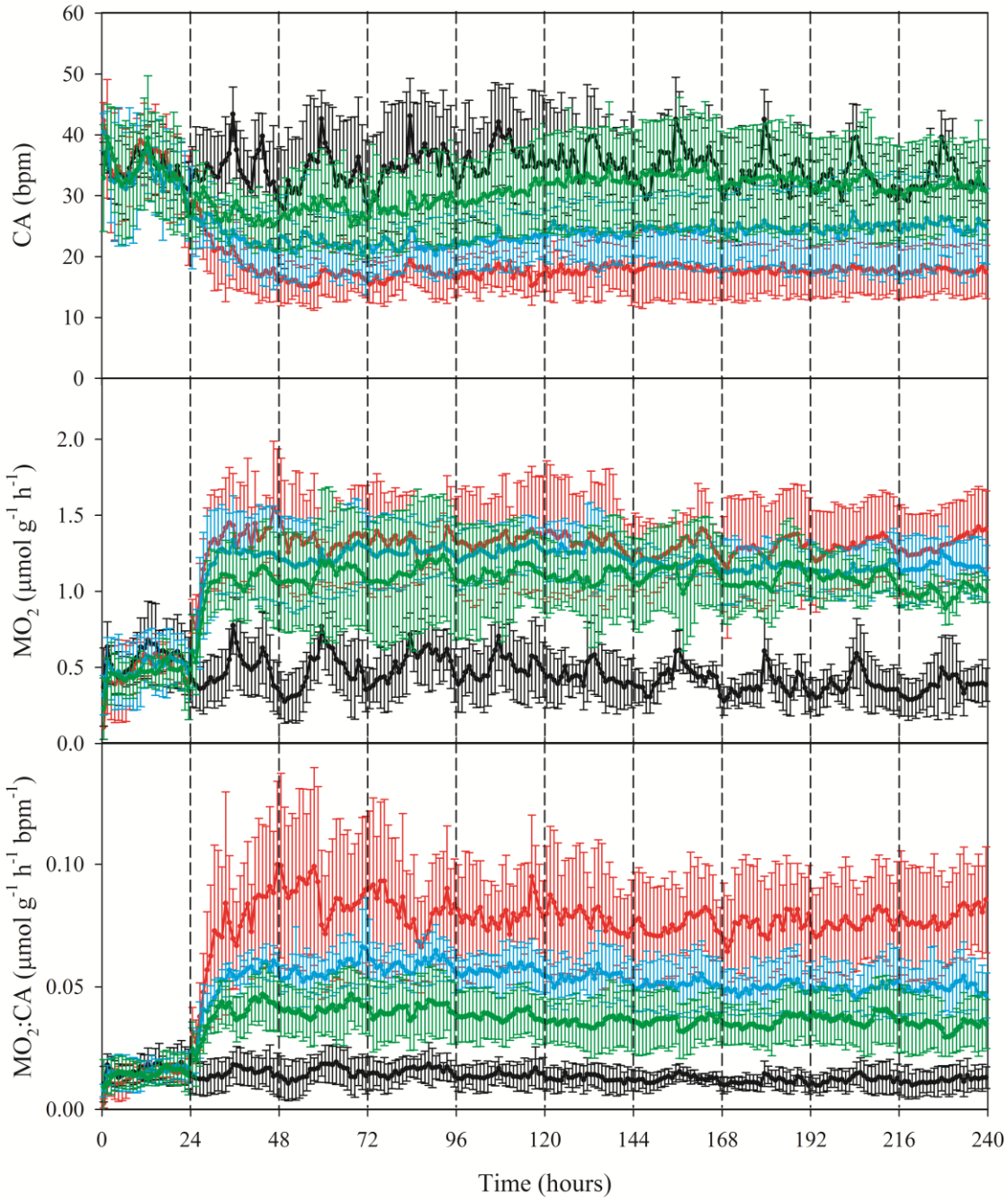

Figure 2.7. Effect of sustained hydrostatic pressure on mean mass-normalised cardiac activity (CA), mean mass-normalised mass-specific molar oxygen consumption (MO_2), and mean MO_2:CA of adult male *Lithodes maja* at 6°C; 0.1 MPa control treatment in black, 7.5 MPa treatment in green, 10.0 MPa treatments in blue, and 12.5 MPa treatment in red (n = 5). Error bars represent 95% confidence intervals.

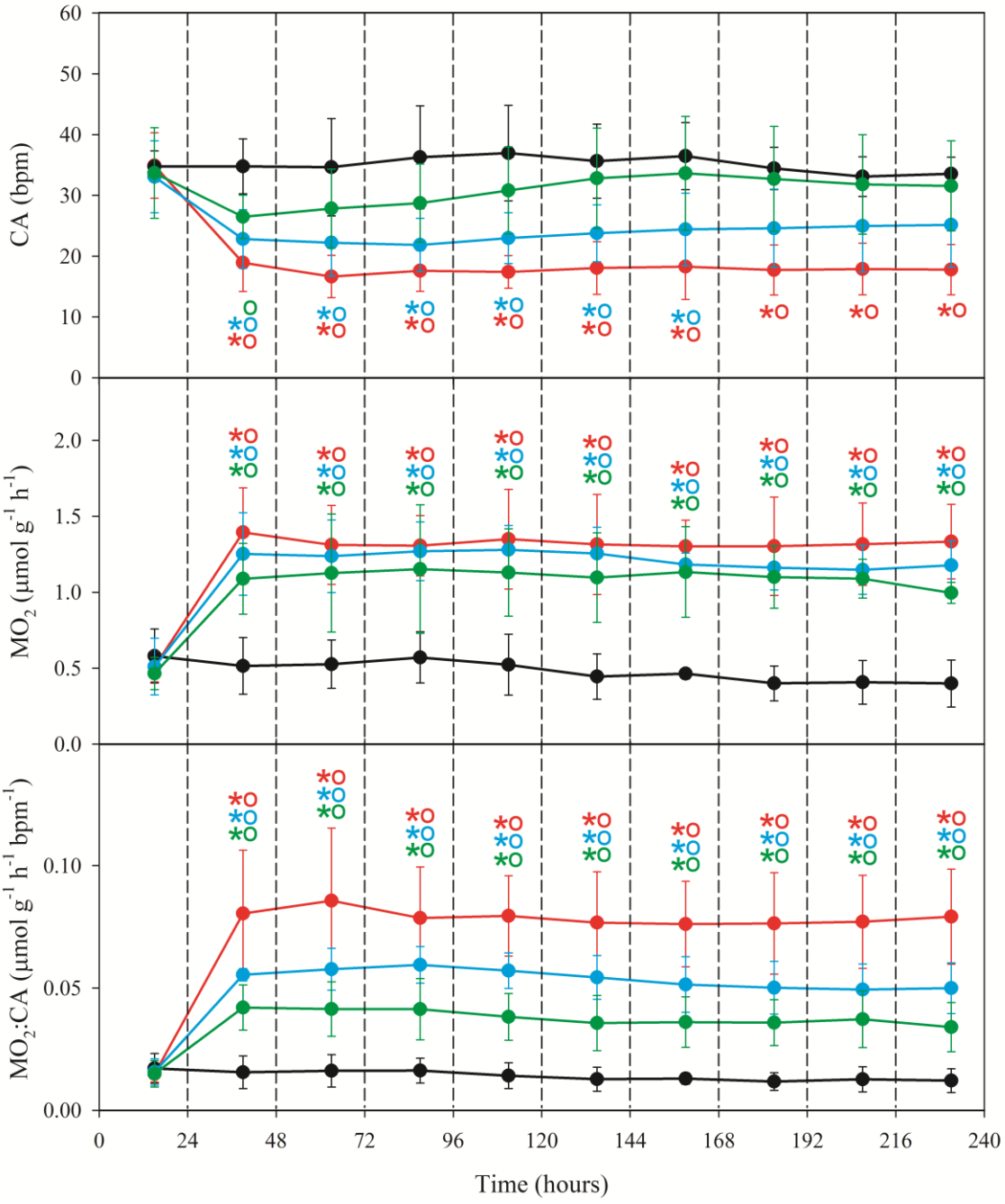

Figure 2.8. Effect of sustained hydrostatic pressure on time-period mean mass-normalised cardiac activity (CA), mean mass-normalised mass-specific molar oxygen consumption (MO_2), and mean MO_2:CA of adult male *Lithodes maja* at 6°C; 0.1 MPa control treatment in black, 7.5 MPa treatment in green, 10.0 MPa treatment in blue, 12.5 MPa treatment in red (*n* = 5). Error bars represent 95% confidence intervals. Significant difference from the same period in the control treatment is indicated by an asterisk; significant difference from the initial 24-hour period of a treatment (= 0.1 MPa in pressure treatments) is indicated by an open circle; symbol colour reflects treatment.

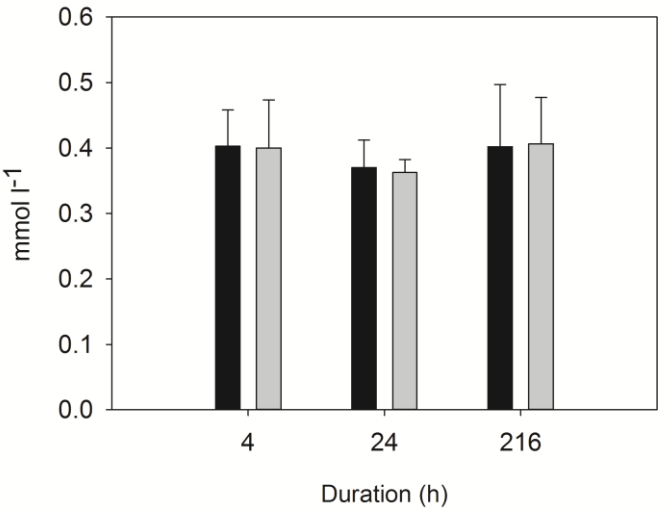

Figure 2.9. Effect of sustained hydrostatic pressure exposure duration on mean haemolymph L-lactate concentration of adult male *Lithodes maja* at 6°C; 0.1 MPa control treatment in black, 7.5 MPa treatment in grey (*n* = 5, except 216-hour durations where replicates are 4 and 3, respectively). Error bars represent 95% confidence intervals. 7.5 MPa treatments do not differ significantly from control treatments.

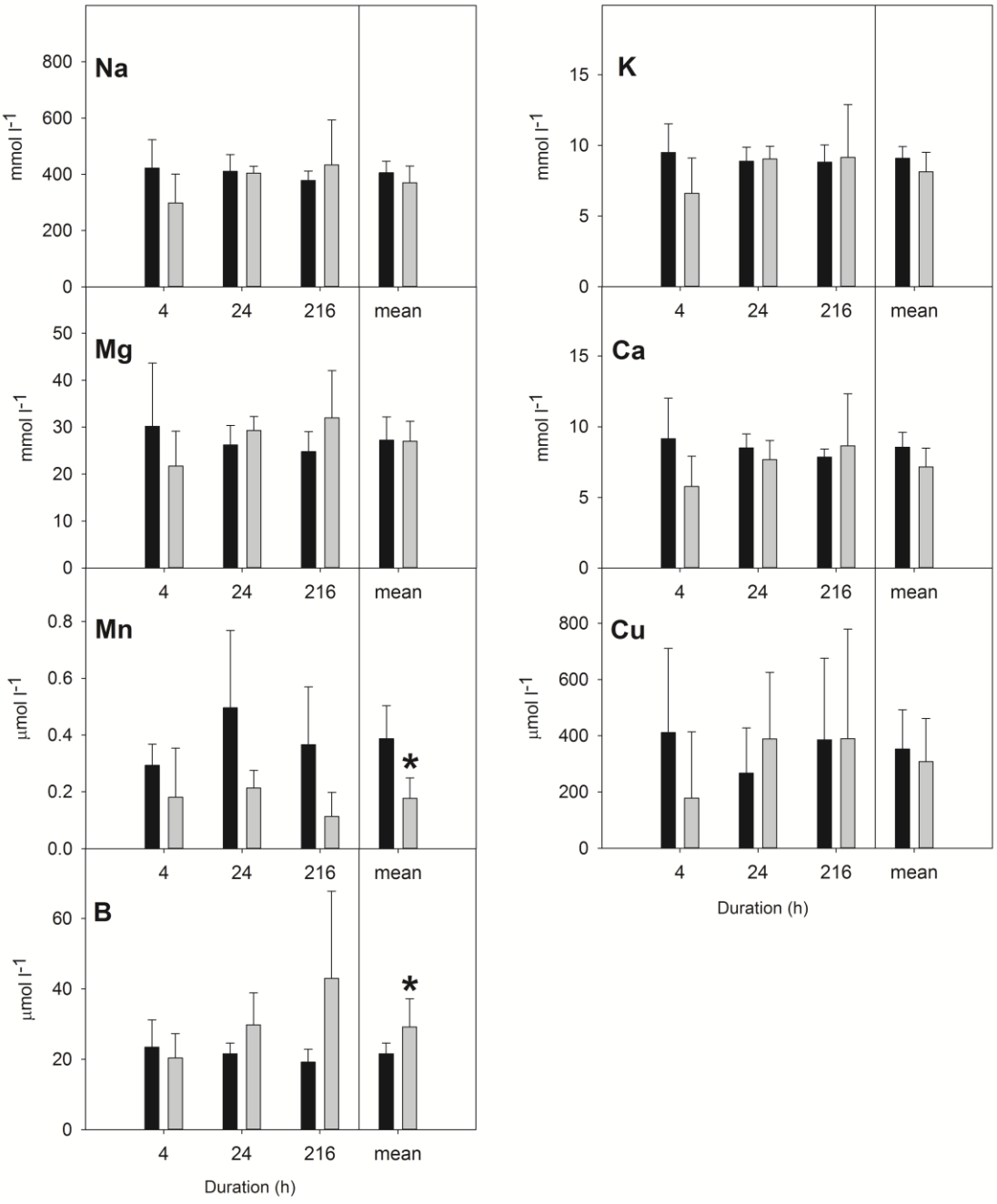

Figure 2.10. Effect of sustained hydrostatic pressure exposure duration on mean haemolymph ion concentration of adult male *Lithodes maja* at 6°C; 0.1 MPa control treatment in black, 7.5 MPa treatment in grey (*n* = 5, except for 216-hour durations where replicates are 4 and 3, respectively). Error bars represent 95% confidence intervals. Significant differences from control treatments are indicated by an asterisk.

2.4 Discussion

Cardiac activity (~28 bpm to ~43 bpm) and oxygen consumption (~0.27 to ~0.77 µmol O_2 g^{-1} h^{-1}) in *Lithodes maja* at 0.1 MPa and 6°C were similar to those reported for other adult lithodids. Cardiac activity was ~21 bpm at rest, ~33 bpm at rest and without pauses, and ~44 bpm after righting trial in *Paralomis granulosa* at 0.1 MPa and 6°C (Wittmann *et al.*, 2012), and ~50 bpm in *Lopholithodes mandtii* at 0.1 MPa and 12°C (McGaw & Duff, 2008). Previous reports of 86.4 bpm cardiac activity in *L. maja* at 0.1 MPa and 10°C (Walters & Uglow, 1981) may result from maintenance at temperatures above those in the species natural distribution (10-15°C), and assessment at the maximum temperature in the species' distribution and/or an invasive methodology. Oxygen consumption was ~0.47 µmol O_2 g^{-1} h^{-1} in *Lithodes longispina* at 0.1 MPa and 5.5°C (Wilson *et al.*, 2013), ~0.53 µmol O_2 g^{-1} h^{-1} in *Paralomis multispina* at 0.1 MPa and 3°C (Childress *et al.*, 1990), and ~0.74 µmol O_2 g^{-1} h^{-1} oxygen in *P. granulosa* at 0.1 MPa and 8°C (Comoglio *et al.*, 2005). However, cardiac activity and oxygen consumption in *L. maja* are evidently pressure sensitive (Figs. 2.5-2.8).

2.4.1 Implications of pressure-sensitive metabolic rate

Interspecific depth-related patterns of mass- and temperature-normalised metabolic rate in benthic invertebrates or demersal fishes reveal either significant declines or no change with depth (Childress *et al.*, 1990; Childress, 1995; Company & Sarda, 1998; Ikeda *et al*., 2006, 2007; Seibel & Drazen, 2007; Brey, 2010, Hughes *et al.*, 2011; Drazen & Yeh, 2012; Ikeda & Takahashi, 2012; McClain *et al*., 2012; Ikeda, 2013*a,b,c*; Wilson *et al.*, 2013, Ikeda, 2014), with the trend typically depending on taxonomic groups reliance on visual interactions. Indeed, limited available data suggest a bathymetric decrease in metabolic rate in anomurans (Fig. 2.11; Table 2.1), consistent with the observations that inspired the visual interaction hypothesis (Childress *et al.*, 1990). However, metabolic sensitivity to hydrostatic pressure in *Lithodes maja* indicates that measuring metabolic rates of benthic deep-sea organisms at surface pressures may not accurately represent *in situ* metabolic rates. Doing so may be analogous to assessing metabolic rates of shallow-water organisms without reference to native

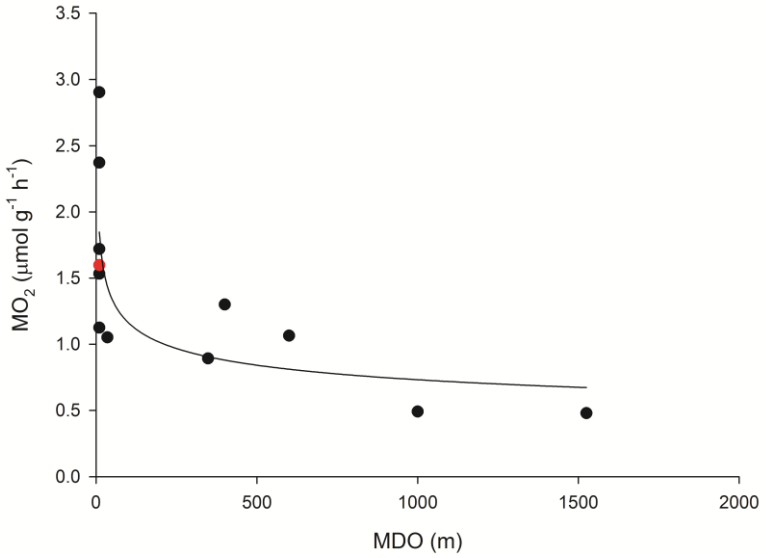

Figure 2.11. Decline in benthic anomuran crustacean mass-normalised mass-specific oxygen consumption (MO_2) as a function of minimum depth of occurrence (MDO) (this study in red; see Table 2.1 for details). Rates adjusted to 10°C and 10 g wet weight according to Childress *et al*. (1990). Nonlinear regression: $MO_2 = 2.94MDO^{-0.21}$; $F_{1,11} = 11.785$, $p = 0.006$; $r^2 = 0.541$.

temperature. Thermodynamic pressure effects may precede effects on conformational structure of proteins or fluidity of biological membranes (see Abe *et al.*, 1999, and references cited therein), and baric acclimatory plasticity, for example in membranes and membrane related function, is likely to be limited. Metabolic rate responds to shifts in pressure in eurybaric adapted taxa too. Metabolic rate in the eel *Anguilla anguilla* decreases to ~65% of the rate at 0.1 MPa with sustained exposure to 10.1 MPa (Simon *et al.*, 1989), which approximates the maximum pressure experienced by the species during its deep oceanic migrations (Aarestrup *et al.*, 2009). The hyperbaric decrease in metabolic rate is putatively due to increased oxidative phosphorylation efficiency with hyperbaric acclimation (Theron *et al.*, 2000), which mimics the ontogenetic plasticity that influences migration (see Righton *et al*., 2012, and references cited therein). Metabolic responses to pressure clearly vary among species.

Although some bathyal fauna tolerate recovery from ~2000 m and survive for sustained periods (e.g. Smith *et al.*, 2013*a*), even these apparently low-pressure tolerant species demonstrate pressure-dependent shifts in metabolic rate. For example, metabolic rate is suppressed in the hydrothermal vent shrimp *Mirocaris*

Table 2.1. Minimum depth of occurrence (MDO), and oxygen consumption rates (MO_2) of post-larval benthic anomuran crustaceans. Minimum depth of occurrence of species occurring at less than 10 m depth is reported as 10 m to facilitate statistical analysis by nonlinear regression. MO_2 adjustment to 10°C and 10 g was made according to Childress *et al*. (1990) based on reported temperature (T) and total wet mass (TWM).

Species	MDO (m)	T (°C)	TWM (g)	MO_2 (µmol g^{-1} h^{-1}) at T and TWM	MO_2 (µmol g^{-1} h^{-1}) at 10°C and 10 g	Source
Galathea strigosa	10	10	32.05	0.89	1.13	Bridges & Brand, 1980
Lithodes longispina	400	5.5	312.8	0.47	1.30	Wilson *et al*., 2013
Lithodes maja	10	6	464.5	0.56	1.60	this study
Lithodes santolla	10	12	3.3	4.01	2.90	Paschke *et al*., 2010
Munida intermedia	35	13	8.75	1.25	1.05	Company & Sarda, 1998
Munida tenuimana	348	13	4.62	1.20	0.89	Company & Sarda, 1998
Munidopsis verrilli	1525	2.8	5.62	0.27	0.48	Childress *et al*., 1990
Pagurus bernhardus	10	10	8.35	1.78	1.72	Bridges & Brand, 1980
Paralomis granulosa	10	8	316.8	1.05	2.37	Comoglio *et al*., 2005
Paralomis multispina	600	3	7.64	0.58	1.06	Childress *et al*., 1990
Pleuroncodes planipes	10	20	3.0	2.85	1.53	Quentin & Childress, 1976
Stereomastis sculpta	1000	4	16.8	0.27	0.49	Childress *et al*., 1990

fortunata following depressurisation (Shillito *et al.*, 2006; Smith *et al.*, 2013*a*). Pressure effects appear mass-dependent; consistent with size-effects on thermal tolerance (Peck *et al.*, 2009), metabolic rate at *in situ* temperature is more severely reduced in larger animals following depressurisation (Shillito *et al.*, 2006). These metabolic impacts are unlikely to result from temperature changes during recovery: *M*. *fortunata* is adapted to tolerate rapidly fluctuating temperatures across the temperature range experienced during retrieval (Desbruyères *et al.*, 2001; Smith *et al.*, 2013*a*). Pressure-related shifts in *M*. *fortunata*'s metabolic rate are not transient or overcome by acclimation to surface pressure. Metabolic rate in *M. fortunata* declines further with sustained exposure to surface pressure (cf. Shillito *et al.*, 2006; Smith *et al.*, 2013*a*), suggesting continuing acclimation. Further, hyperbaric adaptation in mitochondrial function and density may not be detectable at surface pressure. For example, putative intraspecifc cold adaptation in mitochondrial function and density in the killifish *Fundulus heteroclitus* is not apparent when acclimated to warm temperatures (Fangue *et al*., 2009). Abyssal deep-adapted organisms demonstrate low-pressure intolerance through mortality (e.g. Yayanos, 1981; Treude *et al.*, 2002). Consequently, it appears that metabolic rates measured at surface pressure may be unrepresentative of deep-sea *in situ* rates, revealing considerable complexity potentially confounding examinations of depth effects on mass- and temperature-compensated metabolic rates using data generated at non-native pressures (e.g. Seibel & Drazen, 2007, and references cited therein). Despite the plausibility of conclusions drawn in such studies, the data must be regarded with some caution until validated by direct comparison between metabolic rates measured *in situ* or in the laboratory at native pressure, and rates measured in the laboratory at atmospheric pressure. These validation studies may instead confirm the proposal that hydrostatic pressure has a critical role in driving bathymetric patterns in metabolic adaptations in the deep sea (see Smith *et al*., 2015), similar to temperature's role in driving latitudinal patterns in metabolic adaptations (see Clarke, 2003; Pörtner *et al*., 2005). Such metabolic adaptations may precede and select for observed deep-sea adaptations, for example in for fish morphology, life history, and behaviour (Seibel & Drazen, 2007; Drazen & Haedrich, 2012; Neat & Campbell, 2013), in range-edge populations (see Parsons, 1990). Membrane

adaptations to low temperature and high hydrostatic pressure may impose adaptive pressure driving the bathymetric decrease in metabolic rates. The polyunsaturated membrane phospholipids which are increased in cold- and pressure-adapted taxa are substantially more susceptible to oxidative stress damage than monounsaturated membrane phospholipids (see Buttemer *et al*., 2010), and the moderating cellular responses may be energetically unsustainable in the deep sea. Fundamental hyperbaric metabolic adaptations have previously been reported in deep-sea bacteria, for example in the cytochrome respiratory system (Barlett, 2002; Vezzi *et al*., 2005).

2.4.2 Oxygen- and capacity-limited hyperbaric tolerance

Increasing metabolic rate to 12.5 MPa contrasts with progressively decreasing cardiac activity with increasing hydrostatic pressure, revealing decreasing aerobic scope through a developing mismatch in oxygen demand and supply, and thus indicates that hyperbaric tolerance in *Lithodes maja* is oxygen- and capacity-limited. Although MO_2:CA decreases at pressure >12.5 MPa may represent decreasing oxygen utilisation coefficient (Maynard, 1960; Ansell, 1973), potentially revealing the onset of histotoxic hypoxia (see Sébert, 2002), pressure effects do not appear to impose a kinetic bottleneck to respiratory exchange rate (Hofmann *et al.*, 2013*a*,*b*). Increasing pressure decreases the minimum oxygen concentration required to support a given uptake rate (Hofmann *et al.*, 2013*a*, and references cited therein), which appears to be the key limiting chemical exchange rate rather than carbon dioxide exchange rate (Hofmann *et al.*, 2013*b*, and references cited therein). Consequently, diminishing metabolic rate at >12.5 MPa appears to represent a distinct physiological response indicating high stress (Sokolova, 2013). Progressive impairment of aerobic metabolism and increasing dependence on anaerobic metabolism during high stress often result in a general slow-down of activity and suppression of metabolic rate (see Sokolova *et al*., 2012; Sokolova, 2013, and references cited therein). Elevated cardiac activity and metabolic rate following return to 0.1 MPa support this interpretation; similar responses are observed following alleviation of extreme hypoxic stress (e.g. Hervant *et al.*, 1999). Whilst a temporary reduction or suspension of metabolic activity is tolerable, long-term survival in this state is not possible (Sokolova *et al*., 2012; Sokolova, 2013).

Constraint of bathymetric distribution by hydrostatic pressure

Thermal tolerance is oxygen- and capacity-limited too (see Pörtner, 2010, and references cited therein), and consequently marine ectotherms' fundamental ecological niches (defined physiologically by the environmental variables where a species can survive; see Barve *et al.*, 2011; Soberón, 2014) appear constrained by the combined physiological effects of temperature and hydrostatic pressure in a matrix of oxygen- and capacity-limited tolerance (adapted from Pörtner, 2010). Such an environmental tolerance matrix may explain the parabolic thermal depth distribution of *L*. *maja* in normoxic conditions (Fig. 2.12). *Lithodes maja*'s thermal depth distribution is unlikely to be an artefact of thermal depth profiles since water temperatures within *L*. *maja*'s latitudinal distribution remain at or above 10°C at 700 m in both the eastern and western North Atlantic (Locarini *et al*., 2010). Other environmental factors may also integrate into a matrix of oxygen- and capacity-limited tolerance too, for example elevated carbon dioxide concentration and decreased oxygen concentration (Pörtner, 2008, 2010). Thermal envelopes can narrow with increasing carbon dioxide concentration (e.g. Walther *et al.*, 2009) and thermal

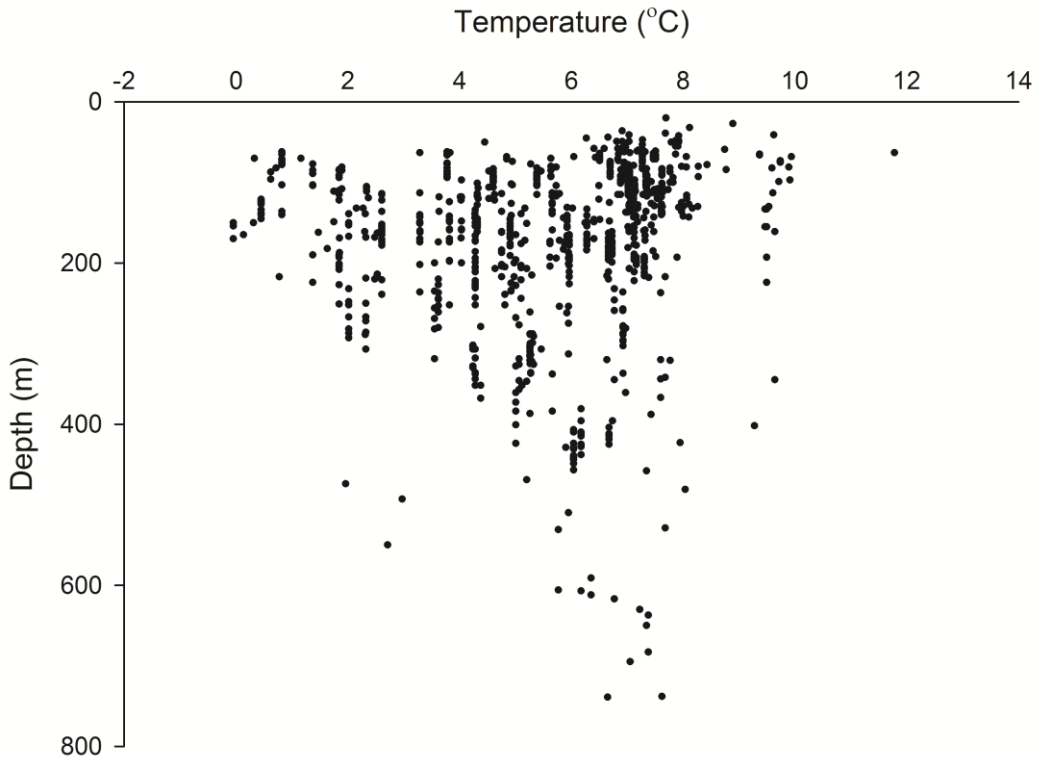

Figure 2.12. Bottom depth and temperature of *Lithodes maja* occurrences. Each point represents one record (OBIS, 2013).

tolerance windows are hypothesised to reduce with decreasing oxygen concentration too (Pörtner, 2010).

2.4.1 The mechanism limiting hyperbaric cardiac performance

The disparate hyperbaric cardiac activity and metabolic rate responses in *Lithodes maja* reveal that hyperbaric tolerance is proximately oxygen-limited but ultimately limited by cardiac capacity. The hyperbaric tolerance of other bathyal crustaceans appears proximately oxygen-limited and ultimately capacity-limited too: cardiac activity decreases in the deep-sea hydrothermal vent crab *Bythograea thermydron* at pressures above and below the species' distribution, with concomitant increases in oxygen consumption (Mickel & Childress, 1982*b*,*c*; Airriess & Childress, 1994). Indeed, cardiac activity decreases by 0.85 bpm MPa^{-1} in *B*. *thermydron* at pressures above maintenance pressure (23.8 MPa; Airriess & Childress, 1994), similar to the 0.89 bpm MPa^{-1} hyperbaric cardiac activity decrease in *L*. *maja*, suggesting a single mechanism may underlie the cardiac responses to elevated hydrostatic pressure in both species.

Thermal cardiac dependence in crustaceans is predominantly mediated by temperature effects on the cardiac ganglion (Worden *et al*., 2006). Negative correlation between cardiac activity and haemolymph $[Mg^{2+}]$ in decapods may depend on calcium channel blockade by magnesium, slowing neuromuscular transmission (see Wittmann *et al.*, 2010, and references cited therein). Neurotransmitter release is reduced at high extracellular $[Mg^{2+}]$ (Parnas *et al.*, 1994) and at low temperature (Dunn & Mercier, 2003); these effects may act together to reduce neuromuscular transmission and consequently cardiac activity (Wittmann *et al.*, 2010). Inability to down-regulate haemolymph $[Mg^{2+}]$ has been specifically implicated in low-temperature intolerance and range limitation in reptant decapods (Frederich *et al.*, 2000*a*,*b*, 2001; Wittmann *et al.*, 2010). Reduction of ambient $[Mg^{2+}]$ can increase performance in reptant decapods (Frederich *et al.*, 2001), including cardiac activity and metabolic rate (Frederich *et al.*, 2000*a*,*b*). Moderating $[Mg^{2+}]$-dependent thermal tolerance may have been critical to the relative success of lithodids at extreme low temperatures (Wittmann *et al*., 2012), which dominate reptant decapod occurrences approaching Antarctica (see Griffiths *et al.*, 2013, and references cited therein) - the

lowest temperature marine environment. Indeed, recent experimental evidence suggests that other factors may be more critical to temperature-dependent physiological function in lithodids than [Mg^{2+}] regulation, including the temperature-dependent mechanism of synaptic transmission, and pre- and postsynaptic transmitter-receptor interaction (Wittman *et al.*, 2011, 2012). However, heartbeat and ventilation beat frequencies in lithodids remain affected by [Mg^{2+}] (Wittmann *et al*., 2012). *Lithodes maja* appears capable of greater haemolymph [Mg^{2+}] down-regulation than other examined lithodids (cf. Wittmann *et al.*, 2010; this study) and is reported in colder waters than Southern Ocean lithodids (cf. -0.05°C in Fig. 2.12 and Hall & Thatje, 2011), possibly reflecting greater cold adaptation arising during and facilitating a relatively ancient trans-Arctic dispersal to the North Atlantic (see Hall, 2010; but see also Anosov *et al*., 2015). Consistent with this hypothesis, cardiac activity is greater in *L*. *maja* than in sub-Antarctic *Paralomis granulosa* at 0.1 MPa and 6°C; respectively ~28 bpm and ~21 bpm at rest (Wittmann *et al*., 2012). Previous indication of apparent inability to down-regulate [Mg^{2+}] in *L*. *maja* (Robertson, 1949; Walters & Uglow, 1981) may reflect the onset of temperature-induced regulatory failure during maintenance at temperatures above those in the species' distribution (i.e. 10-15°C; Fig. 2.12); the 24% reduction in cardiac scope between 6°C and 10°C in *L*. *maja* supports this explanation (cf. Walter & Uglow, 1981; this study). Co-occuring differences in thermal distribution and in [Mg^{2+}] regulation suggest that [Mg^{2+}] regulation may remain capacity-limiting in lithodids.

Decreasing cardiac activity observed in the lithodid crab *Paralomis granulosa* in response to decreasing temperature is similar to the hyperbaric decrease in cardiac activity in *L*. *maja*, implying that [Mg^{2+}] regulation may also critically influence hyperbaric performance by compounding oxygen- and capacity-limitation. Linear regression indicates that cardiac activity decreases by 1.01 bpm $°C^{-1}$ in *P*. *granulosa* (Wittmann *et al.*, 2012) and by 0.889 bpm MPa^{-1} in *L*. *maja*. Indeed, the apparent transient decrease in haemolymph [Mg^{2+}] in *L*. *maja* after 4 hours exposure to 7.5 MPa appears to temporarily counteract decreasing cardiac activity (cf. Figs. 2.5, 2.7, and Fig. 2.10). Disparity between hydrostatic pressure thresholds in relatively effective and ineffective haemolymph [Mg^{2+}]-regulating crustaceans further supports this

interpretation. Hydrostatic pressure tolerances are significantly lower in anomurans and brachyurans (relatively ineffective haemolymph [Mg^{2+}] regulators), than in amphipods, carideans, and isopods (relatively effective haemolymph [Mg^{2+}] regulators) ($F_{1,13}$ = 9.292, p = 0.009; for data see Table 3.1).

Cellular effects may mediate observed systemic responses to hydrostatic pressure too (see Daniels & Grossman, 2010, and references cited therein). Progressive failure of membrane transporter Na^+,K^+-ATPase with increasing pressure may contribute to the gradual decrease in cardiac activity observed at >12.5 MPa in *L*. *maja*. Pressure affects the function of Na^+,K^+-ATPase (Gibbs & Somero, 1989), which is involved in Ca^{2+} homeostasis in crustaceans (see Freire *et al.*, 2008, and references cited therein) and is critical to maintaining membrane potential needed to generate action potentials in excitable cells such as those regulating cardiac function (see Lodish *et al.*, 2003). Na^+,K^+-ATPase dysfunction can eventually render the heart unable to beat (Stillman, 2002). The maximum velocity of Na^+,K^+-ATPase is reduced under pressure resulting in a nonlinear decrease in enzymatic activity (Gibbs & Somero, 1989). Critical pressure effects on Na^+,K^+-ATPase are supported by enzymatic functional pressure-adaptation in deep-sea organisms (Gibbs & Somero, 1989). Decreases in Na^+,K^+-ATPase activity may be mediated by reduction in membrane fluidity (Somero, 1992), potentially by inducing NMDA receptor over-activity (see Carvalho *et al.*, 2012).

NMDA receptors are ligand- and voltage-gated glutamate receptors: ion channels that mediate excitatory neurotransmission (Dingledine *et al.*, 1999). Up-regulation of NMDA receptor-regulated *narg* transcription at 12.5 MPa (Appendix 1 Fig. A1.1) may reflect the onset of compromised neurogenic cardiac activity control resulting from changes in the activity of NMDA receptors. NMDA receptors are highly calcium permeable (Mor & Grossman, 2006) and appear to be present in all major ganglia of decapods, demonstrating strong localisation in synaptosomal membranes (Hepp *et al.*, 2013). Pressure can diminish the efficacy of receptor blockade and augment NMDA receptor synaptic responses, leading to hyperexcitability and potentially to neurotoxicity (Mor & Grossman, 2006, 2007, 2010); pressure induced NMDA receptor over-activity has been hypothesised to cause excitotoxic neuronal injury by allowing excessive cellular influx of Ca^{2+} (Mor & Grossman, 2006, 2010). Concomitant up-

regulation of several other genes is proposed to support the NMDA receptor over-activity hypothesis: increased transcription of *hsp70* suggests macromolecular damage (see Morris *et al.*, 2013), with increases in *β-actin* and *arf* transcription implying damage to actin and DNA respectively, and increased *gapdh* transcription indicating consequent elevation of cellular metabolism (Morris *et al.*, 2015). Significant up-regulation of *narg* in decapods in response to elevated hydrostatic pressure has therefore been interpreted as a response to over-activity of NMDA receptors (Morris *et al.*, 2015). Indeed, down-regulation of *α-tubulin* transcription at 7.5 MPa in *L. maja* (Appendix A Figs. A1.1, A1.2) may reflect the onset of hyperbaric effects on NMDA receptors. NMDA receptor activation can suppress microtubule growth in neurons (Kapitein *et al*., 2011), and NMDA receptor activity stimulation can induce *α-tubulin* degradation in the nervous system (Palumbo *et al.*, 2002), which may influence *α-tubulin* regulation.

Although *narg* regulation appears insensitive to temperature shock (Morris *et al.*, 2015), this may depend on experimental assessments made at temperatures well within the species' eurythermal scope and on the test species' adaptations to rapidly fluctuating thermal environment (see Smith *et al.*, 2013*a*). Shifts in NMDA receptor activity may also occur during cold stress in decapods (inferred from NMDA receptor down-regulation; Stillman & Tagmount, 2009). As discussed, low-temperature performance is increased in reptant decapods by a reduction in ambient [Mg^{2+}] (including cardiac activity and metabolic rate; Frederich *et al.*, 2000*a*,*b*, 2001), putatively due to reduced neurotransmission interference (Wittmann *et al*., 2010). Inhibition of NMDA receptor activity depends predominantly on Mg^{2+} blockade (Blanke & Van Dongen, 2009). Consequently, improvements in low-temperature performance with decreased ambient [Mg^{2+}], despite Mg^{2+} blockade appearing relatively weak in invertebrates (see Xia & Chiang, 2009), infer that low-temperature neurotransmission interference results from NMDA receptor under-activity rather than NMDA receptor over-activity.

Neural and muscular symptoms of temperature and hydrostatic pressure intolerance are also compatible with slowing NMDA receptor kinetics (e.g. Talpalar & Grossman,

2006). Shifts in *narg* transcription may instead be mediated through reduced NMDA receptor activity resulting from impaired NMDA receptor function, consistent with dominant hyperbaric pressure effects on voltage-dependent Ca^{2+} channels (Aviner *et al.*, 2010). Indeed, the hyperbaric cellular stress response differs significantly from the cytosolic [Ca^{2+}]-induced cellular stress response (Elo *et al*., 2000) implied by the NMDA receptor over-activity hypothesis. NMDA receptor subtypes display differential responses to elevated pressure, and ionic currents can be decreased by elevated pressure (Mor *et al.*, 2012). Of eight NMDA receptor subtypes examined for pressure responses, only one produces significantly larger ionic current under high pressure (10.1 MPa): three subtypes appear unresponsive, and four appear significantly depressed (Mor *et al.*, 2012). Acute cold reduces voltage-dependent Ca^{2+} channel currents similarly and prolongs action potential duration, reducing Ca^{2+} flux and depressing cardiac contractility (e.g. Shiels *et al*., 2014). Hydrostatic pressure and temperature effects on membrane properties and ion channel structure (Tillman & Cascio, 2003) may destabilise the intradimer interface in ligand-binding cores of glutamate receptors, enhancing neurotransmitter desensitisation (Sun *et al.*, 2002). Synaptic transmission is consistently depressed under hyperbaria (see Aviner *et al.*, 2010), representing another potential mechanism decreasing NMDA receptor activity: decreased glutamate release may diminish NMDA receptor activity (Duguid & Smart, 2009). Sustained significant reduction in haemolymph [Mn^{2+}] in *L*. *maja* supports the reduced NMDA receptor function interpretation. Mn^{2+} can competitively inhibit Ca^{2+} channels (see Baden & Eriksson, 2006, and references cited therein); consequently, reduced haemolymph [Mn^{2+}] may counteract hyperbaric decreases in NMDA receptor activity mediated by depressed synaptic transmission.

Macromolecular damage and elevation of cellular metabolism suggested to result from Ca^{2+} influx due to NMDA receptor over-activity (Morris *et al.*, 2015) may instead be caused by an increase in cellular oxidative stress (production and accumulation of reactive oxygen species (ROS) beyond the quenching capacity) (Lesser, 2006; Abele *et al*., 2007) under moderate stress conditions (see Sokolova *et al*., 2012; Sokolova, 2013, and references cited therein). Repression of circadian rhythms in *L*. *maja* at 7.5 MPa supports an increased oxidative stress interpretation. Metabolic circadian rhythms are

known for crustaceans (reviewed by Strauss & Dircksen, 2010): for example, nocturnal elevation in metabolism also occurs in the congeneric species *Lithodes santolla* maintained under optimum conditions (Schvezov *et al.*, 2013) and may be a synapomorphic trait. Increased metabolic rate and ROS production (see Welker *et al.*, 2013, and references cited therein) under elevated hydrostatic pressure may moderate the molecular mechanism of circadian regulation (see Schippers *et al.*, 2013, and references cited therein). High hydrostatic pressure can certainly induce elevated ROS generation (see Moisan *et al.*, 2010, and references cited therein), which can adversely impact membranes and membrane proteins (see Halliwell & Gutteridge, 1999).

Sustained down-regulation of *α-tubulin* transcription at 7.5 MPa in *L. maja* (Appendix A Figs. A1.1, A1.2) may also be indicative of the transition to moderate stress, and the shift from cellular growth to cell repair with concomitant changes in phosphorylation (Crenshaw *et al.*, 1996): *α-tubulin* transcription is unresponsive to cold acclimatisation in anomuran *Petrolisthes cinctipes* porcelain crabs but is down-regulated in response to sub-lethal cold stress (Stillman & Tagmount, 2009). Microtubule tubulin polymers are essential cytoskeletal components affecting cell shape, cell transport, and cell division in all eukaryotes: down-regulation of *α-tubulin* may reduce microtubule filament formation, with a multiplicity of cascading cellular effects (see Nogales, 2000). Stress proteins and antioxidant up-regulation in the calanoid copepod *Calanus helgolandicus* during toxic exposures occurs concurrently with reduced *α-tubulin* transcription (Carotenuto *et al.*, 2014). Increased cellular oxidative stress under moderate stress conditions induces antioxidant expression (see Sokolova *et al*., 2012; Sokolova, 2013; Tomanek, 2014), including in lithodids (Romero *et al.*, 2007; 2011; 2013; Schvezov *et al.*, 2013) and in response to low temperature (Schvezov *et al*., 2015). Antioxidants include highly conserved enzymatic (e.g. superoxide dismutase; SOD) and non-enzymatic components (e.g. glutathione) that mitigate oxidative stress by scavenging, transforming, and detoxifying ROS (Halliwell & Gutteridge, 1999). Mitochondrial ROS production is not linearly dependent on electron transport rates but rather depends on the mitochondrial membrane potential (Abele *et al*., 2007), and compensatory mitochondrial H^+ leakiness (Abele *et al*., 2007) may be inhibited by

hyperbaric impacts on mitochondrial membranes. Significantly lower haemolmyph [Mn^{2+}] at 7.5 MPa in *L. maja* may indicate a mismatch in Mn^{2+} supply and demand, suggesting that hyperbaric conditions may result in increased expression of mitochondrial and cytosolic Mn-SOD (Brouwer *et al.*, 2003) to mitigate elevated mitochondrial and cellular oxidative stress (Brand *et al.*, 2004), supporting the increased oxidative stress proposition. Glutathione synthesis may be elevated under hyperbaric oxidative stress too, and glutamate is a critical glutathione component (see Halliwell & Gutteridge, 2009, and references cited therein). Increased glutathione glutamate demand may outstrip the supply capacity delivered through glutamate synthesis, reducing glutamate availability and thereby potentially contributing to diminishing NMDA receptor activity (Duguid & Smart, 2009).

Ultimately, insufficient data are available to allow definitive inference regarding the mode of *narg* regulation, but changes in *narg* transcription reflect shifts in NMDA receptor activity regardless of the mechanism of *narg* regulation. Pressure depresses synaptic transmission at all examined synapses in shallow-water taxa (see Aviner *et al.*, 2010), including the crustacean neuromuscular junction at ~10.0 MPa (Campenot, 1975*a,b*; Grossman & Kendig, 1988; Kendig *et al.* 1988). Shifts in NMDA receptor activity therefore imply adversely affected synaptic transmission mediated by hyperbaric effects on membranes and membrane function. Similar effects are observed in other synaptic processes (e.g. in glutamatergic AMPA, GABAergic, glycinergic, dopaminic, serotonergic, and cholinergenic synaptic procesees; see Grossman *et al*., 2010, and references cited therein). Impacts on synaptic transmission interfere with neurogenic muscle regulation (Anderson & Cooke, 1971; see Friedrich, 2010, and references cited therein) and neurotransmitter desensitisation has been hypothesised to cause observed decreases in excitatory potential amplitude at the neuromuscular junction in the shallow-water brachyuran crab *Libinia emarginata* at 10.0 MPa (Campenot, 1975*b*). Putative pressure adaptations make excitatory potential amplitude in the deep-sea brachyuran crab *Geryon quinquidens* insensitive to hydrostatic pressures up to at least 20.0 MPa (Campenot, 1975*a,b*). Thus significantly up-regulated *narg* expression and significant sustained depression of cardiac activity in *L. maja* at 12.5 MPa suggest sustained adverse pressure effects on synaptic

transmission. Sustained significantly decreased haemolymph [Mn^{2+}] at 7.5 MPa supports this hypothesis: haemolymph [Mn^{2+}] accumulation significantly impairs neuromuscular performance (e.g. Baden & Neil, 1998), and down-regulation of haemolymph [Mn^{2+}] may therefore improve neuromuscular performance.

In contrast, the transient significant depression of cardiac activity at 7.5 and 10.0 MPa suggests transient hydrostatic pressure effects on synaptic transmission in *L*. *maja*, revealing hyperbaric acclimatory capacity. Thermal acclimatory plasticity in cardiac function has previously been identified in decapods (e.g. in the brachyuran crab *Carcinus maenas*; Tepolt & Somero, 2014) and may be linked to membrane and membrane function plasticity, which is predominantly outside of nervous control (Cuculescu *et al*., 1999). Acclimatory capacity can certainly compensate for hyperbaric effects in adapted taxa. Hyperbaric reduction in membrane fluidity can be counteracted through homeoviscous acclimation. For example, membrane function is restored in the eurybaric adapted eel *A*. *anguilla* during sustained exposure to 10.1 MPa by incorporating additional phospholipids in cell membranes (see Sèbert, 2002, and references cited therein). Unsaturation of membrane fatty acids also restores membrane fluidity, improving oxidative phosphorylation efficiency and readjusting relative activities of respiratory chain complexes, and decreasing ROS production (see Sébert *et al.*, 2009, and references cited therein). Homeoviscous modifications during cold acclimation do not appear to occur in the short term (e.g. 24 hours; Ronges *et al.*, 2012) but can occur over slightly longer time-scales (e.g. 72 hours; Waagner *et al.*, 2012). Therefore, homeoviscous acclimation may explain the recovery in CA in *L*. *maja* at 7.5 and 10.0 MPa.

The apparent decrease in haemolymph ion concentration in *L*. *maja* after 4 hours exposure to 7.5 MPa is indicative of acute high-hydrostatic-pressure effects on ion movements and/or transport mechanisms (see Péqueux & Sébert, 2010, and references cited therein). Ionic regulation in crustaceans is mediated by membranes in the multifunctional gills (Freire *et al.*, 2008) under neuroendocrine control (see Charmantier *et al*., 2009, and references cited therein), further supporting critical hyperbaric impacts on synaptic transmission. The transience of the apparent decrease

in haemolymph ion concentration suggests that hyperbaric membrane functionality is restored by membrane acclimatory plasticity more rapidly than during thermal acclimation in decapods, consistent with previous evidence (New *et al.*, 2014). Reduced activity of membrane transporters such as Na^+,K^+-ATPase in *A. anguilla* is compensated by increased Na^+,K^+-ATPase concentration at 10.1 MPa (Sèbert *et al.*, 1991). Elevated haemolymph [B^{3+}] observed in *L. maja* at 7.5 MPa may stimulate Na^+,K^+-ATPase activity (Schon *et al.*, 1990) counteracting hyperbaric reduction in Na^+,K^+-ATPase activity. Increased haemolymph [B^{3+}] effects on membrane characteristics may also moderate hyperbaric effects on transmembrane signalling (see Nielsen, 1991), contributing to the recovery of CA in *L. maja* at 7.5 and 10.0 MPa. Delayed transcriptional responses may contribute to cardiac recovery too; shifts in transcriptome were not detected until 12 hours during cold exposure in the anomuran porcelain crab *Petrolisthes cinctipes* (Ronges *et al*., 2012).

Significantly increased MO_2:CA at 7.5-12.5 MPa in *L. maja* suggests that increasing cardiac stroke volume may ameliorate the hyperbaric declinine in cardiac activity, although increasing oxygen utilisation coefficient may cause or contribute to increased MO_2:CA too (Maynard, 1960; Ansell, 1973). The effects of progressively declining cardiac activity during hypoxic exposures can certainly be ameliorated by increased cardiac stroke volume in decapods, maintaining or even increasing cardiac output, and therefore gill perfusion and respiration (see McMahon, 2001, and references cited therein). Indeed, low rate and high volume cardiac performance appears to be a paradigm of cold adaptation (Tota *et al.*, 1997). However, the compensatory potential of cardiac acclimatory plasticity through this mechanism is clearly limited.

Adaptive capacity in both structure and function in the oxygen-transporter haemocyanin in response to a range of modulators (see Bruneaux *et al*., 2008) suggests that haemocyanin may be adapted to pressure in deep-sea taxa. Haemocyanin displays thermal adaptation (e.g. to relatively stable low temperatures in the lithodid crab *Paralithodes camtschaticus*; Molon *et al*., 2000; Decker *et al*., 2007; Podda *et al*., 2008) and functional depth adaptation certainly occurs in other critical proteins (Somero, 1998). Haemocyanin adaptations are known in deep-sea crustaceans associated with hydrothermal vents (attributed to high variability in

temperature and oxygen concentration; see Chausson *et al.*, 2004, and references cited therein) and pelagic deep-sea taxa associated with oxygen-minimum zones (attributed to low oxygen concentration; Sanders & Childress, 1990), but haemocyanin adaptation in taxa from the wider deep-sea environment remains unexplored. Haemocyanin acclimatory plasticity is also possible (see Bruneaux *et al*., 2008). During hypoxic exposure the king crab *L*. *santolla* compensates for oxygen-limitation by increasing haemocyanin concentration and enhancing haemocyanin affinity (Paschke *et al.*, 2010). Such compensation may be critical for sustaining crustacean cardiac performance under thermal extremes (see Giomi & Pörtner, 2013) and may also occur during hyperbaric oxygen-limitation. Lactate potentiation of haemocyanin oxygen affinity (see Bridges, 2001) during sustained exposure to 7.5 MPa is precluded by the absence of a significant increase in haemolymph L-lactate concentration, and the absence of a significant increase in haemolymph [Cu^{2+}] during sustained exposure to 7.5 MPa suggests that haemocyanin concentration remains unchanged (see Baden & Neil, 1998). However, haemocyanin structure and function are sensitive to environmental parameters and may respond to hydrostatic pressure, affecting respiratory capacity (Giomi & Beltramini, 2007). Haemocyanin subunits in the lobster *Homarus americanus* are partially dissociated by pressure of 20.0 MPa in the presence of metabolites (Gebhardt & Kulozik, 2011). Lower pressures may affect haemocyanin dynamics too (Gebhardt & Kulozik, 2011), likely reducing oxygen-affinity and oxygen-binding cooperativity (see Podda *et al*., 2008).

Integrating available physiological evidence predominantly supports deteriorating cardiac performance as the critical determinant of hyperbaric limitation, mediated by hyperbaric effects on membranes and membrane related functions. Decreasing hyperbaric tolerance during early ontogeny (zoea I to megalopa to crab I) in *L*. *maja* (Munro, 2013) may support this proposition. Neurogenesis extends into larval stages in crabs (e.g. Harzsch *et al*., 1998) with cardiac regulation typically shifting from myogenicity to neurogenecity during early development in crustaceans (e.g. during the first juvenile stage in the direct developing isopod *Ligia exotica*; Yamagishi & Hirose, 1997). The transition to neurogenecity coincides with the onset of cardiac sensitivity to environmental factors (Spicer, 2001). Cardiac region delineation occurs during

development from megalopa to juvenile in the congeneric king crab *L*. *santolla* (McLaughlin *et al*., 2001) and is likely associated with a shift in cardiac function that increases environmental sensitivity (see Spicer, 2001). In constrast, shifts in haemocyanin subunit composition during ontogeny may increase oxygen affinity, decreasing environmental sensitivity. For example, megalopa and early juvenile haemocyanin in *Cancer magister* have an intrinsic oxygen affinity 50% lower than adult haemocyanin (Terwilliger & Dumler, 2001). However, development of cardiac function and haemocyanin ontogeny have not been scrutinised in lithodids, and other ontogenetic structural and functional adjustmensts may influence hyperbaric tolerance too. For example, digestive enzyme expression shifts occur concurrently with gastric region delineation between megalopa and juvenile in *Lithodes* spp., during the ontogenetic transition from endotrophy to exotrophy (Anger, 1996; McLaughlin *et* al., 2001; Lovrich *et al*., 2003; Saborowski *et al*., 2006).

2.4.2 Bathymetric range limitation by metabolic cost

Metabolic responses to moderate environmental stress require flexible energy resource allocation and/or metabolic power (see Sokolova *et al*., 2012; Sokolova, 2013, and references cited therein). In the dynamic energy budget model, energy assimilated by an organism is divided among maintenance, activity, development/growth, and reproduction (Kooijman, 2010). Surplus energy may be deposited as an energy reserve and the reserve rapidly provides energy to meet elevated maintenance metabolic cost during moderate stress. Somatic maintenance is the dominant energy budget component and cannot be reduced below a fundamental level. Maintenance costs comprise energy demands for basal cellular and organismal maintenance supporting key cellular processes (e.g. ion regulation, protein turnover, anabolism) and essential systemic activities such as ventilation and circulation (Sokolova *et al*., 2012; Sokolova, 2013). At the whole-organism level, compensatory mechanisms may involve accelerated oxygen uptake (Willmer *et al.*, 2005). Increasing allocation of energy reserves to maintenance is apparent in significantly elevated and increasing oxygen consumption at 7.5-12.5 MPa in *Lithodes maja*, indicating moderate stress. Moderate stress causes adverse impacts, but these may be moderated by cellular, physiological,

and behavioural mechanisms which impose substantial synthesis and turnover energetic cost (see Sokolova *et al*., 2012; Sokolova, 2013, and references cited therein). Stress typically induces a transition from a state of cellular growth to one of cellular repair (Kültz, 2005; Kassahn *et al.*, 2009). Metabolic compensation often involves elevated protein synthesis and turnover which are amongst the most important ATP sinks in the cell (see Sokolova *et al*., 2012; Sokolova, 2013, and references cited therein). For example, stress proteins are highly conserved molecular chaperones that fold newly synthesised polypeptide chains and repair or stabilise molecular structures (Feder & Hofmann, 1999), even regulating membrane fluidity (Tsvetkova *et al.*, 2002). Pressure of 7.5 MPa can dissociate macromolecular protein assemblages such as cytoskeleton tubulin in shallow-water organisms, affecting basic cell morphology, organisation, and function (Salmon, 1975*a*). Sustained elevated stress protein expression may be induced by sustained hyperbaric exposure. For example, *hsp70* is induced by sustained exposure to 10 MPa in the shrimp *Palaemonetes varians* (Cottin *et al.*, 2012). Production and functioning of stress proteins and antioxidants can strongly increase cellular and whole-organism energy expenditure during stress, requiring the support of high levels of aerobic metabolism (Calow, 1991; Feder & Hofmann, 1999). Inferred moderate stress in *L. maja* at 7.5-12.5 MPa is sustained, suggesting that hyperbaric acclimation (e.g. in ionic regulation and gene transcription) cannot entirely offset pressure effects. However, the absence of a significant increase in haemolymph L-lactate concentration indicates that metabolism remains aerobic, at least at 7.5 MPa. Additional homeostatic energy costs lead to energetic trade-offs with reduced scope for growth, activity and reproductive output (Calow, 1989, 1991; Calow & Forbes, 1998). Increased homeostatic effort incurred through transcriptomic shifts during acclimation to suboptimal temperatures in the Antarctic fish *Pachycara brachycephalum* led to the development of functional imbalances which paralleled deterioration of whole animal performance (Windisch *et al*., 2014).

Sustained elevation of homeostatic effort is consistent with the recent hypothesis of increased metabolic costs imposed on shallow-water organisms during hyperbaric exposure, which may contribute to bathymetric patterns of increasing per offspring energetic investment (Smith *et al*., 2015), including in lithodids (see Hall, 2010).

Although acclimation may reduce metabolic cost and experimental evidence indicates thermal acclimation in Antarctic marine invertebrates requires ≥2 months (Peck *et al.*, 2014), cardiac activity recovery demonstrated here suggests that acclimation to hydrostatic pressure may occur within 9 days. Similarly, the component of thermal plasticity that influences hydrostatic pressure tolerance in *P. varians* appears acclimated after 8-10 days sustained pressure exposure (New *et al.*, 2014). The sustained increase in metabolic rate in *L. maja* contrasts with the hyperbaric response in the eurybaric adapted (Dunel-Erb *et al.*, 1996; Vettier *et al.*, 2005) eel *Anguilla anguilla*. Metabolic rate in *A. anguilla* increases by >100% during compression to 10.1 MPa, before declining progressively over 6-8 days to ~65% of the rate at 0.1 MPa and remaining approximately constant for at least 20 days (Simon *et al.*, 1989).

Environmental temperatures in marginal populations of many aquatic ectotherms correlate with the critical temperatures at which these organisms transition to moderate stress: individual fitness is reduced by increasing homeostatic effort beyond this transition, and species biogeographic limits certainly occur before the transition to extreme stress (Pörtner & Knust, 2007; Pörtner & Farrell, 2008; Sokolova *et al*., 2012; Sokolova, 2013). Indeed, thermal boundaries of species distributions may be determined by the energetic costs involved in moderate stress responses (Somero, 2002; Hofmann & Todgham, 2010; Sokolova *et al*., 2012; Sokolova, 2013). Increased allocation of energy reserves to maintenance during stress diminishes energy available for other functions, for example decreasing capacity to buffer fluctuating food availability to ensure continuous metabolic energy supply or to allocate energy to reproduction (Sokolova *et al*., 2012; Sokolova, 2013).

The transition to moderate stress in *L. maja* is evident at 7.5 MPa and precedes the 790 m bathymetric limit to the species known distribution (Fig. 2.13), suggesting that the metabolic costs associated with increasing moderate stress may constrain bathymetric distribution. Similar increases in metabolic rate occur in the shallow-water benthopelagic anomuran crab *Pleuroncodes planipes* in response to acute exposure to pressure beyond the species' intertidal to 300 m depth distribution (Quentin & Childress, 1976). However, direct experimental examination of energetic limitation of bathymetric distribution is currently precluded by the inability to supply food under

Constraint of bathymetric distribution by hydrostatic pressure

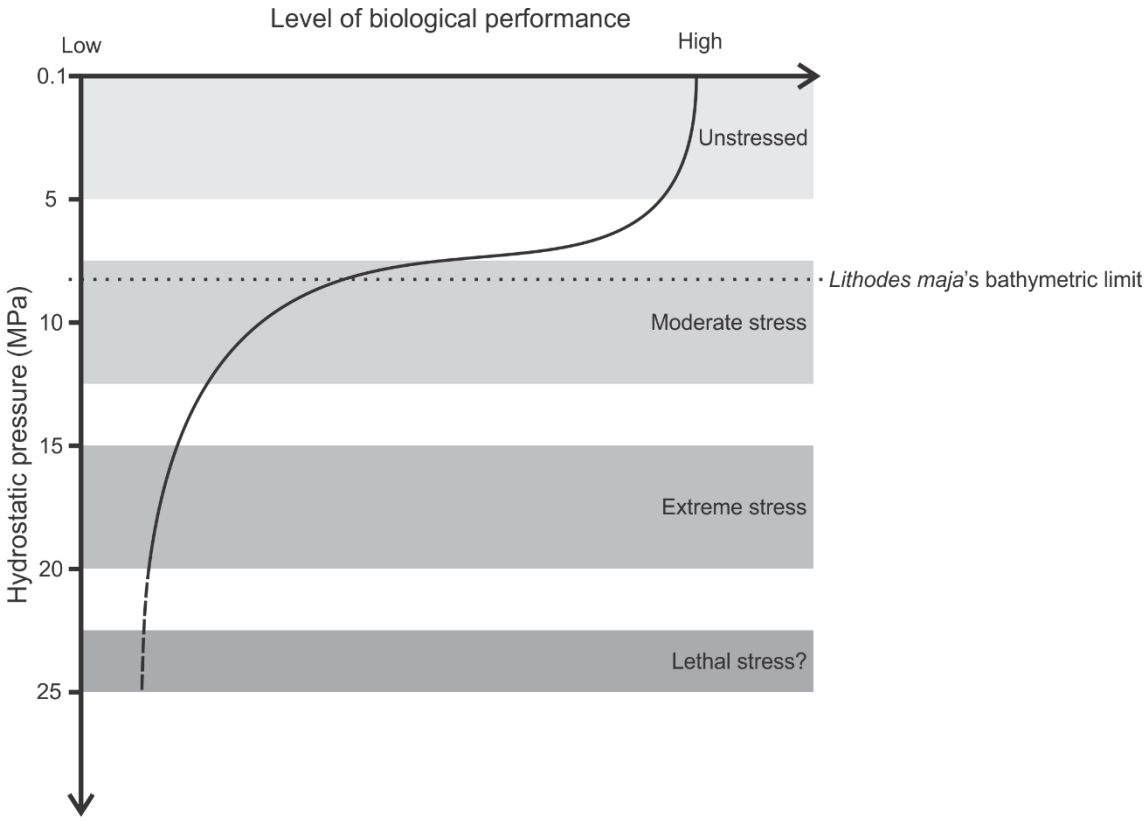

Figure 2.13. Conceptualisation of hyperbaric biological performance in adult male *Lithodes maja* at 6°C. Individuals' biological performance (black line represents experimental data interpretation; dashed line represents extrapolation beyond empirical data) decreases with increasing hydrostatic pressure as hyperbaric impacts mediate transition between stress states (represented by shaded boxes). Elevated maintenance metabolic cost reduces energy available for reproduction, development/growth, and activity. Metabolic costs associated with hyperbaric transition to moderate stress are tolerable, but coincide with the species bathymetric distributional limit, suggesting that increasing metabolic costs may constrain *L. maja*'s bathymetric distribution.

hyperbaric conditions over sustained periods (see Thatje & Robinson, 2011). Energetic costs associated with tolerance of suboptimal environmental conditions are hypothesised to integrate in a matrix of energy-limited distribution (Sokolova *et al.*, 2012; Sokolova, 2013). For example, hyperbaric cardiac acclimation may narrow thermal tolerance, similar to cold cardiac acclimation in the eurythermal fish *Oncorhynchus mykiss* (Aho & Vornanen, 2001). Fundamental ecological niche prediction based on such a matrix would anticipate hyperbaric tolerance peaking at a species' optimum temperature, consistent with *L. maja*'s thermal depth distribution (Fig. 2.12). Species' thermal adaptive history will therefore be key to understanding temperature effects on their hyperbaric tolerance (New *et al.*, 2014).

2.4.3 Ecological and evolutionary implications of energy-limited bathymetric distribution

Energetic limitation of bathymetric range implies that ecological constraints may interact with hyperbaric stress. Estimated organic carbon flux to the seafloor decreases within the bathymetric range of *Lithodes maja* (Corliss & Chen, 1988), implying that a mismatch may develop between bathymetrically decreasing food availability and increasing energy demand with depth. High energy demand in visual predators such as *L*. *maja* are likely to be exacerbated by hyperbaric elevation of metabolic cost, driving the proposed metabolic adaptation of visual taxa in the deep-sea (see Seibel & Drazen, 2007, and references cited therein). Hyperbaric effects on metabolic rate may accentuate increases in total metabolic rate similarly to hypothermal effects, by requiring increased foraging effort to sustain the elevation in metabolic rate (Campbell *et al*., 2007). Seasonal shifts in haemolymph concentration in the North Atlantic endemic bathyal lithodid *Neolithodes grimaldii* are suggested to represent increased activity during periods of low food availability (McAllen *et al.*, 2005), and therefore seasonality may further accentuate the effects of decreasing food availability with depth. Differences in the bathymetric ranges of *Lithodes* spp. may suggest intrageneric variation in hyperbaric tolerance, but all *Lithodes* spp. are constrained to less than ~1250 m (Hall 2010), equivalent to the 12.5 MPa limit to *L*. *maja*'s hydrostatic pressure tolerance. Intrageneric differences in depth limits may instead result from energy limitation, dependent on variation in food availability.

Hyperbaric metabolic costs may contribute to global patterns of standing stock in the deep-sea benthos, which indicate that overall biomass and abundance decrease exponentially with depth as a result of decreasing food availability (Rex *et al.*, 2006). Decreasing food availability with increasing depth results in a bathymetric decline in population density (Rex *et al.*, 2006), which may become too low to remain reproductively viable (Rex *et al.*, 2005*b*). The source-sink hypothesis of abyssal diversity posits that the vulnerability of abyssal populations to chronic abyssal extinction arising from population density effects is only mitigated by immigration from bathyal sources (Rex *et al.*, 2005*b*). Indeed, increased food flux appears capable of mitigating the bathymetric decline in population density and reducing the

dominance of source-sink dynamics (Brault *et al.*, 2013*a*). Energy constraints result in a shift toward dominance of smaller organisms with depth too (Rex *et al.*, 2006); larger organisms have more efficient metabolism per unit weight, but still require more energy than smaller organisms. Consequently, disparity between hyperbaric tolerance and bathymetric range may be expected to increase with increasing size. Decreasing hyperbaric tolerance with increasing size (see Mestre *et al.*, 2013) may have compounded energy limitation, contributing to the prevalence of smaller organisms in the deep sea.

Energy constraints may also contribute to ontogenetic bathymetric distribution patterns reported e.g. for several species of deep-sea lithodids (Somerton, 1981; Somerton & Otto, 1986; Abelló & Macpherson, 1991; Stevens & Lovrich, 2014), as the higher energy requirements of larger individuals may be compounded by ontogenetic decrease in hyperbaric tolerance (see Mestre *et al.*, 2013). Upslope migrations may be an adaptive behaviour reducing adult energetic maintenance costs and allowing increased allocation of energy to reproduction, concomitantly concentrating relatively low density bathyal populations to facilitate reproduction. Reproductive upslope migratory behaviour may also reduce the energetic cost of larval development (see Smith *et al*., 2015) in brooding species.

Projected reductions in the export flux of particulate organic carbon from the surface ocean in response to climate change predict a future decrease in bathyal benthic biomass in most regions, with up to 38% reductions in parts of the northeast Atlantic (Jones *et al.*, 2014). Such decreases in organic inputs to the deep sea provide a natural test for energetic limitation of bathymetric range: maximum depth limits of bathyal fauna can be expected to shallow with decreasing food availability, increasing the inconsistency with critical hyperbaric limits. Similarly, decreasing food input to the deep sea will test the source-sink hypothesis of abyssal diversity. Future reductions in food availability should lead to shallowing of bathyal populations, perhaps resulting in abyssal extirpations. The deep sea has a critical role in global ecological and biogeochemical processes (e.g. Snelgrove, 1999), and deep-sea ecosystem functioning is exponentially linked to benthic biodiversity (Danovaro *et al.*, 2007). Any effects of

changing climate on deep-sea biodiversity may therefore affect the suite of critical services provided by the deep sea (Thurber *et al.*, 2014), with potentially significant anthropic impacts (Mora *et al.*, 2013).

2.4.4 Conclusion

Experimental evidence presented here supports:

Hypothesis 1: that hyperbaric tolerance in *Lithodes maja* is proximately oxygen-limited but ultimately limited by cardiac capacity;

Hypothesis 2: that hyperbaric cardiac capacity is limited through hydrostatic pressure effects on membrane effects;

Hypothesis 3: that *L. maja*'s depth limit coincides with the species' sublethal hyperbaric threshold.

Whether these mechanisms of physiological- and range-limitation are consistent across taxa remains unclear and can only be confirmed by direct experimental exploration. However, the required experimental approaches are extremely time-consuming and resource-intensive. Consequently, an alternative approach is necessary in the short-term to explore the potential ubiquity of these mechanisms.

3. Taxonomic pattern of hyperbaric limitation: support for the hypothesis of oxygen- and capacity-limitation of hyperbaric tolerance*

3.1 Introduction

Extensive experimental assessment of thermal effects on shallow-water marine organisms suggests that thermal tolerance relates directly to an organism's ability to maintain aerobic metabolism (see Pörtner, 2010, and references cited therein). Beyond the range of optimal temperature conditions, biological membrane function is significantly reduced by changes in membrane fluidity, and the efficiency of protein transcription, translation, and replication is reduced by protein denaturation (Pörtner, 2010). The resulting increase in effort required for regulating and maintaining internal conditions (homeostasis) demands increased mitochondrial activity, for example to support elevated synthesis of protein chaperones to counteract interruption of protein structure (Pörtner, 2010). But the increase in oxygen supply delivered by elevated ventilation and circulation does not directly match the increase in mitochondrial oxygen demand (Frederich & Pörtner, 2000; Pörtner, 2010). Consequently, although basic metabolic processes may be maintained beyond optimal conditions, processes not essential to basic life support, such as growth, reproduction, feeding, and voluntary movement, are reduced (Pörtner, 2010). At a population level, this may lead to significant demographic impacts over time; reductions in growth and subsequently in reproductive output may affect species' survival (Pörtner, 2010). For the individual, passing the critical threshold where mitochondrial oxygen demand exceeds the respiratory capacity of the animal results in a mismatch of oxygen supply and demand, and anaerobic respiration ensues ultimately leading to death (Pörtner, 2010).

Available evidence supports a functional association between thermal and hypoxic tolerance (Anttila *et al*., 2013). For example, hyperoxia has been shown to increase the

* Updated from 'Brown, A. & Thatje, S. (2015). The effects of changing climate on faunal depth distributions determine winners and losers. *Global Change Biology* **21**, 173-180'.

critical thermal limit of the Antarctic marine bivalve *Laternula elliptica* (Pörtner *et al*., 2006). Consequently, hypoxia is hypothesised to exacerbate oxygen-limitation of thermal niches, narrowing thermal performance windows and reducing thermal ranges (Pörtner, 2010). Ocean warming and increasing stratification are causing declines in oxygen concentration in the ocean interior, which may be compounded by eutrophication (Keeling *et al*., 2010). Oxygen concentration in the tropical Atlantic and Pacific has declined by 0.9 to 3.4 µmol O_2 kg^{-1} $decade^{-1}$ in the 300 to 700 m layer, with a vertical expansion of the hypoxia zone as the depth of the 60 µmol O_2 kg^{-1} hypoxic horizon shallowed from 245 to 170 m in the eastern Pacific (Stramma *et al*., 2008). In the North Pacific, the best resolved region, declines of 12 µmol O_2 kg^{-1} $decade^{-1}$ occurred between 1987 and 2006 at 1300 m depth off British Columbia (Whitney *et al*., 2007). Similar magnitude declines occurred from 1984 to 2004 off the coast of Southern California, associated with a shallowing of the hypoxic boundary by up to 100 m (Bograd *et al*., 2008). Models predict declines of between 1 and 7% in mean ocean oxygen concentration by 2100, which predominantly affect waters of the upper continental slope and shallower (Keeling *et al*., 2010). Decreasing oxygenation of deeper waters is likely to reduce tolerance of the lower temperatures that prevail with increasing depth, decreasing the depth of fundamental ecological niches (FENs). However, the bathymetric limit of FENs may not be determined by oxygen concentration and temperature alone.

The extant deep-sea fauna are understood to derive predominantly from colonisations and radiations by shallow-water organisms, and consequently widespread patterns of bathymetric zonation on continental margins have been interpreted as evidence of a physiological bottleneck, imposed by the effects of high hydrostatic pressure and low temperature (Brown & Thatje, 2011). Low temperature and high hydrostatic pressure are, therefore, perceived as key factors that physiologically limit submergence of shallow-water taxa. Technical challenges have restricted experimental assessments of physiology under sustained pressure, and there is little evidence of the mechanisms constraining pressure tolerance. However, observations of respiratory and cardiac responses to pressure change appear to support the application of the oxygen- and capacity-limitation hypothesis to hydrostatic pressure tolerance (Mickel & Childress,

1982*b*,*c*; Airriess & Childress, 1994; Thatje & Robinson, 2011; see Chapter 2). There are also consistent indications that voluntary movement and feeding are affected by hyperbaric conditions beyond optimum (Thatje *et al*., 2010; Oliphant *et al*., 2011; Thatje & Robinson, 2011). If oxygen- and capacity-limitation constrains tolerance of increased hydrostatic pressure, *a priori* assumptions suggest that any variation in hyperbaric tolerance among taxonomic groups would be significantly correlated with variation in hypoxia tolerance among taxonomic groups. This hypothesis may be tested by comparing hypoxic thresholds of different benthic marine groups, identified using a data set compiled by Vaquer-Sunyer & Duarte (2008), with hyperbaric thresholds of different benthic marine groups, identified by pooling data from existing hyperbaric studies. Whilst it is not possible to attribute correlation in hypoxic and hyperbaric thresholds to a specific mechanism, this can offer support for the existing hypothesis of oxygen- and capacity-limited hyperbaric tolerance. It is proposed that:

Hypothesis 1: hyperbaric thresholds vary significantly among taxonomic groups of shallow-water fauna;

Hypothesis 2: hyperbaric and hypoxic thresholds of taxonomic groups of shallow-water fauna correlate.

3.2 Materials and Methods

3.2.1 Data collection

Literature searches were performed using the keywords “hyperbaric”, “pressure”, “shallow-water”, and “marine”, and their combinations to guide the search. Published reports of shallow-water marine organisms’ responses to hydrostatic pressure were then examined to identify the most sensitive measure permitting comparison across the broadest range of phyla (following groupings in Vaquer-Sunyer & Duarte, 2008 to facilitate direct comparison of taxonomic patterns in pressure thresholds and oxygen concentrations thresholds; minimum $n = 3$). Experimental assessments examining 1-hour lethal pressure thresholds (statistically derived pressure at which 50% of test animals die; LP_{50}) provided data for the greatest number of species of marine

organisms across the widest range of taxonomic groups. Although multiple values of LP_{50} exist for several species, a single representative value for each species was required to avoid biasing the pressure threshold of any taxonomic group toward an individual species. To eliminate the potential effect of temperature stress the LP_{50} derived at the temperature most similar to maintenance or sampling temperature was selected as the representative value where studies examined thresholds at multiple temperatures. A pattern of decreasing pressure tolerance with advancing ontogeny has recently been highlighted (Mestre *et al*., 2013), therefore the LP_{50} of the most ontogenetically advanced stage was adopted as the representative value for a species where multiple life-history stages were examined. Although patterns in the hyperbaric tolerance of pelagic fauna of different taxonomic groups are expected to be similar to those of benthic and demersal fauna, it is anticipated that absolute tolerances may differ as a result of contrasting adaptations in differing habits, for example diel vertical migration in pelagic taxa. Insufficient data were available to represent pelagic fauna of different taxonomic groups independently from benthic and demersal fauna, and consequently pelagic taxa were excluded to reduce potential confounding by contrasting adaptations to benthic and demersal fauna.

Data for sublethal oxygen concentration thresholds of shallow-water benthic and demersal marine organisms (oxygen concentration at or below which 50% of test animals exhibit sublethal responses, or at or below which there is a significant effect on the response measure; SLC_{50}) were identified from Vaquer-Sunyer & Duarte, 2008. Sublethal thresholds were selected as the most sensitive measure permitting comparison across the broadest range of taxonomic groups. Data were treated as described for lethal hydrostatic pressure threshold data. Selection of data for the most advanced life-history stage was again appropriate, since hypoxia typically impacts larger animals first (Clark *et al*., 2013*a*). Where multiple values for SLC_{50} remained, the lowest sublethal oxygen concentration value was selected as representing the most acclimatised or adapted population of the species. Both field and laboratory studies were included; results are unlikely to be biased by differences in variance and replication (Hillebrand & Gurevitch, 2014).

3.2.2 Statistical analysis

LP_{50} and SLC_{50} data (Tables 3.1, 3.2) were square-root transformed to achieve normality and equal variance (Shapiro-Wilk and Levene tests respectively; $p > 0.05$). ANOVA was used to test for differences in thresholds among taxonomic groups, and the Holm-Sidak post-hoc test was used to determine significant differences between mean threshold values among taxa ($\alpha = 0.05$). Covariance in mean lethal pressure thresholds and sublethal oxygen concentration thresholds was examined by testing their correlation (Pearson product-moment correlation).

3.3 Results

Lethal pressure thresholds and sublethal oxygen concentration thresholds are significantly different among marine taxa ($F_{4,30} = 15.033$, $p < 0.001$, and $F_{4,35} = 12.321$, $p < 0.001$ respectively), and show strikingly similar patterns of variation (Fig. 3.1). Fishes are significantly less tolerant of increased pressure or decreased oxygen concentration than all other taxonomic groups. Crustaceans are significantly more tolerant of increased pressure or decreased oxygen concentration than fishes, but significantly less tolerant of these challenges than polychaetes, molluscs, and echinoderms. There are no significant differences between the tolerances of polychaetes, molluscs, or echinoderms to increased pressure or decreased oxygen concentration. Mean lethal pressure thresholds and sublethal oxygen thresholds are significantly inversely correlated (Pearson's $r = -0.983$, d.f. = 3, $p = 0.003$; $r^2 = 0.966$; Fig. 3.2).

3.4 Discussion

Caution is required in interpreting the significant correlation between hypoxic and hyperbaric thresholds evidenced here, since available data are limited and the taxonomic sampling is not paired, and since correlation does not necessarily indicate causation. However, the correlation supports the application of the oxygen- and capacity-limitation hypothesis, originally conceived to explain thermal tolerance (Pörtner, 2010), to hydrostatic pressure tolerance. It is, perhaps, unsurprising that

Table 3.1. 1-hour lethal pressure threshold (statistically derived pressure at which 50% of test animals die; LP_{50}) data and sources.

Taxon	Species	LP_{50} (MPa)	Source
CRUSTACEA	*Balanus* sp.	89	Selvakumaran *et al*., 1974
	Carcinus maenas	34	Naroska, 1968
	Crangon crangon	23	Naroska, 1968
	Eupagurus bernhardus	12	Naroska, 1968
	Eurypanopeus sp.	23	Menzies & Selvakumaran, 1974
	Gammarus oceanicus	55	Naroska, 1968
	Idotea baltica	50	Naroska, 1968
	Jaera albifrons	77	Naroska, 1968
	Latreutes fucorum	20	Menzies & Selvakumaran, 1974
	Lepidactylus sp	42	Menzies & Selvakumaran, 1974
	Pagurus longicarpus	16	Menzies & Selvakumaran, 1974
	Petrolisthes armatus	19	Menzies & Selvakumaran, 1974
	Sesarma reticulatum	29	Selvakumaran *et al*., 1974
	Sphaeroma quadridentatum	40	Menzies & Selvakumaran, 1974
	Uca pugilator	23	Avent, 1974
ECHINODERMATA	*Amphioplus macilentus*	79	Selvakumaran *et al*., 1974
	Asterias rubens	75	Naroska, 1968
	Psammechinus miliaris	80	Naroska, 1968
FISHES	*Fundulus heteroclitus*	9	Selvakumaran *et al*., 1974
	Platichthys flesus	14	Naroska, 1968
	Pleuronectes platessa	15	Naroska, 1968
	Stephanolepis hispidus	18	Menzies & Selvakumaran, 1974
	Urophycis sp.	2	Selvakumaran *et al*., 1974
	Zoarces viviparus	37	Naroska, 1968
MOLLUSCA	*Aplysia protea*	83	Menzies & Selvakumaran, 1974
	Arctica islandica	73	Naroska, 1968
	Donax variabilis	56	Menzies & Selvakumaran, 1974
	Littorina irrorata	94	Menzies & Selvakumaran, 1974
	Littorina littorea	75	Naroska, 1968
	Modiolus modiolus	75	Naroska, 1968
	Mya arenaria	75	Naroska, 1968
	Mytilus edulis	80	Naroska, 1968
POLYCHAETA	*Arenicola marina*	52	Naroska, 1968
	Nereis diversicolor	78	Naroska, 1968
	Nereis occidentalis	82	Selvakumaran *et al*., 1974

both thermal and hyperbaric tolerance may be limited by the same mechanism; the effects of high hydrostatic pressure on biological membranes and proteins are similar to the effects of low temperature, and low temperature and high hydrostatic pressure both require increases in homeostatic effort (Brown & Thatje, 2014). As previously discussed, oxygen limitation of thermal tolerance depends proximately on capacity-

Table 3.2. Sublethal oxygen concentration threshold (oxygen concentration at or below which 50% of test animals exhibit sublethal responses; SLC_{50}) data and sources, adapted from Vaquer-Sunyer & Duarte (2008). Data recalculated from sources.

Taxon	Species	SLC_{50} (μmol O_2 l^{-1})	Source
CRUSTACEA	*Callinectes sapidus*	64	Pihl *et al*., 1991
	Calocaris macandreae	93	Anderson *et al*., 1991
	Carcinus maenas	116	Hill *et al*., 1991
	Crangon crangon	133	Sandberg *et al*., 1996
	Monoporeia affinis	125	Johansson, 1997
	Munida quadrispina	10	Burd & Brinkhurst, 1984
	Penaeus aztecus	92	Renaud, 1986
	Penaeus monodon	99	Seidman & Lawrence, 1985
	Penaeus schmitti	160	Rosas *et al*., 1997
	Penaeus setiferus	160	Rosas *et al*., 1997
	Penaeus vannamei	61	Seidman & Lawrence, 1985
	Sanduria entomon	64	Johansson, 1997
	Squilla empusa	77	Pihl *et al*., 1991
ECHINODERMATA	*Amphiura chiaje*	25	Rosenberg *et al*., 1991
	Amphiura filiformis	32	Vistisen & Vismann, 1997
	Echinocardium cordatum	25	Nilsson & Rosenberg, 1994
	Holothuria forskali	27	Astall & Jones, 1991
	Luidia clathrata	81	Diehl *et al*., 1979
	Ophiura albida	32	Vistisen & Vismann, 1997
FISHES	*Callionymus lyra*	212	Hughes & Umezawa, 1968*a*
	Diplodus puntazzo	77	Valverde & Garcia, 2004
	Fundulus heteroclitus	144	Voyer & Hennekey, 1972
	Gadus morhua	55	Schurmann & Steffensen, 1997
	Gadus macrocephalus	144	Alderdice & Forrester, 1971
	Hydrolagus colliel	274	Hanson, 1967
	Rhacochilus vacca	253	Webb & Brett, 1972
	Scyliorhinus canicula	140	Hughes & Umezawa, 1968*b*
	Squalus suckleyi	212	Lenfant & Johansen, 1966
MOLLUSCA	*Abra alba*	19	Jorgensen, 1980
	Cerastoderma edule	19	Jorgensen, 1980
	Hydrobia ulvae	19	Jorgensen, 1980
	Mya arenaria	19	Jorgensen, 1980
	Mysella bidentata	32	Nilsson & Rosenberg, 1994
	Octopus vulgaris	145	Valverde & Garcia, 2005
	Theora fragilis	41	Tamai, 1996
POLYCHAETA	*Capitella capitata*	26	Warren, 1977
	Loimia medusa	33	Llanso & Diaz, 1994
	Pectinaria koreni	32	Nilsson & Rosenberg, 1994
	Scoloplos armiger	36	Schottler & Grieshaber, 1988
	Streblospio benedicti	16	Llanso, 1991

limitation of ventilation and circulation (Pörtner, 2002). Progressive failure of the thermally sensitive membrane transporter Na^+,K^+-ATPase contributes to thermal limitation of cardiac capacity (Stillman, 2002); Na^+,K^+-ATPase is critical to maintaining membrane potential required to generate action potentials in excitable cells such as

Constraint of bathymetric distribution by hydrostatic pressure

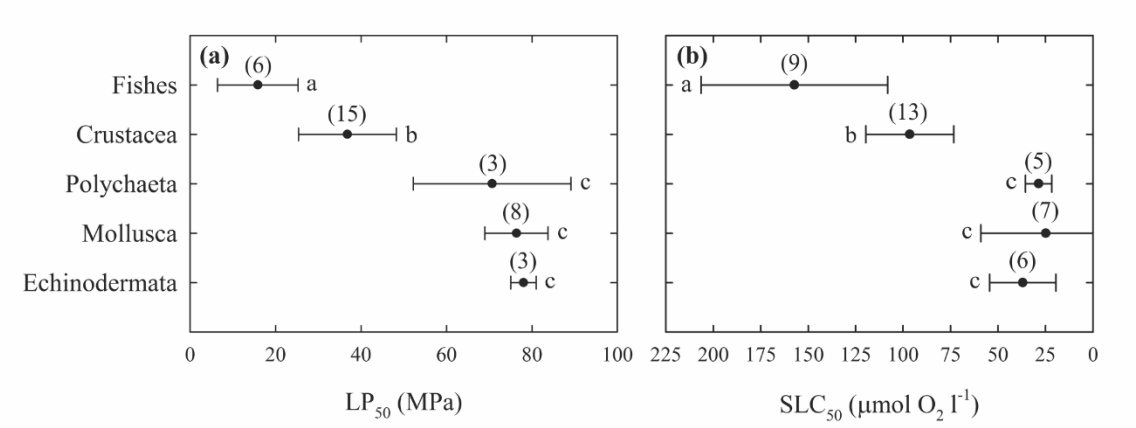

Figure 3.1. Distributions of (a) hyperbaric and (b) hypoxic tolerance thresholds among shallow-water marine benthic invertebrates and demersal fishes. Hyperbaric LP_{50} and hypoxic SLC_{50} tolerance thresholds identified by analysis of pooled data from published studies. Error bars represent 95% confidence intervals; numbers in brackets represent the number of species used to calculate the mean value of each taxonomic group. Letters indicate significant differences.

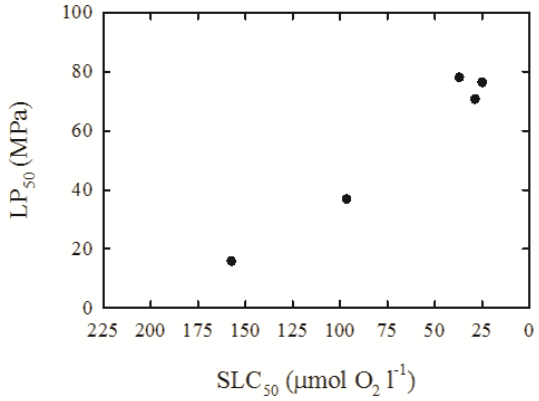

Figure 3.2. Correlation between hypoxic and hyperbaric tolerance thresholds of taxonomic groups of shallow-water benthic invertebrates and demersal fishes; $r^2 = 0.966$.

those regulating cardiac function (see Lodish et al., 2003). Progressive failure of Na^+,K^+-ATPase likely results from thermal effects on membrane fluidity, which stimulate conformational changes in membrane-bound proteins, progressively impacting protein function (Tillman & Cascio, 2003). Lipid bilayers of biological membranes are one of the most pressure sensitive cellular structures (Somero, 1992). Pressure increase reduces the fluidity of biological membranes: a pressure increase of 10 MPa is equivalent to a decrease in temperature of approximately 1.3 to 2.1°C, depending on membrane composition (Somero, 1992). Pressure effects on membrane fluidity stimulate conformational changes in membrane bound proteins too (Tillman & Cascio, 2003),

and Na^+,K^+-ATPase fails progressively under hyperbaric conditions (Gibbs & Somero, 1989), potentially causing observed hyperbaric decreases in excitatory junction potential amplitude at the neuromuscular junction by impeding neurotransmitter release (Campenot, 1975*a,b*), and thus impairing cardiac capacity. Critical pressure effects on Na^+,K^+-ATPase are supported by enzymatic functional pressure-adaptation in deep-sea organisms (Gibbs & Somero, 1989). Mechanisms of thermal and hyperbaric limitation of cardiac capacity are therefore expected to be similar. Whilst increased homeostatic effort may moderate the effects of hydrostatic pressure, for example through elevated protein chaperoning (Cottin *et al*., 2012), increased mitochondrial oxygen demand required for increased homeostatic effort is not matched by the increase in respiratory capacity delivered through elevated ventilation and circulation at low temperature (Frederich & Pörtner, 2000). Ventilation and circulation also fail to deliver required oxygen levels under high hydrostatic pressure conditions (see Chapter 2), and survival will be time-limited beyond the critical threshold where anaerobic mitochondrial respiration ensues. Consequently, the fundamental environmental niches of marine ectotherms therefore appear constrained by a matrix of oxygen- and capacity-limited tolerance combining the physiological effects of temperature, hydrostatic pressure, and oxygen concentration (adapted from Pörtner, 2010). The fundamental assumptions underlying this model imply a taxonomic hierarchy of thermal limitation which may be tested by assessing differences in thermal tolerance thresholds among taxonomic groups. Since species' thermal adaptive histories are likely to affect their thermal scope it will be critical to compare the tolerances of taxonomic groups from different thermal regimes independently (e.g. intertidal, subtidal, tropical, temperate, or polar).

Differences in tolerance thresholds among taxonomic groups reflect the pattern of ionic regulation (ionic regulation is typically slight in echinoderms, molluscs, and polychaetes relative to crustaceans and fishes; Evans, 2009), supporting a critical role for membrane function in environmental tolerance. However, intraspecific ontogenetic variation in temperature or hydrostatic pressure tolerance is putatively due to increasing structural and/or functional complexity (Pörtner & Farrell, 2008; Mestre *et al*., 2013), and disparity in environmental tolerance thresholds may also

depend on structural and/or functional complexity too. For example, differences in hypoxia thresholds among taxonomic groups are proposed to relate to metabolic and behavioural adaptations to low-oxygen conditions (Vaquer-Sunyer & Duarte, 2008). Assessing complexity's contribution to differing tolerance among taxonomic groups is problematic – defining a complexity metric is challenging – but the oxygen- and capacity-limitation hypothesis implies that any attempt to do so must focus on respiratory system complexity. Resource-transport theory may prove apposite in this context (see e.g. Glazier, 2010; Glazier *et al*., 2015).

Variation in sensitivity to oxygen-and capacity-limitation among taxa, revealed here, and in sensitivity to carbon dioxide concentrations (Kroeker *et al*., 2010, 2013; Wittmann & Pörtner, 2013), suggests that phylogenetic physiological variation may have affected the evolution of extant marine community structure, contributing to the reduced relative contribution of fishes and crustaceans to biodiversity in polar regions and in the deep sea (see e.g. Gage & Tyler, 1991). Differences in tolerance thresholds among taxonomic groups also suggest that taxonomic groups will respond differently to ocean warming, deoxygenation, and increasing carbon dioxide concentration. This is already evident from recent analysis of the global imprint of climate change on marine life, where bony fish demonstrate the greatest change in latitudinal distribution among marine animals (Poloczanska *et al*., 2013), and may also be expected in depth distribution.

3.4.1 Conclusion

Evidence presented here supports:

Hypothesis 1: that hyperbaric thresholds vary significantly among taxonomic groups of shallow-water fauna;

Hypothesis 2: that hyperbaric and hypoxic thresholds of taxonomic groups of shallow-water fauna correlate.

Consquently, hyperbaric tolerance appears oxygen- and capacity-limited across taxonomic groups. Evidence elucidating the mechanism limiting hydrostatic pressure

tolerance is a significant development in hyperbaric physiology, and may also provide a fundamental step to understanding the role of hydrostatic pressure in the evolution and radiation of organisms colonising the deep sea.

4. Synthesis

4.1 Do lineage-specific tolerances contribute to global phylogenetic bottlenecks?

Evidence presented here indicates that hyperbaric tolerance is oxygen- and capacity-limited, contributing to a matrix of oxygen- and capacity-limited environmental tolerance that defines the physiological limits to organisms' distributions. Lineage-specific physiological adaptation may be expected to contribute to global phylogenetic bottlenecks, and thereby structure lineage-specific distributions. Indeed, patterns of bathymetric zonation suggest a role for evolutionary adaptation to environmental conditions (Brown & Thatje, 2014; Wagstaff *et al*., 2014). Assessing the contribution of physiological limitation to global phylogenetic bottlenecks requires a taxonomic group with a well-constrained evolutionary history and phylogeography. The lithodid crabs represent such a group: the phylogeny and biogeography of the Lithodidae are well resolved (Hall & Thatje, 2009; Hall, 2010).

Thermal tolerances appear to have played a critical role in determining the global distribution of lithodids (Hall & Thatje, 2009), and appear to continue to do so, for example by restricting colonisation of the Antarctic continental shelf (Hall & Thatje, 2011). Adults of North Pacific lithodid taxa occur in regions with water temperatures between 0 and 25°C, whereas deep-water lithodid lineages are excluded from regions with water temperatures greater than 13°C (Hall & Thatje, 2009). In contrast, differences in thermal tolerances among lithodid lineages do not appear to explain contrasting bathymetric distributions. Adverse effects of hydrostatic pressure on the shallow-water anomuran crabs *Pleuroncodes planipes* and *Pagurus cuanensis* at pressures greater than ~300 m water depth (Quentin & Childress, 1976; Thatje *et al*., 2010) may be indicative of anomuran synapomorphic (see Bracken-Grissom *et al*., 2013) pressure intolerance, which may persist in shallow-water lithodids and contribute to depth restriction. In contrast, *Lithodes maja* is not significantly affected by hydrostatic pressures equivalent to ~500 m water depth, indicating physiological adaptation to greater hydrostatic pressure. Intergeneric differences in bathymetric

range are evident amongst deep-sea lithodids too (Hall, 2010), and may reflect further differences in hyperbaric tolerance suggesting progressive adaptation to hydrostatic pressure. Whilst *Lithodes* spp. are limited to less than ~1250 m depth, *Paralomis* spp. are limited to less than ~2000 m depth, and *Neolithodes* spp. are limited to less than ~3300 m depth (Hall, 2010), suggesting genera-specific dispersal pathways along depth strata. Intraspecific adaptation across thermal gradients indicates that adaptation in cardiac physiology is critical to invasion of novel thermal conditions (Tepolt & Somero, 2014) and hyperbaric adaptation in cardiac function may also be necessary for successful colonisation of the deep sea. Adaptation in membranes and membrane-related functions may have a critical role in cardiac function plasticity through effects on synaptic processes (see Somero, 2002), and also appear fundamental to improving hyperbaric metabolic efficiency (see Sébert *et al*., 2009), but adaptation in haemolymph vascular system morphology (Keiler *et al*., 2013) may moderate the effects of reducing cardiac activity by increasing stroke volume. Clearly, additional comparative hyperbaric physiology experiments on shallow-water lithodids are required to establish unequivocal differences in hyperbaric limits among lithodid taxa, but it appears that hydrostatic pressure tolerance represents a phylogenetic bottleneck that has contributed to the phylogeography of lithodids.

However, adult lithodid tolerances do not reflect larval lithodid tolerances: North Pacific shallow-water lithodid species' distributions are limited to regions with water temperatures below 16°C during periods of larval development despite tolerance of higher temperatures in adults, indicating that larval high-temperature tolerance is biogeographically limiting in shallow-water lithodid species (Hall & Thatje, 2009). Shallow-water lithodid taxa appear unable to develop at constant low temperatures representative of the deep North Pacific (e.g. larval development to juvenile appears restricted to temperatures ≥8°C in *Paralithodes camtschaticus*; Kurata, 1960; Swingle *et al*., 2013). In contrast, larval thermal tolerance does not appear to limit shallow-water latitudinal biogeography in deep-sea lithodid lineages: deep-water lineage adults are exluded from waters exceeding temperatures of 13°C (Hall & Thatje, 2009) despite larval thermal tolerance of higher temperatures in at least some species (e.g. Anger et al., 2003, 2004).

Lineage-specific differences in lithodid larval thermal tolerance co-occur with differences in larval developmental mode. Deep-sea taxa have evolved lecithotrophic and abbreviated development, whereas shallow-water taxa predominantly retain planktotrophic development (Hall, 2010). Lecithotrophy (inferred from egg size >1.2 mm; Hall, 2010) in some shallow-water *Paralithodes* spp. suggests that endotrophic larval development may have evolved prior to the lithodid deep sea invasion, and therefore that lecithotrophy is not sufficient to remove restrictions to depth distributions alone. Further increases in per offspring investment (POI) may have been required to permit bathymetric range extensions (Smith *et al*., 2015).

Thermal plasticity in POI emerges from differing thermal effects on egg production (differentiation) and yolk production (growth), and has been examined in the shallow-water shrimp *Palaemonetes varians* (see Oliphant & Thatje, 2013). Energy loss through development is greater at temperatures below optimum due to increased developmental time and increased metabolic cost, suggesting greater allocation to maintenance effort (Oliphant & Thatje, 2014). Offspring benefit from greater POI, reaching juvenile with greater mass (and presumably fitness) (Oliphant & Thatje, 2013), therefore natural selection operating on temperature-driven plasticity in POI at low temperature may result in increasing POI and eventually lecithotrophy (Oliphant, 2014). Increasing POI also results in abbreviated development (see Oliphant & Thatje, 2013; Oliphant *et al*., 2014). Developmental plasticity in at least some shallow-water anomurans appears similar to that demonstrated in *P. varians* (Boyd & Johnson, 1963; Diaz & Costlow, 1987; Oliphant *et al*., 2013), and developmental variability is retained in at least some deep-sea lithodids with lecithotrophic development (Shirley & Zhou, 1997), possibly relating to significant intraspecific variability in POI (e.g. Anger, 1996; Thatje & Mestre, 2010). This plasticity and variability provide a mechanism for the evolution of lecithotrophy in lithodids, generating potential for increases in POI through natural selection during deep-sea colonisation. Similar factors are perceived to drive developmental selection in polar and deep-sea environments (Thorson, 1950; Thiel *et al*., 1996), and decreasing temperature and diminishing seasonal thermal variation with increasing depth (e.g. see Hall & Thatje, 2009) may therefore have selected for elevated POI and lecithotrophy in *Paralithodes californiensis* and *P*.

rathbuni. *Paralithodes* spp. retaining planktotrophic development are known to migrate seasonally to shallower depths during periods of larval release (e.g. *P*. *camtschaticus*; Stone *et al.*, 1992), but whether *P*. *californiensis* or *P*. *rathbuni* migrate bathymetrically remains unknown.

The bathymetric temperature trend appears likely to have selected for elevated POI and lecithotrophy, and abbreviated development in the incipient deep-sea lithodids. Lithodid egg size, a proxy for POI, certainly increases with depth (Morley *et al*., 2006; Hall, 2010). However, it remains unclear whether hydrostatic pressure effects have also contributed to the depth-related trend in egg size. Experimental assessment of hyperbaric impacts on lecithotrophic development in the whelk *Buccinum undatum* has revealed that elevated hydrostatic pressure decreases developmental rate and increases homeostatic effort similarly to low temperature, yielding reduced metabolic developmental efficiency (Smith *et al*., 2015). Consequently, natural selection acting on variation in fitness at elevated hydrostatic pressure related to developmental plasticity may additionally favour increases in POI (Smith *et al*., 2015). Thermal effects on variability in egg size may be enhanced by the bathymetric adaptation in temperature normalised metabolic rate (Fig. 2.9).

An increasing mismatch in developmental time and food availability may also select for increasing POI (Thatje *et al*., 2005*a*), thus decreasing food availability at increasing depth may compound temperature-driven selection for increasing POI. However, the paradigm of a food poor deep-sea environment requires revision (see Danovaro *et al*., 2014, and references cited therein). Laterally advected organic flux to continental margins can be high (see Danovaro *et al*., 2014), including on the northeast Pacific continental slope (e.g. Bishop *et al*., 1999), the putative habitat of the incipient lithodines (Zaklan, 2002*a*). Food bioavailability can also be elevated relative to continental shelf habitat (see Danovaro *et al*., 2014). Despite this, temporal variation in food input is sufficient to stimulate seasonal responses in abundance and biomass, and in reproduction in some bathyal species on continental slopes (see Gooday *et al*., 2002, and references cited therein), indicating that the potential for mismatches in developmental time and food inputs remain.

The potential contribution of hypothermal and hyperbaric physiological demands to bathymetric range limitation and to natural selection for lecithotrophy represent physiologically induced phylogenetic bottlenecks, with fundamental consequences for lithodid phylogeography. Hyperbaric-intolerant taxa remain constrained to shallow-water North Pacific, whereas those tolerant of greater hydrostatic pressure have dispersed globally (see Hall & Thatje, 2009). Early life history patterns appear to play a key role in defining radiation and speciation potential too (e.g. giving rise to the endemism and diversity of Antarctic benthic invertebrates; Clarke & Crame, 1992; Thatje, 2012), and it therefore seems likely that developmental mode and traits may influence bathymetric speciation patterns. Although poor decapod taphonomy precludes conclusive palaeontological assessment of variation in speciation rates among lithodid genera, differences in extant lithodid lineages are strongly suggestive of phylogenetic impacts of lineage-specific tolerances. Whilst shallow-water lithodid radiation appears evolutionary in shallow-water, the deep-sea lithodid radiation appears predominantly adaptive with phylogenetic niche conservatism (see Pryon *et al*., 2014). Greater genus level innovation in the notably morphologically diverse shallow-water lithodids (11 genera; 21 species) contrasts with the significantly greater species level innovation in deep-sea lithodids (4 genera; 108 species) (McLaughlin *et al*., 2010). However, considerable variation in species diversity persists in the deep-sea genera: there are 29 extant *Lithodes* species and 66 extant *Paralomis* species (McLaughlin *et al*., 2010), despite *Lithodes* diverging ~10 myr earlier (Bracken-Grissom *et al*., 2013). Only 12 *Neolithodes* species are known (McLaughlin *et al*., 2010).

Intraspecific and intrageneric genetic diversity are low in examined lithodines relative to other anomurans (Hall, 2010; da Silva *et al*., 2011). Relatively low genetic diversity likely reflects the recent rapid lithodid radiation (<19 myr; Bracken-Grissom *et al*., 2013), but may also result from low mutation rate and/or high gene flow in deep-sea lithodid taxa (Hall, 2010). Substantial dispersal potential can increase gene flow and may be conferred by relatively protracted larval development at representative bathyal temperatures (~87 days at 6°C and 0.1 MPa in *L*. *maja*; own unpublished data) and demersal drifting (Thatje *et al*., 2005*a*; Hall, 2010). High dispersal potential may also have facilitated large geographic ranges in deep-sea lithodids (see Zaklan, 2002*b*)

and rapid global dispersal. Lithodid larval dispersal capability may have been underestimated previously as a result of experimental influences on larval activity. *Lithodes maja* larvae reared in 24-hour darkness appear qualitatively more active than previously examined deep-sea lithodid larvae reared under 12:12-hour light:dark cycling (e.g. Anger *et al*., 2003; Calcagno *et al*., 2003; Thatje *et al*., 2003; S. Thatje, personal communication). Brooding female *L*. *maja* also orient in a rostrum upward aspect when releasing larvae in aquaria (personal observation), which may export larvae from the slowest part of the benthic boundary layer in the wild, increasing dispersal potential. Females releasing larvae at elevation will similarly improve larval dispersal capability, and lithodids on the Antarctic continental slope are often observed elevated above the surrounding seabed, perched on dropstones (personal observation). Adult lithodines themselves are capable of covering long distances too (e.g. 13.1 km day^{-1} in *P*. *camtschaticus*; Stone *et al*., 1992), and female lithodines brood larvae for sustained periods (e.g. >300 days at 6°C and 0.1 MPa in *L*. *maja*, own unpublished data), potentially further enhancing dispersal potential.

It remains unclear whether hyperbaric physiological limitation contributes to the maximum depth limit of the only other species with a shallow to deep distribution in which hyperbaric tolerance has been examined extensively. Although embryonic development to veliger in the whelk *Buccinum undatum* occurs at ecologically relevant temperatures at hydrostatic pressures equivalent to ~2000 m depth (considerably beyond the species' known 465 m bathymetric distribution; Fig. 4.1), it demands higher metabolic cost and proceeds at a slower rate than at surface pressure (Smith *et al*., 2015). In accordance with the general pattern of increasing hyperbaric sensitivity with ontogeny (see Mestre *et al*., 2013) juvenile *B. undatum* are less tolerant of hydrostatic pressure than veliger *B*. *undatum* (Smith & Thatje, 2012), suggesting that the pressure tolerance of adult *B*. *undatum* may be lower still. Distributional limits in *B*. *undatum* may reflect constraints to adult survival because larval development is intracapsular attached to the substratum, and individuals hatch as crawling juveniles (Smith & Thatje, 2013). However, the species' maximum depth distribution does occur close to the species experimentally determined 6°C larval thermal optima (Fig. 4.1; Smith & Thatje, 2012; OBIS, 2013; Smith *et al*., 2013*b*), suggesting that temperature

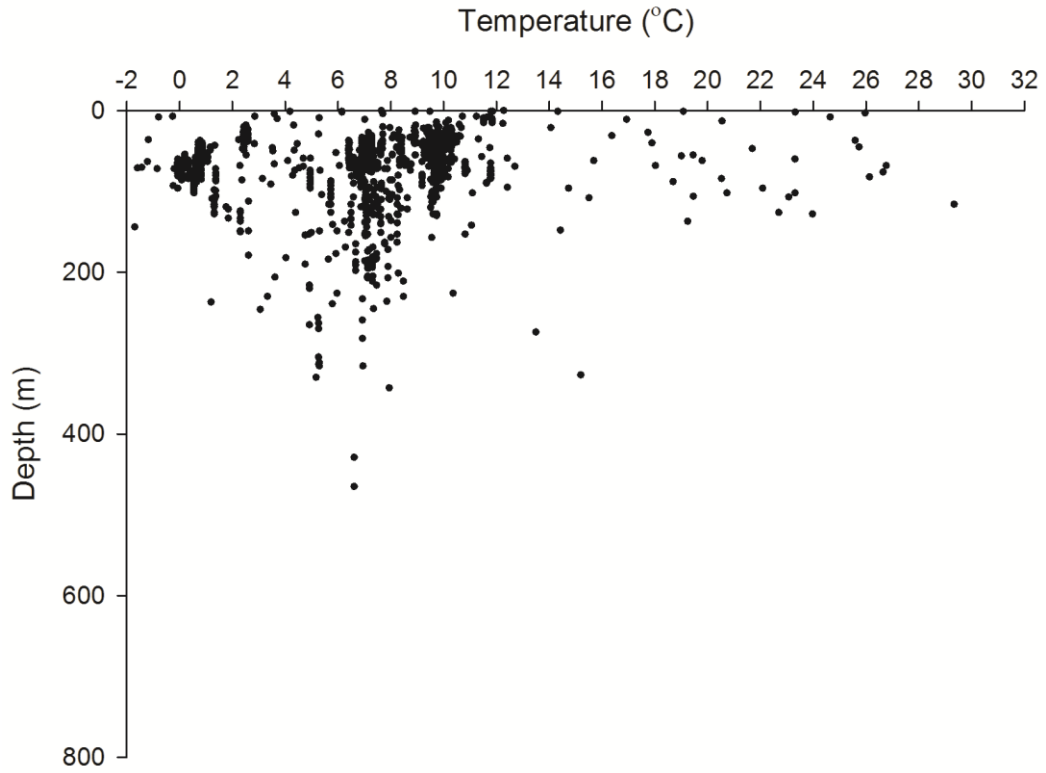

Figure 4.1. Bottom depth and temperature of *Buccinum undatum* occurrences. Each point represents one record (OBIS, 2013).

and hydrostatic pressure do interact. The persistence of some populations of *B. undatum* through the Last Glacial Maximum (LGM; approximately 20 thousand years ago) may have depended on this pressure tolerance (Pálsson *et al*., 2014), providing an additional mechanism through which lineage-specific physiological tolerances may contribute to phylogenetic bottlenecks: hydrostatic pressure intolerant lineages may have been locally extirpated or entirely eradicated as postulated for the Antarctic continental shelf fauna during glacial periods (Thatje *et al*., 2005*b*). *B. undatum*'s wide thermal range approaching its maximum depth limit may also have contributed to the persistence of populations during the LGM.

It appears that lineage-specific physiological tolerances, which may be identified through phylogeographic bottlenecks, do indeed contribute to phylogenetic bottlenecks. Hyperbaric limitation of bathymetric range demonstrated and discussed here supports the proposed critical role of hydrostatic pressure in structuring bathymetric zonation in the deep sea. However, it is not immediately clear how the

physiological effects of hydrostatic pressure or low temperature could contribute to the unimodal pattern of diversity with depth, despite diversity typically peaking at similar depths to the proposed physiological bottleneck, even though the area represented by these depths is relatively low. Is there other evidence suggesting a cause for this bathymetric diversity phenomenon?

4.2 Bathymetric variation in evolution*

Variation in the production of novel taxa has been identified as causative of another, somewhat analogous, evolutionary pattern in biodiversity. A meta-analysis of nearly 600 latitudinal biodiversity gradients assembled from the literature has corroborated the high generality of the latitudinal diversity decline, including for the marine environment of both hemispheres (Hillebrand, 2004*a*,*b*), a phenomenon, which has prompted much discussion (e.g. see Rohde, 1992). Mid-domain effects have been shown to be inconsistent with these broad-scale patterns of species richness (Currie & Kerr, 2008). Recent assessment of the global patterns and predictors of marine biodiversity identified sea surface temperature as the only environmental predictor related to diversity across all taxa examined (Tittensor *et al*., 2010), in agreement with the "out of the tropics" dynamic (Jablonski *et al*., 2006) and the time hypotheses (Mittelbach *et al*., 2007). Since biodiversity patterns appear to be driven by speciation rates (Allen & Gillooly, 2006), kinetic effects of temperature on rates of genetic divergence and speciation have been proposed as the mechanism by which temperature plays a fundamental role in structuring cross-taxon marine biodiversity (Allen *et al*., 2002, 2006; Brown *et al*., 2004; Tittensor *et al*., 2010). These are likely manifested through the effects of metabolic rate on generation time (Thomas *et al*., 2010; Lehtonen & Lanfear, 2014) and mutation rate (Gillooly *et al*., 2001, 2005; Savage *et al*., 2004; but see Held, 2001; Lanfear *et al*., 2007; Matsuba et al., 2012), two fundamental variables influencing the rate of evolution (Kimura, 1983). Studies of incipient speciation and microevolution have shown faster rates of microevolution in

* Updated from 'Brown, A. & Thatje, S. (2014). Explaining bathymetric diversity patterns in marine benthic invertebrates and demersal fishes: physiological contributions to adaptation of life at depth. *Biological Reviews* **89**, 406-426'.

marine foraminiferans, plants and mammals occupying low latitudes (Allen *et al*., 2006; Wright *et al*., 2006; Gillman *et al*., 2009); phylogenetic and palaeontological evidence on rates of diversification and origination also support this hypothesis (Mittelbach *et al*., 2007). It has been argued that tropical diversification is further increased by positive feedback from sympatric speciation once standing diversity reaches a particular threshold (Briggs, 2003, 2007). Despite the complex Cenozoic history of the marine environment, tropical origination rates have left a permanent mark on the taxonomic and biogeographic structure of the modern biota (Krug *et al*., 2009), including the deep sea (e.g. Macpherson *et al*., 2010). Clearly, although it may have some bearing on latitudinal gradients that have been reported in deep-sea species diversity (Rex *et al*., 1993), such a temperature-dependent evolutionary-rate mechanism is unlikely to explain the unimodal bathymetric pattern of diversity alone since temperature typically decreases with increasing depth. Other factors have been suggested to influence speciation rates that may be ecologically relevant in a deep-sea context (see McClain *et al*., 2009), however little consideration has been made of the potential role of hydrostatic pressure. Despite depth often being the best predictor of diversity, and apparently contributing to an evolutionary biodiversity bottleneck, it is generally believed that depth is not itself a primary driver of diversity (Levin & Dayton, 2009).

Although evolutionary rates reflect the interplay of mutation with selection and genetic drift (Kimura, 1983; Baer *et al*., 2007), molecular and morphological analyses across a range of invertebrate and fish taxa have indicated that there is greater potential for population differentiation and speciation at bathyal depths between ~500 m and ~3300 m (France & Kocher, 1996*a*,*b*; Chase *et al*., 1998; Etter *et al*., 1999, 2005, 2011; Kojima *et al*., 2001; Quattro *et al*., 2001; Oliver *et al*., 2010; Syme & Oakley, 2012; Priede & Froese, 2013; Corrigan *et al*., 2014; but see Havermans *et al*. 2013). For example, speciation in cylindroleberidid ostracods living deeper than 1000 m is estimated to be twice as rapid as in cylindroleberidids living shallower than 1000 m (Syme & Oakley, 2012). Other taxa appear to have speciated at least as rapidly in deep habitats as in shelf habitats, despite decreasing temperature with depth (e.g. elasmobranch sharks; Sorenson *et al*., 2014). There are persistent suggestions of

cryptic speciation, which may be consistent with directional selection on ecological traits and is suggested to be non-random with regard to biome (Bickford *et al*., 2007), in addition to reports of strong genetic variation over relatively small distances, consistently associated with differences in depth (Doyle, 1972; Siebenaller, 1978; Bucklin *et al*., 1987; France, 1994; France & Kocher, 1996*a*,*b*; Creasey *et al*., 1997, 2000; Chase *et al*., 1998; Creasey & Rogers, 1999; Etter *et al*., 1999, 2005, 2011; Morita, 1999; Kojima *et al*., 2001; Quattro *et al*., 2001; France & Hoover, 2002; Goffredi *et al*., 2003; Rogers, 2003; Weinberg *et al*., 2003; Howell *et al*., 2004; Le Goff-Vitry *et al*., 2004; Baco & Shank, 2005; Held & Wägele, 2005; Zardus *et al*., 2006; Raupach *et al*., 2007; Hunter & Halanych, 2008; Brandão *et al*., 2010; Cho & Shank, 2010; Reveillaud *et al*., 2010; White *et al*., 2010, 2011; Baird *et al*., 2011; Boyle, 2011; Ingram, 2011; Miller *et al*., 2011; Morrison *et al*., 2011; Schüller, 2011; Baco & Cairns, 2012; Knox *et al*., 2012; Havermans *et al*., 2013; Jennings *et al*., 2013; Quattrini *et al*., 2013; Brix *et al*., 2014; Cowart *et al*., 2014; Glazier & Etter, 2014; Shum *et al*., 2014).

Similar vertical partitioning and variation in evolutionary rate have been reported between mesopelagic (500-1000 m depth) and bathypelagic (1000-2000 m depth) copepods (Laakmann *et al*., 2012), suggesting that these patterns may also occur in pelagic fauna. Although many shelf fauna appear to penetrate to great depth in the Antarctic (Brey *et al*., 1996), it has been suggested (Rogers, 2007) that cryptic species with distinct bathymetric ranges (e.g. Held & Wägele, 2005; Raupach *et al*., 2007; Brandão *et al*., 2010; Schüller, 2011) may challenge the concept of extended eurybathy reported for Antarctic fauna (Brey *et al*., 1996). Indeed, as genetic analyses proliferate cryptic speciation is increasingly reported in the Antarctic fauna (e.g. Allcock *et al*., 1997; Rogers *et al*., 1998; Page & Linse, 2002; Held, 2003; Raupach & Wägele, 2006; Linse *et al*., 2007; Raupach et al., 2007; Wilson *et al*., 2007; Hunter & Halanych, 2008; Lörz *et al*., 2009; Krabbe *et al*., 2010) on the depressed continental shelf (mean depth 450 m, extending to over 1000 m in some places; Clarke & Johnston, 2003). It has been suggested that Antarctica may be a hotspot for this phenomenon (Grant *et al*., 2011). By contrast, extremely low genetic diversity has been identified in abyssal organisms (Bisol *et al*., 1984; France & Kocher, 1996*a*,*b*; Etter *et al*., 2011). Similar trends have been reported in phenotypic variation (Etter & Rex, 1990; Rex & Etter, 1990, 1998; Rex

et al., 1999). There are also suggestions of low mutation rates in the deep-sea lithodid crab sub-family, Lithodinae (Hall, 2010; da Silva *et al.* 2011). This pattern has prompted the proposition that the continental margins may be the primary site of adaptive radiation in the deep sea (Etter *et al*., 2005) and the establishment of the "depth-differentiation hypothesis" focusing on spatial and temporal environmental heterogeneity as the primary driver of evolution (Etter *et al*., 2011). Links have been proposed between genetic diversity and species diversity with congruent patterns of phenotypic and genetic divergence (see Rex & Etter, 2010, and references therein). The implied elevation in speciation rate at bathyal depths between ~500 and ~3000 m would subsequently lead to higher diversity (Mittelbach *et al*., 2007), consistent with the unimodal bathymetric biodiversity pattern reported for, for example, the lithodid king crabs (Zaklan, 2002*b*; Hall & Thatje, 2009; Mclaughlin *et al*., 2010), and notothenioid (Clarke & Johnston, 1996) and macrourid fishes (Merrett & Haedrich, 1997, and references therein), amongst others (e.g. Priede & Froese, 2013). Indeed, diversification rates increased at or near the origination of Antarctic notothenioids which may have coincided with the evolution of antifreeze proteins (Colombo *et al*., 2014), but also coincided with deep-sea colonisation (see Clarke & Johnston, 1996, and references therein). Assessing duration of speciation events in related shallow- and deep-water lineages may provide a robust method for identifying composite environmental effects on speciation rates (see Etienne *et al*, 2014), but this cannot resolve the distinct contributing components.

Over time a bathyal peak in evolutionary rate clearly could result in a unimodal pattern of diversity peaking at these depths, despite the relatively small area they represent. Consequently, it appears a distinct possibility that an evolutionary role for high hydrostatic pressure and low temperature may have been neglected. Indeed, reanalysis of existing diversity–depth data using quadratic depth and temperature functions may offer evidence for such a role. But how could these factors stimulate the rate of evolution at bathyal depths?

4.3 The stress-induced evolutionary mechanism in the deep sea*

Existing evidence suggests that adaptive radiation is the predominant mode of biological diversification (see Glor, 2010, and references therein). It seems apparent from species invasions that adaptive change can occur rapidly and that severe population bottlenecks do not preclude rapid adaptation (Sax *et al*., 2007). Links between evolutionary innovation and environmental stress have been proposed several times (see Jablonski, 2005) and molecular evidence has suggested potential mechanisms for such a stress–novelty link. Intragenomic site-specific mutation rates can vary across orders of magnitude and it has subsequently been suggested that mutation rates may be higher in sequences critical for adaptation, leading to rapid divergence even among closely related species (King & Kashi, 2009). It has been proposed that the absence of a stress response during embryogenesis and subsequent increased mutation in germ cells resulting from DNA damage may accelerate evolutionary processes (Epel, 2003). Although adaptation can arise due to a new mutation (see Rosenberg, 2001, and references therein), most adaptive alleles among identified adaptive loci seem to have been present as standing genetic variation (see Stapley *et al*., 2010, and references therein). Bathymetric macroecological patterns may also derive from the stress–novelty link through inactivation of a canalisation system by physiological stresses during embryonic or larval development, induced by the effects of high hydrostatic pressure and low temperature around the suggested physiological bottleneck at bathyal depths (Tables 1.1, 1.2; Fig. 1.2). This adaptive canalisation has been suggested to occur in extreme environments (Eshel & Matessi, 1998). The physiological effects of bathyal hydrostatic pressures and temperatures suggest that the deep sea constitutes such an environment for shallow-water species (Hall & Thatje, 2009; Thatje *et al*., 2010; Brown & Thatje, 2011).

The ubiquitous cellular stress response affords cells a transient increase in tolerance to any form of damage-inflicting environmental challenge in larval and adult organisms, allowing time for stressor-specific adaptation to re-establish cellular homeostasis

* Updated from 'Brown, A. & Thatje, S. (2014). Explaining bathymetric diversity patterns in marine benthic invertebrates and demersal fishes: physiological contributions to adaptation of life at depth. *Biological Reviews* **89**, 406-426'.

(Kültz, 2003, 2005). Such adaptive variation may be achieved by a single amino acid substitution in a protein and in response to only moderate environmental change (Somero, 2012). Rates of DNA evolution appear linked to DNA damage by mutagenic by-products of oxygen metabolism (the metabolic-rate hypothesis; see Martin, 1999, and references cited therein; but see Held, 2001), suggesting that significantly elevated metabolic rate resulting from hyperbaric stress is likely to increase DNA damage. Theoretical assessment indicates that stress-induced mutagenesis increases the rate of complex adaptation without reducing reducing population mean fitness (Ram & Hadany, 2014). Exposure to high pressure has been shown to trigger an increase in the expression of stress proteins in organisms adapted to atmospheric pressure (Welch *et al*., 1993; Takahashi *et al*., 1997; Kaarniranta *et al*., 1998, 2000, 2003; Elo *et al*., 2000, 2003, 2005; Sironen *et al*., 2002; Wemekamp-Kamphuis *et al*., 2002), apparently as a sustained response (Cottin *et al*., 2012). It has also been suggested that molecular chaperones such as these stress proteins, expressed when an organism is exposed to environmental extremes (Feder & Hofmann, 1999), can act as evolutionary capacitors regulating hidden variation or mutagenic activity, occasionally resulting in complex adaptive phenotypes (e.g. Rutherford & Lindquist, 1998; Bergman & Siegal, 2003; Madlung & Comai, 2004; Sangster *et al*., 2004; Aertsen & Michiels, 2005*a*; Jarosz & Lindquist, 2010; Chen *et al*., 2012; Trotter *et al*., 2014; for review see Jarosz *et al*., 2010; Taipale *et al*., 2010). Although the absence of a heat shock response has been reported for some Antarctic marine invertebrates in response to temperature (Clark *et al*., 2008*c*), this is not a universal phenomenon (Clark *et al*., 2008*b*,*d*, 2011) and has been attributed to constitutively high levels of inducible isoforms (Place *et al*., 2004; Place & Hofmann, 2005; Clark *et al*., 2008*a*) thus maintaining the possibility of contribution to a stress–evolution mechanism in that habitat. Indeed, given the unusually deep Antarctic continental shelf, the stress–evolution mechanism induced by high pressure and low temperature may also contribute to the high diversity and cryptic speciation among the taxa present in the Southern Ocean relative to latitudinal trends (Brandt *et al*., 2007; Grant *et al*., 2011); perhaps in concert with the frequent fluctuation in the extent of the grounding line of the continental ice sheet across the continental shelf during Late Cenozoic glacial periods (Clarke & Crame, 1997; Thatje *et al*., 2005*b*). Environmental stress may also activate or release transposable elements

and it has been argued that these represent a source of significant evolutionary innovation (e.g. McClintock, 1984; McDonald, 1990, 1995; Kidwell & Lisch, 1997, 2001; Shapiro, 1999, 2005; Lisch, 2009; Casacuberta & González, 2013; Belyayev, 2014). Transposable elements certainly have a role in environmental adaptation, for example increasing resistance to oxidative stress (Guio *et al*., 2014). Up-regulation of reverse transcriptase may be implicated in transposable element activity (see Casacuberta & González, 2013), and occurs in sub-lethally cold stressed anomuran *Petrolisthes cinctipes* (Stillman & Tagmount, 2009). Exposure of organisms to high pressure has resulted in such mobilisation of transposable elements (Aertsen & Michiels, 2005*b*; Lin *et al*., 2006), generating diversity in response to hyperbaric stress. Alteration of methylation patterns of mobile elements has also been reported following hydrostatic pressurisation (Long *et al*., 2006).

Mutation in germ cells, adaptive canalisation during embryonic or larval development, release of hidden genetic variation or mutagenic activity, or activation or release of transposable elements in larvae or adults, all increase genetic or phenotypic variation. Elevated variation unrelated to hydrostatic pressure tolerance may promote increased parapatric or sympatric speciation into vacant niches, whilst taxa remain bathymetrically constrained (Fig. 4.2). Models of range restriction by gene flow along gradients, in the absence of sharp environmental boundaries, suggest increased adaptation in peripheral populations in the absence of competition, as may have been the case during colonisation of the deep sea following mass extinctions (see Carney, 2005, and references therein). By contrast, variation that results in increased tolerance of hydrostatic pressure may promote parapatric or peripatric speciation past the high-hydrostatic-pressure and low-temperature induced bottleneck at bathyal depths, simultaneously reducing environmental stress and the subsequent evolutionary response, and returning speciation to the background rate or perhaps even constraining it further. These varied forms of evolution constitute important sources for marine biodiversity (Briggs, 2006). Affected genes may represent speciation genes (see Nosil & Schluter, 2011). Such speciation would be consistent with the ecological hypothesis of speciation (Schluter, 2001). Under such circumstances elevated rates of evolution may occur at genus and species level at bathyal depths. This is consistent

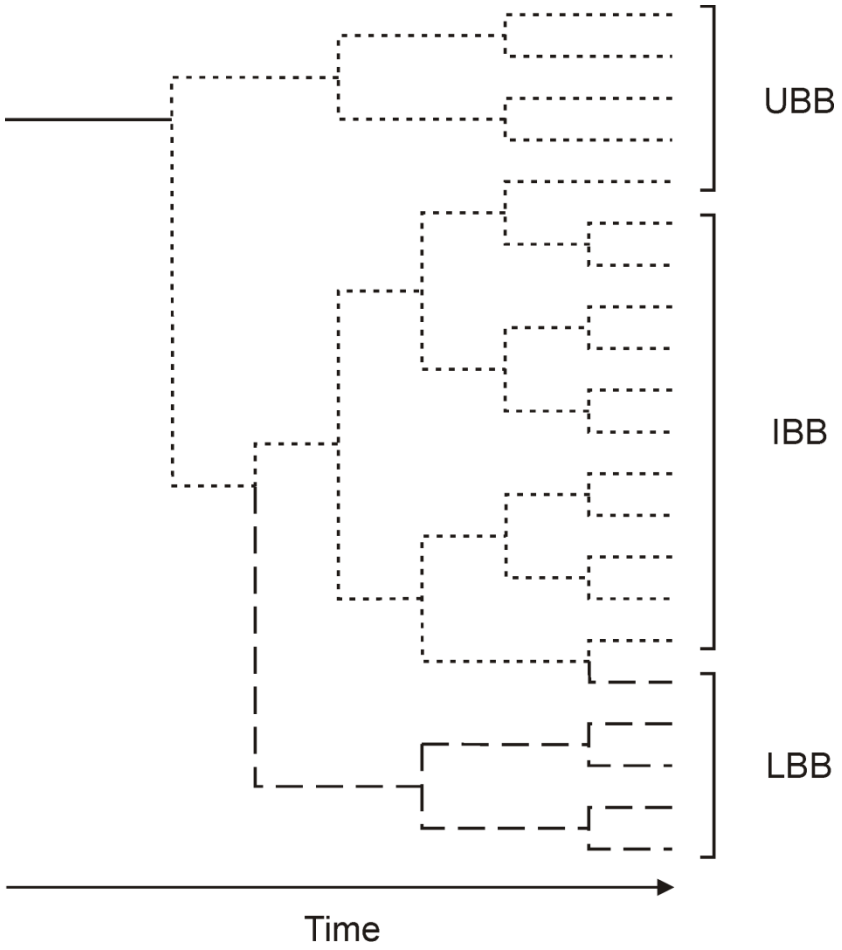

Figure 4.2. Scenario for colonisation of the deep sea following dysoxic mass extinction. An enduring upper boundary species extends its distribution downslope to a taxon-specific maximum depth at the physiological bottleneck determined by interacting effects of high hydrostatic pressure and low temperature (solid line). At this limit hyperbaric and thermal stress effects increase genetic or phenotypic variation. Greater variation unrelated to hydrostatic pressure or temperature tolerance results in significantly increased parapatric or sympatric speciation of upper boundary species or inter-boundary species into niches left vacant by mass extinction (dotted lines). These remain bathymetrically constrained by the combined effects of high hydrostatic pressure and low temperature. Species remain under stress promoting continuing elevated variation, and speciation rate remains increased. Variation increasing tolerance of high hydrostatic pressure or low temperature instead results in parapatric or peripatric speciation of lower boundary species (dashed lines). Increased tolerance of high hydrostatic pressure or low temperature diminishes the stress effect and returns variation to background rate. The differential rates of speciation over time result in the unimodal pattern of biodiversity with depth. Experimental evidence indicates consistently that critical pressure and temperature conditions for shallow-water benthic invertebrate species equate to the bathyal environment; the unimodal diversity–depth pattern typically peaks at these depths.

with an elevated origination observed on the deep continental margin (Fig. 4.2), yielding a unimodal pattern of diversity with depth. High incidence of cryptic repeated elements in regions flanking microsatellites, which are associated with transposable elements, has been reported in examined genomes of deep-sea galatheid squat lobsters (Bailie *et al*., 2010), hinting at recent elevated transposable element activity or mutation. Analysis of the genus *Paramunida* suggests a period of rapid diversification following origination between 17 and 21 MYA (Cabezas *et al*., 2012), and the deep-sea galatheids of the Pacific Ocean continental slope display a unimodal pattern of diversity peaking at around 650 m (Macpherson *et al*., 2010). Molecular phylogeny suggests that galatheiod squat lobsters colonised the deep sea from shallow water (see Bracken-Grissom *et al*., 2013) with diversity increasing in deep waters (e.g. in munidopsids; Ahyong *et al*., 2011). Stress-protein-regulated genetic variation appears to preserve phenotypic robustness in addition to providing a broad conduit to diversification (e.g. Jarosz & Lindquist, 2010), and this mechanism may also offer an explanation for lower production of ordinal-level novel taxa in the deep sea. Although the stress effects of high pressure and low temperature could be compounded by other factors, for example deep-water hypoxia in oxygen minimum zones, it is unlikely that stress-induced variation alone is responsible for bathymetric macroecological patterns. Any effects on variation could be enhanced by the vicariance-mediated speciation effect proposed for transient oxygen minimum zones for example (White, 1987; Rogers, 2000; Levin & Sibuet, 2012), or hydrodynamic characteristics that affect larval transport and retention (Mantelatto *et al*., 2014), amongst other potential barriers to gene flow on continental margins (see Rex & Etter, 2010). Similarly, according to the species-energy hypothesis (Allen *et al*., 2007), reductions in food supply with depth and consequently smaller population sizes may lead to reductions in speciation rate in abyssal depths. The resulting bathymetric diversity pattern may be amplified by increased evolutionary pressure from biotic interactions under relatively stable deep-sea environmental conditions (see Currie *et al*., 2004).

Significant variation in hyperbaric sensitivity among taxonomic groups suggests that the stress-induced evolutionary mechanism will be invoked at significantly different depths in shallow-water fishes, crustaceans, and other taxa invading the deep sea, and

may provide an opportunity to test the hypothesis of stress-induced. However, there are currently insufficient studies of bathymetric diversity patterns in different taxonomic groups, colonising the deep-sea from similar shallow-water thermal environments, over similar periods, to facilitate such an analysis. It is clear that understanding marine evolutionary dynamics demands increased knowledge of links between continental margin fauna (Clarke & Crame, 2010). Demonstration of intrinsic or emergent tolerance to high pressure and low temperature in taxa with well-constrained radiation and speciation from shallow water into the deep sea, for example the Lithodidae, may ultimately help to explain the evolution of bathymetric biodiversity patterns in the deep sea. Given that we may be within a sixth mass extinction (Barnosky *et al*., 2011) better understanding of the evolutionary impact of stress-driven adaptation is of paramount importance for assessing both the potential resilience and recovery of marine biodiversity. Whilst marine ectotherms' capacity for sufficiently rapid adaptive responses to changing climate remains uncertain (Munday *et al*., 2013), assessing potential impacts on species distributions may provide some indication of the risk posed to marine taxa by projected climate change.

4.4 The effects of changing climate on faunal depth distributions determine winners and losers*

Global climate change is warming the oceans (Collins *et al*., 2013). Depending on the emission scenario, projections of oceanic warming under the moderate greenhouse gas growth scenario predict an increase in global mean sea surface temperature ranging from about 1°C under Representative Concentration Pathway 2.6 (RCP2.6; approximate total radiative forcing of 2.6 W m^{-2} in 2100 relative to 1750) to more than 3°C under RCP8.5 for the period 2081-2100, relative to the period 1986-2005 (Collins *et al*., 2013). Increasing temperature will affect taxa on the continental shelf and upper continental slope first, but the whole ocean will eventually warm up reasonably uniformly by the amount of the surface increase (Li *et al*., 2013), affecting even abyssal organisms. Marine ectotherms tend to fully occupy their thermal niches (Sunday *et al*.,

* Updated from 'Brown, A. & Thatje, S. (2015). The effects of changing climate on faunal depth distributions determine winners and losers. *Global Change Biology* **21**, 173-180'.

2012), and it is well recognised that organisms' latitudinal ranges are responding rapidly to geographical shifts in their fundamental ecological niches (FENs) forced by changing climate (Pinksy *et al*., 2013). However, changes in the depth of organisms' FENs remain little considered in projections, despite clear supporting evidence for responses in shallow-water (<200 m depth) (e.g. Perry *et al*., 2005; Dulvy *et al*., 2008; Nye *et al*., 2009; Engelhard *et al*., 2011; Pinksy *et al*., 2013; Hiddink *et al*., 2015) and deep-sea (>200 m depth) taxa (e.g. Yasuhara *et al*., 2009).

Constructing a conceptual model allows examination of shifts in hypothetical oxygen- and capacity-limited FENs of shallow-water taxa in different thermal zones in response to predicted changes in ocean temperature and oxygenation under stabilising emissions scenarios. Despite increasing recognition of the potential impact of changing carbon dioxide concentration (e.g. Brewer & Peltzer, 2009), zonal mean sections (latitude vs. depth) identifying carbon dioxide concentrations changes projected by global ocean models under stabilising emissions scenarios are not currently available. Incorporating effects of changing carbon dioxide concentration on FENs into a conceptual model is, therefore, not possible. Projected increases in temperature (°C) and decreases in oxygen concentration (μmol kg^{-1}) by 2100 under stabilising emissions scenarios were identified from Collins *et al*., 2013 (RCP4.5) and Matear & Hirst, 2003 (IS92a). Marine taxon range shifts are tightly coupled to shifts in thermal envelope (Pinksy *et al*., 2013). Therefore, the magnitude of anticipated climate warming effects on thermally constrained latitudinal ranges of hypothetical oxygen- and capacity-limited FENs was estimated based on the best supported mean temperature effects on leading-edge expansions and trailing-edge contractions of distributions (30.6 ± 5.2 km dec^{-1}) (Poloczanska *et al*., 2013), as temperatures have increased (0.07°C dec^{-1}) (Burrows *et al*., 2011). Mean rates were used because the disparity in leading-edge expansions and trailing-edge contractions likely derives predominantly from geographic variation in climate velocity (Pinksy *et al*., 2013; Burrows *et al*., 2014). This yielded leading-edge expansion and trailing-edge contraction rates of ~440 km $°C^{-1}$; latitudinal shifts were rounded to the nearest 10 km. The magnitude of anticipated thermal effects on hyperbaric limits to hypothetical oxygen- and capacity-limited FENs was estimated based on mean experimentally determined temperature effects on the

critical hyperbaric thresholds of post-larval shallow-water tropical (+90 m depth $°C^{-1}$; *n* = 3; three crustaceans) (Menzies & George, 1972), temperate (+50 m depth $°C^{-1}$; *n* = 2; two crustaceans) (Thatje *et al*., 2010; Oliphant *et al*., 2011), and polar (-330 m depth $°C^{-1}$; *n* = 2; one mollusc and one crustacean) (George, 1979; Smith & Thatje, 2012) taxa; bathymetric shifts were rounded to the nearest 10 m. Insufficient data were available to estimate the magnitude of effects of ocean deoxygenation on FENs, therefore arbitrary effects of oxygen changes (10 km latitude μmol^{-1} O_2, and 1 m depth μmol^{-1} O_2) were modelled to provide an indication of relative impacts on FENs in different thermal zones. Hypothetical FENs initially span 10° (polar) or 20° (temperate and tropical) latitude and are 300 m deep and were selected to represent hypothetical oxygen- and capacity-limited species with distributions in the areas of the global oceans most severely impacted by changing climate. Shifts in cross-sectional area of FENs in response to projected ocean warming were calculated, but no calculation was made for responses to projected ocean deoxygenation or combined ocean warming and deoxygenation, since deoxygenation impacts were assigned arbitrarily.

Latitudinal limits to all FENs shifted poleward under projected ocean warming (Fig. 4.3a). Both the upper and lower bathymetric limits of the tropical FEN were depressed (Fig. 4.3a). The lower bathymetric limit of the temperate FENs was also depressed, but the lower limit of the polar FENs shallowed (Fig. 4.3a). Tropical and temperate FENs increased in cross-section in response to projected ocean warming, whereas the polar FENs decreased in cross-section (Table 4.1). Latitudinal limits to all FENs were constricted under projected ocean deoxygenation (Fig. 4.3b). Both the upper and lower bathymetric limits of the tropical FEN were depressed (Fig. 4,3b). In contrast, the lower bathymetric limits of temperate and polar FENs shallowed (Fig. 4.3b). The effects of ocean deoxygenation compounded ocean warming induced shifts in trailing-edges of FENs, but moderated shifts in leading-edges (Fig. 4.3c). Whilst shifts in bathymetric limits of tropical and polar FENs induced by ocean warming were compounded by ocean deoxygenation, ocean deoxygenation moderated shifts in the bathymetric limits of temperate FENs caused by ocean warming (Fig. 4.3c).

Distinct adaptations of organisms in tropical, temperate, and polar regions suggest that their FENs will be affected differently by ocean warming (Somero, 2012) (Fig.

Constraint of bathymetric distribution by hydrostatic pressure

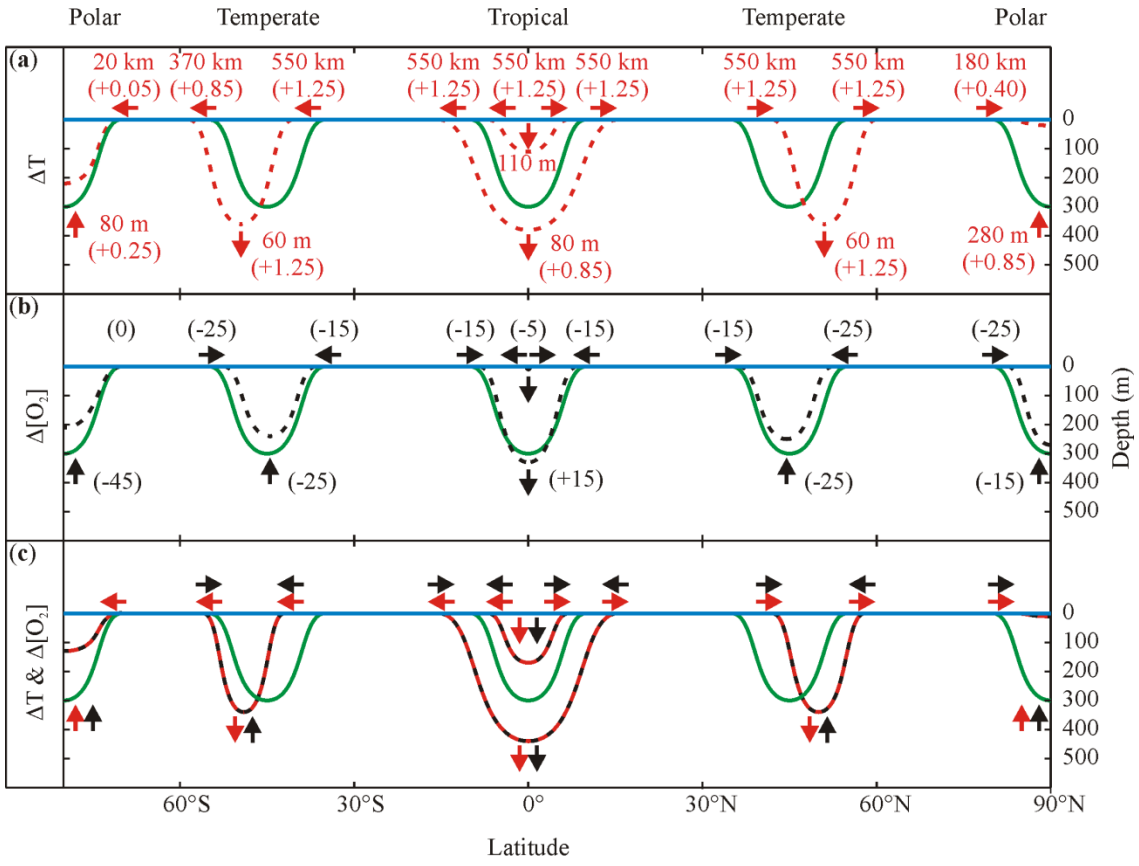

Figure 4.3. Projected shifts in fundamental ecological niches (FENs) in response to (a) increasing ocean temperature, (b) decreasing ocean oxygenation, and (c) increasing ocean temperature and decreasing ocean oxygenation, assuming oxygen-limitation of thermal, hyperbaric and hypoxic tolerance. FENs initially span 10° (polar) or 20° (temperate or tropical) latitude and are 300 m deep. Arrows indicate projected effects of increasing temperature (—) and decreasing oxygenation (—) on hypothetical FENs (—) in tropical, temperate, and polar zones. The magnitude of anticipated thermal effects on latitudinal and bathymetric limits to FENs is indicated alongside arrows, with dashed lines indicating resulting FENs. Projected increases in temperature (°C) and decreases in oxygen concentration (µmol O_2 kg^{-1}) by 2100 are indicated in brackets.

4.3a). Temperature increases will shift the upper bathymetric boundaries of the tropical FEN downwards, but increasing temperature will also mediate the effects of high pressure on tropical fauna, increasing hyperbaric tolerance and shifting the lower bathymetric limit downwards, too. Consequently, ocean warming will expand the latitudinal range of the tropical FEN substantially (Table 4.1). The poleward shift in temperate FENs will also be accompanied by depression of the lower bathymetric limits (Fig. 4.3a). For example, the pressure tolerance of the temperate shallow-water hermit crab *Pagurus cuanensis* increases from the equivalent of 200 m water depth to

the equivalent of 500 m water depth with a 5°C temperature increase (Thatje *et al*., 2010). Based on this, the 1.0-1.5°C temperature increase predicted for 200 to 400 m water depth in the northern temperate zone by 2100 (Collins *et al*., 2013) will shift the lower bathymetric limit of the FEN downwards by 60 to 90 m water depth. Although this increase appears modest, depths between 200 and 300 m constitute ~1% of the total surface area of the global ocean (Brown & Thatje, 2014). Depths from the sea surface to 200 m constitute ~6% of the total surface area of the global ocean (Brown & Thatje, 2014). Clearly, a 60-90 m downward shift in depth limit represents a substantial expansion in FEN. Such an expansion is sufficient to offset latitudinal contraction of the southern temperate FEN in the model, resulting in an overall increase in both temperate FENs (Table 4.1). Whilst the hyperbaric tolerance of tropical and temperate taxa is increased by increasing temperature (Brown & Thatje, 2014), the hyperbaric tolerance of cold-adapted polar taxa appears reduced (Smith & Thatje, 2012). The consequent shallowing of the lower bathymetric limits of polar FENs, together with a poleward shift in the latitude of upper thermal limits, mean that polar FENs will contract with increasing temperature (Table 4.1).

Tropical, temperate, and polar FENs will all contract latitudinally and bathymetrically as a consequence of decreasing ocean oxygenation (Fig. 4.3b). But the magnitude of the resulting FEN contraction remains entirely uncertain in the absence of experimental studies assessing the impact of deoxygenation on marine organisms' thermal and hyperbaric limits, and will depend on the degree of deoxygenation, which varies regionally. Nevertheless, the hypothesised deoxygenation-related FEN contraction will compound the contractive effect of ocean warming on the FENs of polar taxa, and will mediate the expansion of temperate and tropical FENs caused by

Table 4.1. Impact of predicted ocean warming to the year 2100 on the cross-sectional area of hypothetical oxygen- and capacity-limited fundamental ecological niches (FENs) from the year 2000. Areas were calculated from Fig. 2.

	Cross-sectional area			
FEN	2000 (km^2)	2100 (km^2)	Change (km^2)	% Change
Polar northern hemisphere	165	9	-156	-94
Temperate northern	330	396	+66	+20
Tropical	330	597	+267	+81
Temperate southern	330	364	+34	+10
Polar southern hemisphere	165	119	-46	-28

rising temperature. Consequently, it appears clear that FENs in tropical, temperate, and polar zones will be affected differently by components of climate change (Fig. 4.3c). Including depth in the model confirms previous predictions that polar taxa are most vulnerable to the effects of climate change, made based solely on consideration of the latitudinal effects of ocean warming (e.g. Cheung *et al*., 2009), with Arctic fauna experiencing the greatest habitat contraction. In contrast, the inclusion of depth in the model reveals for the first time that temperate fauna as well as tropical fauna may experience substantial FEN expansion with ocean warming and deoxygenation, rather than FEN maintenance (northern hemisphere temperate and tropical) or contraction (southern hemisphere temperate) suggested by solely considering latitudinal range shifts (Fig. 4.3).

Climate projections to 2300 predict that sea surface and subsurface temperatures will rise by between 6 and 7°C at low latitudes and ~10°C at high latitudes, with the deep sea warming by 2 to 5°C (Schmittner *et al*., 2008). By the year 3000 global mean oxygen concentration will decline by 30%: shallow subsurface ocean oxygen concentration in the eastern tropical Pacific and Atlantic will decrease by more than 80%, and the eastern North Pacific margin will suffer a 40 to 80% oxygen reduction (Schmittner *et al*., 2008). The deep ocean will experience a decrease of more than 35% (Schmittner *et al*., 2008). Occurrences of widespread hypoxia are predicted to increase, as the volume of the total ocean that is hypoxic (defined in this case as ≤80 μmol O_2 kg^{-1}) rises from 9.1% currently, to 61% around 5000 (Shaffer *et al*., 2009). Based on these projections, changing climate will continue to force shifts in FENs for millennia.

Whether, and to what extent, taxa are able to shift their distribution to match the shift in their FEN depends on habitat availability (e.g. continental shelf and slope area for benthic species), suitability (e.g. primary productivity and food availability, seasonality), and accessibility (e.g. dispersal ability, current direction), amongst other factors (see Barve *et al*., 2011, and references cited therein). Light penetration may be a particularly critical limitation for bathymetric range increases in animals with vision too; adaptations in vision shift with bathymetric changes in light parameters (reviewed

by Herring, 2002; Warrant & Locket, 2004). Further, there is great regional variability in changing climate (e.g. Pinksy *et al*., 2013). Undoubtedly, differences in FEN shifts, and in organism's ability to respond to such shifts, will contribute to the development of no-analog communities, with the potential for changes in ecosystem functioning (see Williams & Jackson, 2007). Ultimately, shifts in bathymetric ranges of taxa such as deep-sea fish may impact global biogeochemical cycling by e.g. affecting benthic production and long-term carbon storage on continental slopes (see Trueman et al., 2014). Such changes may affect humans significantly (Mora et al., 2013) by impacting the critical services that the deep sea provides (Thurber et al., 2014).

Although the effects of changing climate inferred here from the matrix of thermal, hyperbaric, and hypoxic oxygen limitation do not incorporate preadaptation or adaptation to changing climate, it remains unclear whether adaptation in marine ectotherms can match rapid change in climate over time (Munday *et al*., 2013): the most warm-adapted organisms may not have capacity for any further thermal adaptation (Storch *et al*., 2014). Environmental preferences certainly appear stable across millions of years of profound climatic change in at least some species (e.g. Saupe *et al*., 2014). During past extinction events, temperature, oxygen concentration, and carbon dioxide concentration all experienced significant perturbations (McClain & Hardy, 2010; Hönisch *et al*, 2012; Bijima *et al*., 2013). The magnitude of future disturbance in these factors and their interaction with hydrostatic pressure effects may make a mass extirpation of life in deep waters inevitable in the long term (adapted from Jackson, 2010), but the results presented here suggest that, at least in the shorter term, there will be winners as well as losers.

4.5 Future research

The significant developments in hyperbaric physiology and ecology presented here enhance understanding of the hyperbaric contribution to the evolution and radiation of deep sea life (e.g. Brown & Thatje, 2014). Exploring *Lithodes maja*'s cardiac transcriptomic responses to hyperbaric exposure may yield further insight into the cellular mechanisms effecting cardiac capacity limitation (see Stillman & Tagmount, 2009). Simultaneous examination of cardiac proteomic responses to elevated pressure

may also prove enlightening (see Tomanek, 2011). Such investigations may identify suitable target genes for exploring physiological adaptation in other shallow- and deep-lineage lithodids.

A shallow-deep lineage comparative physiological approach remains critical to resolving hydrostatic pressure's contribution to bathymetric limitation in shallow-water fauna. The striking trend towards lecithotropic development in deep-sea taxa strongly suggests that a mismatch between prolonged development at lower temperature and decreasing larval food availability with increasing depth may also limit successful colonisation of deep water by shallow-water taxa (Thatje *et al*., 2005*a*). Exploring the pressure tolerance of closely related shallow- and deep-water species which demonstrate contrasting developmental modes may reveal hyperbaric exaptation in shallow-water taxa, and will identify the relative importance of developmental adaptation for colonisation of the deep sea. The general distinction between planktrophic development in shallow-water lithodid species and lecithotropic development in deep-sea lithodid species and their well-resolved evolutionary origin (see Hall, 2010) make lithodids an ideal model family for such a macrophysiological approach (see Chown *et al*., 2004; Gaston *et al*., 2009): mapping comparative physiology onto phylogeography to bridge the gap between observational and experimental sciences (Dawson, 2014). Comparing deep-lineage physiological data presented here with physiological data from similar experimental hyperbaric exposures using a shallow-lineage lithodid species such as *Acantholithodes hispidus* will achieve this.

Differences in developmental mode or hydrostatic pressure tolerance between lineages may be attributable to positive selection and habitat-driven adaptation in gene sequences with known functions, linking genotypes to phenotypes and fitness (Dalziel *et al*., 1995), and these may be identified through whole genome scans and candidate gene analysis (e.g. Oliver *et al*., 2010; see Wray, 2013). Shallow-water *Paralithodes californiensis* and *P. rathbuni* appear to have evolved lecithotrophic larval development (inferred from egg size >1.2 mm; Hall, 2010) and may represent an intermediate stage in deep-sea colonisation; whole genome scans in these species may

reveal independent evolutionary responses in larval and adult life-history phases (see e.g. Oliver *et al*., 2010; Aguirre *et al*., 2014). Data from multiple species genome scans could additionally be analysed to assess mutagenic or transposable element activity, testing the hypothesis of the stress-induced evolutionary mechanism in the deep sea. Undertaking a population genetics approach using a widely dispersed lithodid taxa with lecithotrophic development (e.g. *Lithodes maja*) may resolve debate regarding the dispersal potential of deep-sea king crab.

Although knowledge of the mechanism constraining pressure tolerance allows predictions of interactions with other physiologically-impacting physical conditions that vary with depth (e.g. temperature, oxygen concentration, and carbon dioxide concentration; Brown & Thatje, 2015), the effect of these environmental factors on bathymetric limitation remains unquantified, and their relative importance remains unknown. Examining distribution limits based on species' reported occurrences may offer a method to explore the impacts of individual environmental factors, but this approach can only utilise species which are not limited by biotic interactions. Projections of increasing ocean temperature, deoxygenation, and elevated carbon dioxide concentration, must make experimental studies to resolve these uncertainties a priority. The physiological study presented here provides a baseline for these further investigations, and *L*. *maja*'s biogeographic distribution (shallow/bathyal and temperate/polar) make it apposite for such studies. The regions and depths *L*. *maja* inhabits are being impacted by changing climate (e.g. Jones *et al*., 2014; Mackenzie *et al.*, 2014), providing an opportunity to compare observed shifts in distribution with predictions for shifts in FENs based on experimental physiological studies.

Lithodids are predators as well as scavengers (see Smith *et al*., 2014, and references cited therein), and have the potential to restructure entire ecosystems, even where other skeleton-crushing predators are present (e.g. Falk-Petersen *et al*., 2011). Consequently, latitudinal shifts and adjustments to bathymetric ranges of *Lithodes maja* may have significant impacts on marine communities, making it of inherent interest. Similarly, ongoing Antarctic emergence of lithodid crabs (Thatje & Arntz, 2004; Thatje *et al*., 2005*a*; Smith *et al*., 2012; but see Griffiths *et al*., 2013) is widely regarded as a potential threat to the unique ecosystem of the Antarctic continental

shelf, which evolved in the absence of durophagous predators (Thatje *et al*., 2005*a*; Aronson *et al*. 2007; Whittle *et al*., 2014). Restriction of Antarctic slope lithodids to bathyal depths (Griffiths *et al*., 2013) is thought to result from intolerance of decreasing temperature with shallowing depth (Hall & Thatje, 2011). Ocean warming (see Schmidtko *et al.*, 2015) may diminish thermal stress, facilitating lithodid emergence onto the Antarctic continental shelf (Hall & Thatje, 2009, 2011). However, emerging lithodid species are from genera that penetrate greater ocean depths (*Paralomis* spp. and *Neolithodes* spp.) and consequently may be adapted to greater pressure than *Lithodes* spp. Decreasing pressure may therefore impose physiological stress on emerging species contributing to physiological constraint of upper bathymetric limits, although potential hypobaric stress can evidently be overcome. For example, *Paralomis granulosa* occurs intertidally in warmer waters having emerged into Tierra del Fuego's Beagle Channel (see Zaklan, 2002*b*, and references cited therein) after the marine incursion ~10,000 years ago that followed the last deglaciation (see Ponce *et al*., 2011). Assessment of thermal and hypobaric interactions on Antarctic lithodid physiology is required to clarify the risk king crabs present to Antarctic continental shelf communities.

4.6 Conclusion

Hydrostatic pressure undoubtedly contributes to physiological constraint of marine ectotherms' bathymetric distribution, and the effects of physiologically-limited environmental tolerance influence the evolution and ecology of species and ecosystems. Revealing these influences is critical to understanding patterns and processes in deep-sea biodiversity, and elucidating hyperbaric effects across all hierarchical organisational levels must remain a priority to inform prediction of climate change impacts.

Appendices

Appendix 1 Gene expression

Transcription analysis was conducted by J. P. Morris*.

A1.1 Materials and Methods

Endogenous reference genes and genes of interest were identified from the literature (Cottin *et al.* 2012; Munro, 2013; Morris *et al.*, 2015). Three reference genes (*eef1a*, *rpl8*, and *α-tubulin*) were selected based on previous use in assessing transcriptional responses in *Lithodes maja* (Munro, 2013): *eef1a* codes for eukaryotic translation elongation factor 1 alpha; *rpl8* codes for ribosomal protein L8; and *α-tubulin* codes for α-tubulin microtubule subunits. The most widely studied marker of hyperbaric stress physiology in marine invertebrates is the 70kDa heat shock protein family (HSP70) (e.g. Cottin *et al.*, 2012; Morris *et al.*, 2015). Heat-shock proteins are molecular chaperones and up-regulation of HSP70 fundamentally signifies alteration of proteins' native conformation (Morris *et al.*, 2013). However, such generalised stress markers are involved in the ubiquitous cellular stress response and are highly reactive to a multitude of potential stressors (Feder & Hofmann, 1999), therefore interpreting shifts in transcription may be challenging (Morris *et al.*, 2013). In contrast, transcription of *narg*, coding for an *N*-methyl-D-aspartate (NMDA) receptor-regulated protein, has been identified as a hyperbaric neurophysiological stress marker (see Morris *et al.*, 2015) and used to assess pressure tolerance across early ontogeny of *Lithodes maja* (Munro, 2013). NMDA receptors are ligand- and voltage-gated glutamate receptors: ion channels that mediate excitatory neurotransmission (Dingledine *et al.*, 1999).

1.5 ml centrifuge tubes containing individual tissue samples were removed from -80°C storage and immersed in liquid nitrogen. The tissue sample was immediately transferred into 2 ml of *TRI-Reagant* (Sigma-Aldrich) and homogenised using an *Ultra Turrax* (Ika). The manufacturer's standard protocol was followed during total RNA extraction. A *Nanodrop* spectrophotometer (Thermo Fisher Scientific) was used to test

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total RNA purity. All samples recorded a 260/280 ratio above 1.8, and a 260/230 ratio above 1.9. *Experion* (Bio-Rad) was used to evaluate RNA integrity and concentration: all samples recorded an RQI above 8.5 and concentration above 300 ng/µl. RQ1 RNase-free DNase (Promega) was used according to the manufacturer's protocol to DNase treat a volume containing 1.5 µg of total RNA. *Superscript IIITM* (Invitrogen) and oligo (dT)18-23 primers were used according to the manufacturer's protocol to reverse transcribe 5 µl of the subsequent mixture (0.75 µg total RNA) in a 20 µl reaction.

Primer sequence data for quantitative polymerase chain reaction (qPCR) were obtained from Munro (2013). Reaction amplification was optimised by testing each qPCR assay at a variety of mixed primer concentrations. Melt curve analysis generated a single and discrete peak for all primer-sets tested. Following determination of optimal concentrations for both FWD and REV primers, qPCR reaction efficiency between 90-105% and linearity greater than $r^2 = 0.98$ were confirmed for each primer-set across the predicted experimental cDNA concentration range by eight 10-fold serial dilutions performed on a cDNA template, as set out by the MIQE guidelines (Bustin *et al.*, 2009).

The *Rotor-Gene 3000* (Qiagen) was used for all qPCR reactions. Each 25 µl reaction contained 12.5 µl of *Precision 2X qPCR Master mix* with *SYBR Green* (Primer-Design, UK), and 2 µl of template cDNA (34 ng). qPCR conditions were: 1 cycle of 95°C for 10 minutes followed by 40 cycles of [95°C 10 seconds, 60°C 1 min]. Each reaction was duplicated for technical replication. Specificity of the qPCR products was demonstrated by performing a melt curve analysis after each run.

A1.2 Statistical analysis

The relative stability of reference genes across experimental treatments was analysed using the geNorm function within *qBase+* software (Biogazelle). The best normalisation strategy was delivered by the combination of *eef1a* and *rpl8*. *qBase+* was used to calculate normalised relative quantities. Statistical significance was identified by one-way ANOVA ($\alpha = 0.05$). The post-hoc Tukey-Kramer test within *qBase+* software was used to determine which treatments differed significantly from the corresponding control treatment.

A1.3 Results

Unexpectedly, *α-tubulin* transcription in the 0.1 MPa control treatment was time sensitive and therefore not an appropriate reference gene. However, *α-tubulin* transcription was significantly lower 7.5, 10.0 and 12.5 MPa than at 0.1 MPa in sustained hydrostatic pressure treatments at (Fig. A1.1). In contrast, *narg* transcription was not significantly different at 7.5 or 10.0 MPa in sustained hydrostatic pressure treatments, but was significantly greater at 12.5 MPa (Fig. A1.1). Analysis of hyperbaric acclimation treatments indicated that transciptional responses in *α-tubulin* or *narg* at 7.5 MPa were not significantly affected by duration of exposure (Fig. A1.2).

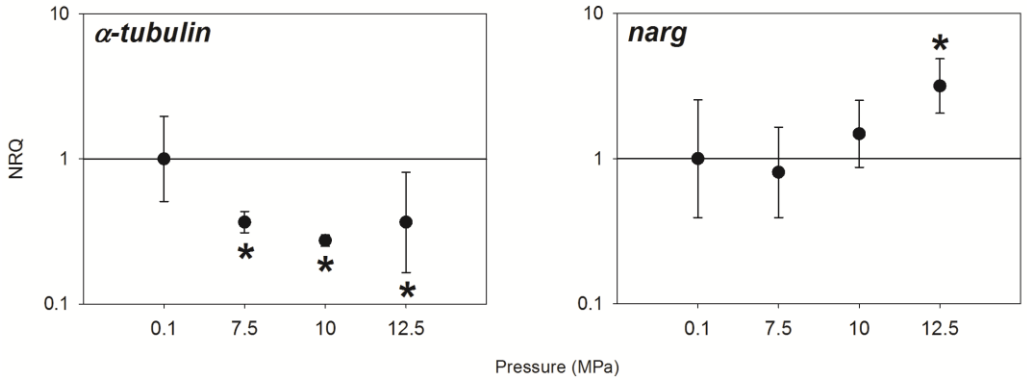

Figure A1.1. Effect of sustained hydrostatic pressure on mean normalised relative quantities (NRQs) of *α-tubulin* and *narg* transcripts of adult male *Lithodes maja* at 6°C (*n* = 5). Error bars represent 95% confidence intervals. Relative quantities are normalised to 0.1 MPa. Significant differences from 0.1 MPa, determined using a one-way ANOVA and a post-hoc Tukey-Kramer test calculated in qBase+ software, are indicated by an asterisk.

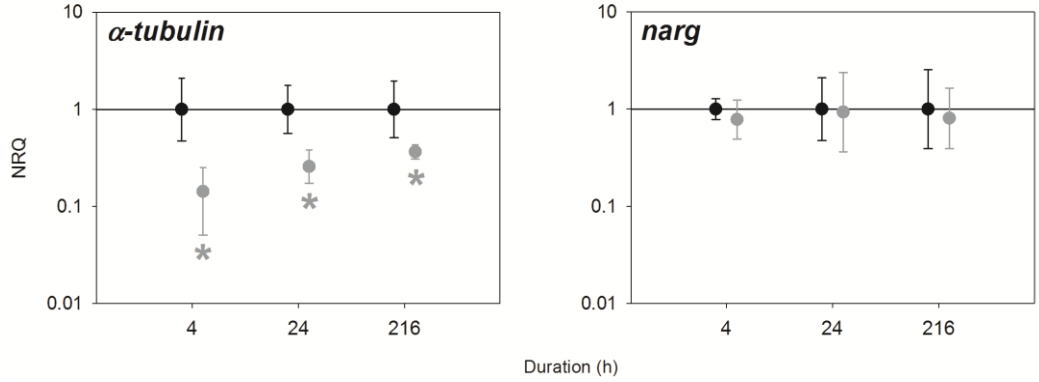

Figure A1.2. Effect of sustained hydrostatic pressure exposure duration on mean normalised relative quantities (NRQs) of *α-tubulin* and *narg* transcripts of adult male *Lithodes maja* at 6°C; 0.1 MPa control treatments in black, 7.5 MPa treatments in grey (*n* = 5). Error bars represent 95% confidence intervals. Relative quantities are normalised to 0.1 MPa in each exposure duration. Statistical comparisons were made within exposure durations, not between exposure durations. Significant differences from 0.1 MPa, determined using a one-way ANOVA calculated in qBase+ software, are indicated by an asterisk.

Appendix 2 List of publications

Contributions to thesis:

Brown, A. & Thatje, S. (2014). Explaining bathymetric diversity patterns in marine benthic invertebrates and demersal fishes: physiological contributions to adaptation of life at depth. *Biological Reviews* **89**, 406-426.

Brown, A. & Thatje, S. (2015). The effects of changing climate on faunal depth distributions determine winners and losers. *Global Change Biology* **21**, 173-180.

Other works:

Brown, A. & Thatje, S. (2011). Respiratory response of the deep-sea amphipod *Stephonyx biscayensis* indicates bathymetric range limitation by temperature and hydrostatic pressure. *PLoS ONE* **6**, e28562.

Brown, A. & Wentworth, J. (2013). Selection of Marine Conservation Zones. Parliamentary Office of Science and Technology note **437**, 4 pp.

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