UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

Ocean and Earth Science

The physiological viability of bathymetric range shifts in marine ectotherms

By

James Peter Morris

Thesis for the degree of Doctor of Philosophy

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"The thing that is important is the thing that is not seen . . ."

The Little Prince,

Antoine de Saint-Exupéry, 1943

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ABSTRACT

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Organisms occupying an environment where change is occurring must move, acclimatise, or adapt in order to prevent extinction. Despite recent evidence, the potential for species to change their distribution range across depths is regularly overlooked as an alternative to well documented latitudinal shifts. The aim of this thesis was to investigate the physiological viability of bathymetric range shifts by assessing the impacts of elevated hydrostatic pressure (HP) and changing temperature in two marine ectotherms: the shallow-water caridean shrimp *Palaemonetes varians*; and the continental slope-depth anomuran crab *Lithodes maja*. Gene biomarkers were characterised in both species and their transcriptional regulation was assessed by qPCR. Acute and long-term exposures revealed that elevated HP produced significant perturbations at the transcriptional level in *P. varians*, and were corroborated by measurements of behaviour and respiratory response. HP-associated pathologies were exacerbated by decreasing temperature, and ameliorated by increasing temperature. Results gave a novel insight into the hierarchy and kinetics of HP-induced stress responses, and suggested that the combination of HP and temperature, in part, determines the physiological limitations to bathymetric migration in shallow-water ectotherms.

To further test the role of HP and temperature in setting depth distribution limits, gene biomarkers were characterised in a continental slope-depth crab *Lithodes maja*. Transcriptional regulation and respiration rate was quantified throughout larval ontogeny, and in mature adults. Early life stages showed HP tolerance beyond that of the species natural distribution limits. The observed HP tolerance window reduced through ontogeny. Reductions in HP tolerance with life stage may be analogous to established thermal tolerance reductions with ontogeny. Further, sustained HP exposures suggest that bathymetric range limits in *L. maja* are constrained by increased metabolic costs, nervous system sensitivities, and macromolecular damage under elevated HP.

HP and temperature, and particularly the combination of the two, play an important role in setting the depth distribution limits of marine ectotherms. For temperate shallow-water adapted marine ectotherms, water temperatures towards the upper limit of their thermal scope may facilitate bathymetric range shifts, whilst colder waters further exacerbate the negative physiological effects of elevated HP. Bathymetric range shifts may, under certain scenarios, offer an important, yet underestimated, alternative to latitudinal shifts in light of contemporary climate change. Further, the combination of HP and temperature may have played an important role in the colonisation of the deep sea from shallow waters by marine ectotherms.

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List of abbreviations and definitions

ACP – Annealing control primer

ADP – Adenosine diphosphate

AGPC – Acid guanidinium thiocyanate phenol chloroform

ANOVA – Analysis of variance

Arf – Adenosine diphosphate-ribosylation factor

Atm – Atmospheres (1 atm = 0.101 MPa)

Bar – No SI unit of pressure (1 bar = 0.1 MPa)

BLAST – Basic local alignment search tool

Bp – Base pair

CCD – Conserved domain database

cDNA – Complementary deoxyribonucleic acid

CHR – Cellular homeostatic response

CI – Confidence interval

cNRQ – calibrated normalised relative quantity

CNS – Central nervous system

CO1 – Cytochrome oxidase subunit 1

CS – Citrate synthase enzyme

CSR – Cellular stress response

Cq – Cycle quantification

CV – Coefficient of variance

DEG – Differentially expressed genes

degenPCR – Degenerate polymerase chain reaction

DEPC – Diethylpyrocarbonate

DNA – Deoxyribonucleic acid

DNase – Deoxyribonuclease

dNTP – Nucleoside triphosphate

DTT – Dithiothreitol or Cleland's reagent

EDTA – Ethylenediaminetetraacetic acid

EEF1α – Elongation factor 1-alpha

EMBL-EBI – European molecular biology laboratory, European bioinformatics institute

ER – Endoplasmic reticulum

EST – Expression sequence tag

GAPDH – Glyceraldehyde-3-phosophate dehydrogenase

gDNA – Genomic deoxyribonucleic acid

GLM – General linear model

GOI – Gene of interest

HP – Hydrostatic pressure

HPNS – High pressure neurological syndrome

HSE – Heat shock element

HSF – Heat shock factor

HSR – Heat shock response

HSC – Heat shock cognate

HSP – Heat shock protein

HSP70 – Heat shock protein family of ~70kDa in atomic mass

IPCC – Intergovernmental panel on climate change

IPOCAMP - Incubateur pressurisé pour l'observation et la culture d'animaux marins profonds

IPTG – Isopropyl β-D-1-thiogalactopyranoside

IRC –Inter-run calibrator

JM109 – Strain of *Escherichia coli* competent cells for cloning techniques

kDa – Kilodalton = 1000 daltons - SI unit for molecular mass

LB – Lysogeny broth

LDH – Lactate dehydrogenase enzyme

LOE – Loss of equilibrium

MIQE – Minimum information for publication of quantitative real-time PCR experiments

MO_2 – Oxygen consumption (μmol O_2 mg^{-1} h^{-1})

MPa – Megapascal = 1,000,000 pascal – SI derived unit for pressure

mRNA – Messenger ribonucleic acid

MSP – Minimal stress proteome

NCBI – National center for biotechnology information

NMDA – N-methyl-D-aspartate

NMDAR – N-methyl-D-aspartate receptor

NOCS – National Oceanography Centre, Southampton

NRQ –Normalised relative quantity

NTC – No template control

OBIS – Ocean biogeographic information system

ORF – Open reading frame

OSR – Overall stress response

PAGE – Polyacrylamide gel electrophoresis

PARP – Poly (ADP-Ribose) polymerase

PCR – Polymerase chain reaction

PVC – Polyvinyl chloride

qPCR – Quantitative polymerase chain reaction

RF – Relative fold

RFC – Relative fold change

RNA – Ribonucleic acid

RNase – Ribonuclease

rRNA – Ribosomal ribonucleic acid

RPL8 – Ribosomal protein L8

RPS26 – Ribosomal protein S26

RQ – Relative quantity

RQI – RNA quality indicator value

ROX – Passive reference dye

SBD – Substrate binding domain

SD – Standard deviation

SYBR – Asymmetrical cyanine dye

TAE – Tris-acetate-EDTA

TAQ – Thermostable DNA polymerase

Tukey HSD – Tukey's honest significant difference test

X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside

DECLARATION OF AUTHORSHIP

I, James Peter Morris, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

"The physiological viability of bathymetric range shifts in marine ectotherms"

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

4. 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;

5. I have acknowledged all main sources of help;

6. 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;

7. Parts of this work have been published as:

- Morris JP, Thatje S, Hauton C (2013) The use of stress-70 proteins in physiology: a re-appraisal. Molecular Ecology 22:1494-1502
- Morris JP, Thatje S, Ravaux J, Shillito B, Fernando D, Hauton C (2015) Acute combined pressure and temperature exposures on a shallow-water crustacean: Novel insights into the stress response and high pressure neurological syndrome. Comparative Biochemical Physiology: Part A 181:9-17
- Morris JP, Thatje S, Ravaux J, Shillito B, Hauton C (in press) Characterising multi-level effects of an acute pressure exposure on a shallow-water invertebrate: insights into the kinetics and hierarchy of the stress response. Journal of Experimental Biology
- Morris JP, Cottin D, Oliphant A, Brown A, Ravaux J, Shillito B, Thatje S, Hauton C. (in review) The physiological viability of climate-driven bathymetric range shifts in a shallow-water ectotherm. Open Science
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Signed:

Date:

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Chapter 1

Introduction

1. INTRODUCTION

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1.1 The history & concept of stress

The term stress is used in an ever widening variety of circumstances, and as such has become increasingly difficult to define. Used frequently and somewhat differentially in both biological and psychological sciences, there are a number of commonalities that highlight the generally accepted fundamental aspects of the concept. At its root stress describes a concept that impacts on an organism's physiological or mental condition (Selye 1956). Beyond this, it becomes difficult to underpin values of the term that are shared between its many usages, particularly, whether or not stress is seen as a cause, an effect, or the process by which the two are connected. Dealing with increasingly complex organisms, stress can encompass an entirely concrete or abstract meaning, and, particularly with humans, be highly subjective (Selye 1956).

Derived from the Latin *stringere* or *strictus*, meaning "to draw tight" and adapted into the Middle English form *destresse*, "the cause/infliction of pain or suffering" (Keil 2004), the pre-1920s term had very few of its modern connotations (Stevenson 2010). It had been used scientifically in physics to describe the internal distribution of a force on a substrate, resulting in strain. From the 1920s onwards the term began to appear in the vocabulary used in biological and psychological circles, referring to a mental strain or an environmental agent that brought about illness. In 1926, Walter Cannon used it to denote a disruption of homeostasis by external factors (Cannon 1929). The first truly novel usage of stress in biology and psychology is attributed to Hans Selye, an endocrinologist who began to use the term to refer to the state of the organism as it responded and adapted to the environment (Selye 1956).

The concept of homeostasis has been central to the idea of stress since Walter Cannon and Hans Selye (Koolhaas et al. 2011). Homeostasis in biology can be thought of as the drive to maintain a steady state or equilibrium: in most cases the concept exists as more of an ideal than an achievable prolonged condition (Filaretova 2012). An organism can be thought of

as being in a constant state of instability, oscillating about a homeostatic point that is the organism's optimal living condition. Environmental factors and internal or external stimuli, no matter how large or small, are continually perturbing the steady state of the organism, and it is these factors that cause an organism to move away from its homeostatic point. As such, it could be argued that any factor that causes a disruption of homeostasis can be interpreted as stress. In this case, stress is seen from a causal perspective. Attempts by the organism to continually restore or maintain homeostasis requires energy that could otherwise be used for processes such a growth or reproduction (Sokolova 2013). This energy cost can also be interpreted as stress, and in this regard stress is seen from an effectual perspective. Being able to define stress from both cause and effect standpoints renders the term somewhat ambiguous, a fact that was recognised by Hans Selye in 1926 when he described stress "… in addition to being itself, was also the cause of itself, and the result of itself…". Selye's most generally accepted definition which to some extent still prevails to this day demarcated stress as "the non-specific response of the body to any demand placed upon it" (Selye 1976).

Defining stress is still a contentious topic today, particularly in the field of biology. Many conflicting definitions are accepted in differing circumstances. An evolutionary biologist will have a different perspective on how to define stress from a molecular physiologist, for example. The biological level of interest (molecular, cellular, organismal, or population) will also have a bearing on its operational definition (Bijlsma and Loeschcke 2005). More importantly for biologists, stress is an attribute of the stressor (environmental factor) and the stressed (the biological component: a cell, an organism, or a population etc.) and therefore the environmental factor and the level at which that factor exerts itself can only be defined in relation to the particular biological component of interest. Despite stress being used most commonly in the context of an environmental stimuli, from an evolutionary perspective environmental change and the subsequent response that it triggers should be considered as inherently connected, i.e. changes in the level of stress felt by an organism can be a consequence of changes of either the stressor, the stressed or both (Bjilsma and Loeschcke 1997). A number of definitions fit these conditions: "an environmental condition that, when first applied, impairs Darwinian fitness" (Sibly and Calow 1989), "an environmental factor causing a change in a biological system which is potentially injurious" (Hoffmann and Parsons 1991; 1997), and "any environmental change that acts to reduce the fitness of organisms" (Koehn and Bayne 1989). Each definition is centred on the idea of a reduction in fitness of the biological component of interest.

Hoffmann and Parsons (1991) suggest that if stress was simply "a reduction in fitness" then wild organisms would constantly experience stress at some level. Many scientists prefer the term stress to be applied only when a particular intensity has been reached (Koolhaas et al. 2011). However, stress intensity varies on a continuous scale, is factor specific and can normally only be judged in hindsight, making it difficult to decide what level of intensity can be denoted as stress rather than just natural variation (Koolhaas et al. 2011). Stressors are most commonly considered to be environmental, or at least of an external nature to the biological component; however, recent molecular research has found that internal factors such as genetic mutation or inbreeding can act as a stressor (Bijlsma et al. 2000). This must be taken into consideration as, for example, inbred populations may show high levels of stress in response to changes in the environment that would be perceived by outbred populations as only mildly stressful.

The fact that science, despite our huge leaps in understanding since the 1920s has not been able to agree on clear conceptual or operational definitions of stress, has led many to believe that any definition should remain ambiguous (Bijlsma and Loeschcke 2005). Although a single definition will evidently not aptly satisfy all connotations of the term, it is important to clearly state how you are defining stress prior to its use in literature. As this thesis covers aspects of molecular biology, cellular physiology and evolutionary ecology, no one single definition of stress so far proposed has encompassed all aspects of the term that may be covered herein, and as such a number of previous definitions have been adapted and combined to satisfy the needs of research conducted in this thesis. From here on in stress will be defined as:

"an environmental or genetic factor that causes a change in a biological system which is potentially damaging, and which has some consequence on the organisms' Darwinian fitness"

Stress will be viewed exclusively from a causal perspective. Effectual stress, or the energy used by the biological component to counteract stress, will be termed the stress response, thus allowing for clear differentiation between the two components.

1.2 The stress response & physiology

All organisms will at some point during their lifespan encounter environmental conditions that challenge the physiological functioning of their cells. When such an effect becomes severe enough to require counter measures in order to maintain cellular homeostasis, it may be considered stressful. Organisms have many common responses to stress (Kültz 2005): at the whole organism level these can be controlled by hormones which initiate classical responses such as the "fight or flight response" (Cannon 1929) and the "general adaptation syndrome" (Selye 1973). Generalised responses at a cellular level are known as the "cellular stress response" (CSR), which can be defined as "a defence reaction to a strain imposed by environmental forces on macromolecules" (Kültz 2005). Stress at this level commonly results in damage to macromolecules (Kültz 2003). The CSR covers a number of processes that assess and counteract stress induced damage, provide short term increased tolerance against such damage, and initiate apoptosis in terminally damaged cells. The specific functions of the CSR include cell cycle control, protein repair, protein folding and translocation, as well as certain features of cell metabolism (Kültz 2005). The CSR is thought to have originated in the earliest examples of life on Earth and is now characteristic of all cells because of stabilising selection (Koonin 2003). The proteins at the heart of the functioning of the CSR are also highly conserved in all organisms. Proteomic analysis of organisms across all three super-kingdoms: archaeal (Macario et al. 1999), eubacterial (Petersohn et al. 2001) and eukaryotic (Pearce and Humphrey 2001), found 44 that were highly conserved in all and were found to have functions related to the CSR (Kültz 2003). The CSR's ubiquity across all forms of life is due to the fact that stressors, despite being numerous and diverse, tend to result in non-specific macromolecular damage. The 44 proteins that make up the universal stress response have been termed the "minimal stress proteome" (MSP) (Kültz 2005). In any given organism many more genes/proteins are involved in the CSR than those that comprise the MSP, however, these are likely to play important roles in stress-specific responses such as, for instance, a response to hydrostatic pressure (HP) change in aquatic organisms that is not often of relevance in a terrestrial lifestyle. The 44 proteins can be clustered according to their common functions, each cluster representing a different aspect of the CSR (Figure 1.1). For a detailed review of individual members of the MSP see Kültz (2005) and Wang et al. (2009).

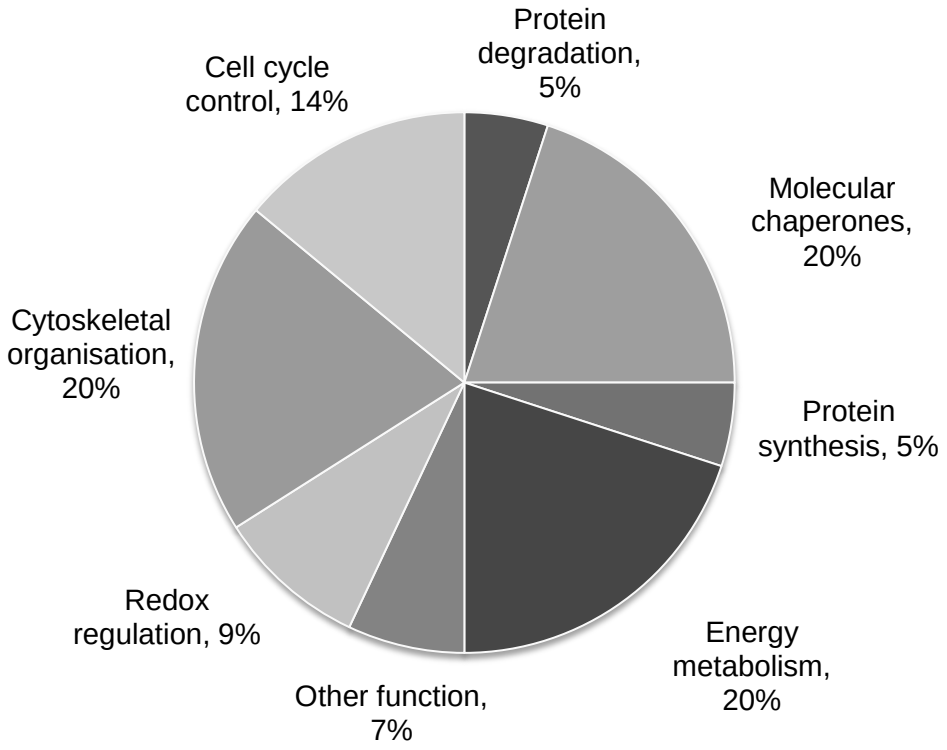


Figure 1.1 Clustered functional classifications of the 44 proteins that comprise the MSP. Proteins with multiple functions were assigned to the group that was considered their primary function. Adapted from Kültz (2005) and Wang et al. (2009)

Because of the generality of the damage caused by stress within cells, and the ubiquity of the 44 proteins that make up the MSP, such proteins have been advocated as good biomarkers of stress. Effective stress biomarkers need to meet certain criteria: they must be quantifiable, universal within the study group, sub-lethal, and reliable for interpretation (Ingeborg Werner 1999; Lewis et al. 1999). The key features that make the proteins in the MSP such attractive research targets as potential biomarkers of stress are:

- Their role and function in one organism is likely analogous in all organisms.
- A large proportion of the 44 proteins in the MSP respond to a diverse set of stressors through transcriptional up-regulation.
- Compared to other stress markers, quantifying components of the MSP provides a quick and easy method for assessing stressful situations.

Undoubtedly the most famous and widely studied group of proteins involved in the CSR are known collectively as the heat shock proteins (HSPs) (Feder 1999; Feder and Hofmann

1999). Heat shock proteins and heat shock cognates (HSCs) are a large subset of proteins termed molecular chaperones. Their name (chaperone) is taken from their function in the prevention of inappropriate protein aggregations; they also play other vital roles in: folding and unfolding, assembly and disassembly, transport, and degradation of misfolded/aggregated proteins (Benarroch 2011). Such tasks are imperative under normal cellular conditions; however, during periods of stress when the potential for proteins and other macro-molecules to function irregularly is amplified, the need for molecular chaperones is also increased. It is important to restate that non-specific macromolecular damage activates the CSR. In the case of heat shock regulation this is controlled by the "cellular thermometer" model outlined by Tomanek and Somero (2002). These authors have proposed that under non-stressful conditions heat-shock factor-1 (HSF1) monomers are associated with molecular chaperones including members of the stress-70 family (HSP70s and HSC70s). However, under stressful conditions as misfolded protein levels increase in the cytosol, the chaperone molecules dissociate from HSF1 freeing it to move into the nucleus and bind to heat shock element (HSE) promoter regions to promote HSP70/HSC70 gene transcription (Tomanek and Somero 2002). Therefore, HSP70 transcription is not activated directly by stress but simply an increase in macromolecular damage.

The first genes coding for HSPs were discovered in *Drosophila buscki* in 1962; seen as chromosomal puffing (as a result of RNA synthesis) after exposure to increased temperatures (Ritossa 1962). Subsequent studies have found that an ever-increasing suite of stressors induces a heat shock response (HSR) (Feder and Hofmann 1999). As a result the HSR is now considered to be a fundamental component of an organism's CSR. The best studied family of heat shock proteins are known as the stress-70 family and are named in relation to their molecular weight (70kDa). This family comprises of three main isoforms (Sorensen 2010):

- Solely constitutive: present during normal cell functioning, among other roles they carry out folding of nascent polypeptides under normal cellular conditions (known as heat shock cognates e.g. HSC70) (Boutet et al. 2003).
- Solely inducible: up-regulated in cells in response to stressful stimuli (Ravaux et al. 2007).
- Constitutive and inducible: expressed during normal cell functioning and also up-regulated in response to stressful stimuli (Callahan et al. 2002).

The HSP70 group shows unique versatility, demonstrating the ability to assist in a large number of protein folding processes (Richter et al. 2010), from *de novo* polypeptide folding to the translocation of proteins across membranes (Benarroch 2011). The ability to perform such a large array of tasks arises from the fact that, unlike many other HSPs which fully or partly enclose their substrates, the HSP70 family member binds via the C-terminal substrate-binding domain (SBD) with only a short segment of the target polypeptide (Lewis et al. 1999). This allows HSP70 to bind to a variety of substrates of differing sizes and configurations. Such flexibility reflects in the frequency of up-regulation and concentration during stress situations; HSP70 being the most commonly up-regulated of all HSPs in most organisms in response to stress (Lewis et al. 1999).

1.3 HSP70 as a stress biomarker

HSPs, and in particular the HSP70 family, have been suggested by many in the past two decades as a good universal biomarker for stress, and superficially it is easy to see why. Effective stress biomarkers need to meet certain criteria: are quantifiable, universal within the study group, sub-lethal, and reliable for interpretation (Ingeborg Werner 1999; Lewis et al. 1999). HSP70 family proteins are ubiquitous and highly conserved in nearly all organisms, making the genes encoding them easy to isolate and identify within a genome. They are responsive to a large variety of stresses: in fact the majority of known/tested stresses will at some magnitude induce a HSR (Lindquist 1986; Sorensen 2010). Induction of HSP70 within organisms is much more sensitive to stress than other more traditional markers such as growth inhibition, lethal dose measurements, or fertility (Sorensen et al. 2003). HSP70 has the largest activity range in comparison to all other stress proteins (Mayer 2010), and will therefore, in most cases, be the easiest to detect.

Since the discovery of HSP70 and the identification of its role in the cellular stress response a plethora of research has used HSP70 differential expression as a marker of stress. During this period technological advances have drastically altered our understanding of the HSP70 family. As an example, we can now probe with great detail how individual proteins within the heat shock collective alter their structural conformation in order to successfully perform tasks within the cell (Mayer 2010). Work on certain model species (*Drosophila* sp., *Arabidopsis* sp., or *Danio rerio* etc.) is continually pushing forward our understanding of the relationship between stress-70 family expression and organismal

stress (see Calabria et al. (2012) as an example). In doing so, such research is also determining previously unidentified limitations in the use of HSP70 as a stress biomarker. Nevertheless, a large portion of the current HSP70 stress biomarker research is carried out on non-model organisms; in many of these cases the way stress-70 differential expression is viewed has not kept a pace, and remains rather simplistic. Our now in-depth understanding of the complexities of HSP70 expression in key model taxa (examples to follow) appears, in a large number of other cases, to have been overlooked in studies using HSP70 as a marker of stress. This may be in the form of incorrect assumptions of what the differential expression of a particular stress-70 family member represents, or inappropriate experimental design. The following sections will highlight the disparity between our current understanding of stress-70 family expression at the cutting edge of the science, and their improper use as stress biomarkers.

1.3.1 Examples of common usage

It has now become common practice to expose organisms to a potential stressor under laboratory conditions and subsequently use the transcriptional up-regulation or protein level increase of a specific member of the HSP70 family as evidence of stress, and the activation of the CSR. Many authors also compare the relative levels of up-regulation to infer the degree of stress experienced by the organism. For example, an organism showing a 20-fold induction of a particular member of the HSP70 family may be thought to be experiencing a higher degree of stress than if only a 4-fold induction was seen. In 2000, Tomanek and Somero conducted experiments on two congeneric marine snails (Genus *Tegula*) that occupy different intertidal zones in order to determine whether the vertical limits of their distributions were set by thermal stress (Tomanek and Somero 2000). Accordingly they exposed individuals from both species to acute heat shock treatments and plotted relative expression levels of a number of HSPs including two HSP70 proteins over a 50-hour recovery period. Both species showed evidence of an up-regulation in different HSPs during the post exposure recovery periods. This up-regulation was suggested as evidence of heat stress. The low-intertidal/sub-tidal species synthesised a higher level of HSP70 over the recovery period than the mid-intertidal species. It was concluded that the low-intertidal/sub-tidal species experienced a higher degree of thermal stress and incurred greater thermal damage than the mid-intertidal species (Tomanek and Somero 2000). In another example, Hauton et al. (2009) conducted sea-water acidification experiments on the marine amphipod *Gammarus locusta* choosing to use HSP70 as a marker of pH stress

caused by disruption of an organisms internal acid/base balance (Hauton et al. 2009). An HSP70 gene sequence was found and tested to determine whether it was an inducible form. After acute heat shock treatment the HSP70 showed a 2000-fold increase in expression, proving that it can be stress induced, at least in response to heat exposure. In the main experiment however, no significant change in expression of the HSP70 gene was resolved in samples taken 14 and 28 days after initial exposure. From the data it was concluded that prolonged exposure to a reduced pH environment did not induce a sustained acid/base imbalance (Hauton et al. 2009). HSP70 has been used in these ways or similar for many years with very few questioning the validity of such methods and analysis. We now contend, however, that because the up-regulation of HSP70, or lack of, occurs in response to so many varying factors, that to make even simplistic assumptions may be subject to many complications. In such cases it can be questioned what exactly HSP up-regulation signifies, and whether experiments designed in this way can elucidate those answers.

1.3.2 Multi-stressor: laboratory versus field measurements

The majority of HSP70 stress biomarker studies have investigated the link between a single stressor and levels of HSP70 as controlled laboratory exposures. The results from such studies are commonly used to explain field observations or scenarios. The natural environment however, is characterised by having many parameters that affect organisms simultaneously: in fact, in the field, multiple stressors are the rule rather than the exception. A vast array of stressors has been shown to provoke HSP70 induction (Figure 1.2a). It is known that HSP70 levels can display a 'toxic cocktail' style phenomenon where a number of minor stressors occurring simultaneously produce higher inducible HSP70 levels than the sum of each stress individually (Hamer et al. 2004). Lockwood and Somero (2011) recently compared gene expression profiles for salinity stress and heat stress and found that 45 genes changed expression significantly in response to both stressors. Interestingly, the most strongly down-regulated heat stress "genes-in-common" were the most strongly up-regulated in response to salinity stress. This differential expression profile between the two stressors may be due to the antagonistic effects of salinity and temperature on cross-membrane ion transportation (Lockwood and Somero 2011). This is a clear example of the differential effects of certain stressors on the expression of stress-inducible genes. The above examples highlight that the usefulness of HSP70 as a stress biomarker in field studies or research involving multiple stressors is significantly curtailed.

1.3.3 Within-organism variation

HSP70 expression has also been found to be tissue-specific in some species. Rabergh et al. (2000) found a variation in HSR among certain tissues of *Danio rerio* (zebrafish). On exposure to heat stress it was found that HSP70 levels were higher in the fish's gonad under all conditions than the liver and gills (Rabergh et al. 2000). It is apparent that certain stresses are going to have greater effects on certain tissues, i.e. heat stress is likely to affect, and therefore induce a greater HSR, in tissues that are more heat sensitive such as fish gonads. In such cases different organs from a single organism might give very different HSP70 expression profiles that without prior knowledge can alter the analysis of perceived stress (Figure 1.2b). An understanding of these potential inter-organ variations is particularly important when working with smaller organisms where whole organism homogenisations may be employed. In such cases stress signals from small but significant tissues may be masked by larger trivial signals (Rabergh et al. 2000; Pyza et al. 2007).

Stress-70 family proteins function in all major subcellular compartments of the cell. As such, distinct isoforms can be differentiated and split into: mitochondrial members, endoplasmic reticulum (ER) luminal members, chloroplastic members (in plants), or cytosolic members. Each isoform may have very similar but distinct roles, and obviously differ in terms of subcellular spatial distribution (Sung et al. 2001). Cytosolic HSP70 members, for example, may play an important role in HSR regulation through the negative repression of heat shock factor (HSF) – mediated transcription (Shi et al. 1998). Evidence is mounting that suggests many species' genomes contain a large number of HSP70 genes: the human genome is thought to contain at least 11 HSP70 genes (Tavaria et al. 1996); the yeast stress-70 family has 14 known members (Rassow et al. 1997), and recently it was discovered that the genome for the Pacific oyster *Crassotrea gigas* contains 88 HSP70 genes (Zhang et al. 2012). In contrast, the *Escherichia coli* genome has only two stress-70 family members (Bardwell and Craig 1984; Seaton and Vickery 1994). Sung et al. (2001) compiled an expression profile analysis of 12 HSP70 genes in an *Arabidopsis* plant, and Li et al. (1999) brought together a similar expression analysis of 10 HSP70 members in spinach (Li et al. 1999): both studies found similar results. In response to elevated temperatures, all stress-70 family members examined showed coordinated up-regulation. Conversely, subsequent repression profiles varied amongst isoforms with some members showing rapid repression after 60-minutes whilst others still showed maximal expression after 90-minutes. This temporal profile of expression itself raises significant problems when considering

experimental and sampling designs (as discussed below). In response to lowered temperatures both studies found no coordination in stress-70 member expression profiles; some members showed no induction, other members showed similar expression to elevated temperature. Sung et al. (2001) found induction by low temperature was limited to cytosolic and mitochondrial members of the stress-70 family, and induction and repression timescales varied individually. Sung et al. (2001) also found differential expression of stress-70 family members in different organs: all members were expressed in the root, with higher levels shown in one mitochondrial and one cytosolic member. In contrast, two chloroplastic members showed higher levels in leaf tissue. These findings highlight that HSP70 expression varies not only between organs, but also at a subcellular level. Variation occurs not only in magnitude but also temporally in terms of induction and repression. This is of fundamental importance in the application of stress-70 members as stress biomarkers. A large portion of the research that uses HSP70 as a stress marker makes no reference to which of the potentially numerous members of that species' stress-70 family was analysed and whether that particular member can be thought of as characteristic of the species' stress response. Analysing different members of the stress-70 family may lead to different interpretations of the level of stress an organism is being exposed to. In order to produce reliable results more than a single member of the stress-70 family should be characterised and analysed in order to determine which, if any, members accurately portray the study organisms' level of stress.

1.3.4 Experimental design

The timing of sampling during or post stress can have a large bearing on the way stressor impact is observed. The sampling target, be it messenger RNA (mRNA) or protein can also have a significant bearing on perceived stress levels (Figure 1.2b and c). HSP70 is involved in repair and restoration, not direct cell death prevention. When an acute stressor is nearing lethal severity cellular mechanisms are primarily concerned with the avoidance of stress-induced cell death. At this point the cost of mounting an HSR outweighs the benefits and all available energy is directed towards cell survival. Only when imminent death is prevented are the mechanisms of repair and restoration set in motion: DiDomenico et al. (1982) found the up-regulation of an HSP70 was delayed by around an hour after a 39°C heat shock in comparison to a 36.5°C heat shock. Sampling during or immediately after a near-lethal acute stressor exposure is unlikely yield an HSP70 up-regulation signature

despite the fact that the cells in question are likely to be severely stressed. Buckley et al. (2006) compared mRNA expression and protein production of an HSP70 in the goby *Gillichthys mirabilis* over timescales during and post stress exposure; they found that an increase in mRNA levels correlated with an increase in protein production in all cases. More interestingly however, they found that mRNA versus protein production varied in relative timing, magnitude and according to the organ sampled. For example, a 5-fold increase in HSP70 mRNA in gill tissue peaking at 180-minutes into heat shock was followed by a 12-fold increase in protein levels peaking at 350-minutes into heat shock. By contrast, in muscle tissue an 18-fold increase in mRNA peaking at 220-minutes into heat shock was followed by a 3-fold increase in protein levels peaking at 300-minutes (Buckley et al. 2006). These data suggest a mismatch between mRNA and protein levels; a concept that is argued in a wider transcriptomics vs. proteomics context also (Kristensen et al. 2013; Li and Biggin 2015). The timing of mRNA or protein measurement during or after stress exposure is also of significance as the peak magnitude of fold increase varied between mRNA and protein and also between tissues sampled. With this in mind, I caution against single "snapshot" time point measurements of either mRNA or protein levels, as this method will likely misrepresent organismal stress levels. This snapshot style measurement is prevalent in many HSP70 stress biomarker studies (Hauton et al. 2009, for example).

It is common practice in acute stress studies to allow a recovery period before preserving samples for HSP70 analysis (Cottin et al. 2010): this is usually a minimum of 30-minutes after acute stressor exposure, followed by regular sampling from a few hours to several days after exposure. In the early 1980s when HSP70 research was still in its infancy, DiDomenico et al. (1982) showed that the severity of a heat stress treatment affected the time taken to express HSP70 and subsequently recover to basal levels. Results from Tomanek and Somero (2000) clearly show that a stress-induced HSP70 is up-regulated substantially during the first 5-hours of recovery and reaches a peak after 15-hours. In contrast, an HSP77 was also analysed and showed no significant up-regulation until 12-hours after exposure. Once again this is an example of the differential behaviour of members of the stress-70 family. Had Tomanek and Somero (2000) only sampled one stress-70 member, as many HSP70 studies do, their evaluations of the study organisms' stress would have been very different (Figure 1.2c). Hauton et al. (2009) only sampled one HSP70 and did so only at 14-days and 28-days after initial exposure. When no significant HSP70 up-regulation was found it was concluded that no sustained acid/base imbalance stress was occurring. In fact it seems more likely that at some point, probably before the

14-day sampling, HSP70 was up-regulated in response to the pH change, particularly as the experimental organisms were subjected to an instantaneous pH change with no acclimation period at the start of the experiment. It may have been that this acute pH change invoked an HSP70 response, which was later replaced by more cost effective stress-specific mechanisms. As highlighted above, different members of the stress-70 family will react to stressors over different time-scales, so sampling only one HSP70 member may lead to misinterpretation of true organismal stress levels. The effective use of HSP70 as a stress biomarker requires the use of numerous stress-70 members alongside a rigorous post-exposure time-series sampling process at the very least: a procedure very few HSP70 biomarker studies conform to.

1.3.5 Non-environmental HSP70 variation

Studies have found that many non-environmental cues can induce and affect the way HSP70 is expressed (Figure 1.2a). Genetic stressors, such as mutation (Zhao et al. 2002) and inbreeding (Kristensen et al. 2002) have been shown to influence levels of HSP70 expression. Kristensen et al. (2002) found that inbred larvae of *Drosophila buzzatii* expressed higher levels of an HSP70 in response to all but the very highest of temperatures within the larvae's thermal limits compared to outbred larvae. Inbreeding depression and the subsequent expression of deleterious alleles may lead to an increase in misfolded proteins at a cellular level, which in turn would up-regulate HSP70 (Kristensen et al. 2002). HSP70 levels are also dependent on the general fitness of the organism regardless of any stressors acting on the organism at a given time. In such cases the costs of mounting a generalised stress response may outweigh the benefits and other forms of response may be more appropriate. This has been shown in populations that are exposed to frequent or continuous stress, which have been observed to produce lower levels of certain HSP70 proteins than populations who only experience stress infrequently (Lansing et al. 2000); effective stress responses are potentially achieved in such populations by other more cost effective and stressor-specific means (Kohler et al. 2000).

Each of the aforementioned factors can profoundly alter the way in which HSP70 is up-regulated within organisms. Up-regulation signals from non-environmental cues may be incorrectly interpreted as an environmental stress signal. Such non-environmental parameters are inherently hard to quantify, can vary significantly amongst individuals even

within a single population, and may distort any stress related HSP70 induction. HSP70 has many well-documented roles within the cell, research has also found that extracellular HSP70 (eHSP70) can act as a highly effective pro-inflammatory response (Basu et al. 2000; Srivastava 2002). Such a response is of particular relevance to this discussion because eHSP70, and therefore also intracellular HSP70, has been shown to be up-regulated in response to perceived danger. Fleshner et al. (2004) found that exposure to an exclusively psychological stressor triggered both intracellular and extracellular HSP72 up-regulation. In the study adult male rats were exposed to a cat, but without any physical contact, thus a stressor of purely psychological origin (Fleshner et al. 2004).

HSP70 expression has been shown to vary according to the life stage and sex of the organism in question. Garbuz et al. (2008) demonstrated that HSP70 expression levels under non-stressful conditions were twice as high in the larvae of soldier flies than in related adults. This pattern maybe due to a reduced need for thermo-tolerance in adults of the species because increased mobility allows them to escape potential stressors with greater ease (Garbuz et al. 2008). Constitutive expression of stress-70 family members has also been shown to vary considerably according to life cycle in *Arabidopsis* plants (Sung et al. 2001). Sex has also been shown as a source of variation in HSP70 expression within species, Sorensen et al. (2001) found that HSP70 expression varied between males and females in two *Drosophila buzzatii* populations, this may be a result of differing sensitivities to stress between the sexes, or the differential cost of HSP70 expression on reproduction. These results have implications for the selection of study organism life stage and sex, as well as subsequent organism handling and the environment within which the experiment is conducted; all of which may have profound effects on HSP70 expression.

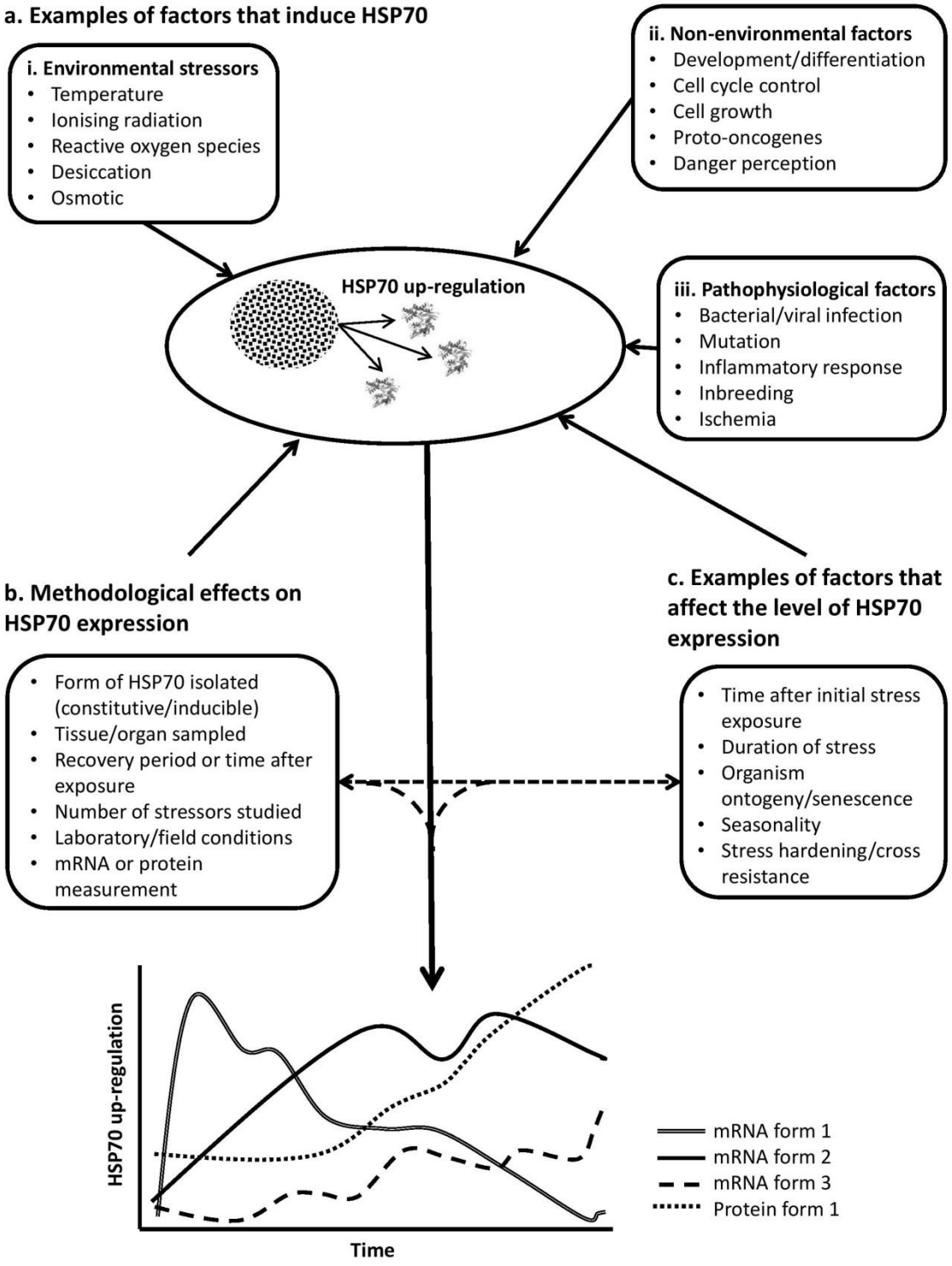
1.3.6 Constitutive versus inducible HSP70

Members of the stress-70 family have been split into two functionally distinct groups: those that are constitutively expressed (HSC70), and those that are stress inducible (HSP70). However, as of yet no clear evidence has been presented that allows any consistent inter-species differentiation between the two groups, either functionally or structurally. Some research has suggested that genes encoding HSP70 lack introns as opposed to those encoding HSC70 which possess them (Gunther and Walter 1994; Boutet et al. 2003). It was suggested that the lack of introns in the stress inducible form allowed for a more rapid response to stress because RNA splicing was not necessary. However, recently it has

become clear that the presence or absence of introns cannot be used to differentiate between HSP70 and HSC70. Qin et al. (2003), amongst others, found introns within genes encoding stress inducible HSP70. Leignel et al. (2007) examined other characteristic regions of genes encoding HSC/HSP70 in hydrothermal vent and coastal crabs and found no structural characteristics that could differentiate between inducible and constitutive forms.

From a functional perspective, differentiation has also been problematic. Research conducted on silver sea bream found that, although structurally distinct in this case, HSP/HSC70 both showed stress-associated induction (Deane and Woo 2005). A number of studies on molluscs have found high levels of both HSP70 and HSC70 in apparently unstressed individuals (Buckley et al. 2001). Franzelliti and Fabbri (2005) found that gene sequences in a Mediterranean mussel (*Mytilus galloprovincialis*) for HSP70 and HSC70 were more closely related to other bivalve HSP and HSC70s, respectively, than to each other. This inter-specific gene homology was suggested to indicate that HSC/HSP70 could be paralogs: performing slightly different but highly related roles within the cell (Franzellitti and Fabbri 2005). Nowhere is the functional confusion of HSP/HSC70 better underlined than in the Antarctic (highlighted in Clark and Peck 2009). In the past decade many studies have found that Antarctic fish (*Trematomus baernacchii*) lack the ability to up-regulate some HSP70s in response to external stresses (Hofmann et al. 2000). To further complicate matters, a number of studies found several of the fish examined showed permanent expression of an inducible HSP70 (Clark et al. 2007; Clark and Peck 2009), which appeared to assume the same role as the constitutively expressed HSC70. What would normally be considered an inducible HSP70 was in this case non-inducible, but was permanently (i.e. constitutively) expressed. This phenomenon is by no means restricted to the Antarctic; Gehring and Wehner (1995) demonstrated that two species of Saharan ants, known to be able to survive body temperatures above 50°C, both accumulate high levels of two inducible HSP70 (i.e. express constitutively) isoforms regardless of whether they are experiencing temperatures outside of their optimal range or not. Similar patterns were found in nine Saharan lizard species (Ulmasov et al. 1992). Another more recent study was conducted on four Stratiomyid larvae (Soldier fly) occurring in the vicinity of hot volcanic springs (Garbuz et al. 2008). Once again, each of the four larval species showed high constitutive expression of a number HSP70 isoforms including inducible forms. This pattern of chronic HSP70 expression is certainly not the norm (continuous or super-expression of HSP70 has been suggested to be harmful (Krebs and Feder 1997)), however

as the above examples reveal, chronic HSP70 expression may be adaptive in thermally "extreme" environments such as the Sahara or Antarctica. Certainly in the above examples, the benefits of increased thermo-tolerance acquired from high constitutive HSP70 levels must outweigh the deleterious effects of high cellular HSP70 levels (Garbuz et al. 2008). In contrast to high constitutive HSP70 expression in "extreme" but stable environments, species occupying highly variable environments have also been shown to constitutively express high levels of HSP70. Dong et al. (2008) showed that limpets occupying the more variable high-intertidal zones had higher constitutive expression of a reported HSP70 than limpets occupying mid- or low-intertidal zone. One of the high intertidal species (*Lottia scabra*) also showed no induction of this particular HSP70 in response to elevated temperatures (Dong et al. 2008). It was suggested that this may be an adaptive "preparative defence" strategy to cope with highly variable and unpredictable environments such as the high intertidal zone. The examples from stable "extreme" and highly variable/unpredictable environments given above have important implications for the use of HSP70 as a stress biomarker. For example, contrary to conventional interpretations, high levels of HSP70 may have no relation to any recent stress exposure or the stress status of the organism and just simply reflect an adaptive strategy to the environment under study. Clearly the structure and function of individual genes within the stress-70 family varies inter-specifically and between habitats, and at present, to extrapolate characteristics of one species' stress-70 family to others may lead to misinterpretations of stress levels. Also the differing strategies employed by species from different environments may lead to incorrect assumptions of the condition of the organism. These matters undoubtedly complicate the interpretation of stress-70 family proteins as a stress biomarker. Strategies such as "preparative defence" and others mentioned in this article may be ecologically important in allowing species to occupy certain habitats. However, some inferred strategies seem convoluted (see Clark and Peck 2009) and may have been developed by authors in order to explain stress-70 expression in the absence of appropriate knowledge of the stress-70 members being studied. With better understanding of the stress-70 family, and their roles, more simplistic and intuitive biological theories may be established.



Legend overleaf

Figure 1.2 An illustration showing the variety of factors that can induce or alter the expression of members of the stress-70 family, as well as experimental and methodological factors that can affect the way HSP70 up-regulation is observed during analysis. **a.** Highlights some examples of the vast array of factors that can induce HSP70 expression. HSP70 expression can be induced by: **i.** environmental factors; **ii.** external non-environmental factors; and **iii.** internal non-environmental factors. **b.** Gives examples of methodological/experimental approaches that may alter the observed HSP70 expression during subsequent analysis. **c.** Highlights how the current state of the test organism and its life history may alter the way it expresses HSP70. The diagram draws attention to the interconnected nature of all the above factors, which belies the simple interpretation of HSP70 expression profiles regularly found in HSP70 stress biomarker research. HSP70 up-regulation must be analysed and interpreted with thorough consideration of this complexity

1.3.7 Summary of HSP70 as a biomarker of stress

At its most fundamental level, the up-regulation of HSP70 signifies the presence of proteins whose native, functional conformations have been altered by stress. When using HSP70 as a stress biomarker we make assumptions that this is the result of stress and that the level of HSP70 expression at any time-point is related to the level of protein non-conformity, and thus the level of stress. The vast array of factors that affect the level and timescale at which HSP70 is up-regulated in response to diverse stressors, and the way the experimental design and analysis can alter the way stress is perceived. Many of these factors break our simplistic assumptions. Without any prior understanding of how each of these factors alters the way HSP70 is expressed individually, and when combined, environmental stress levels may be misinterpreted. In many cases, uncertainty remains over the differential role of members of the stress-70 family. What is clear is the function and structure of specific members of the stress-70 family varies between species and regions. Without using a number of members of the stress-70 family, both inducible and constitutive; and profiling their expression levels over an appropriate timescale, stress signals can be easily missed or misinterpreted. Stress-70 family expression can be validated by quantification of other stress response mechanisms. Future research may benefit from eliciting stress-specific responses as well than generalised ones. Stress- or taxa- specific biomarkers are unlikely to be subject to such in-depth validation as is required for HSP70. A stress-specific approach to the search for stress biomarkers may yield more insightful progress in our understanding of stress physiology.

1.4 More than a single stress response system

Two systems for responding to stress were proposed at the cellular level by Dietmar Kültz (2003, 2005) (Figure 1.3). The first, the CSR, has received considerable attention, and the term has proliferated throughout stress research (as highlighted previously). The second, the cellular homeostatic response (CHR), has remained esoteric. The CHR is not transient in nature like the CSR, and thus remains permanently switched on until either acclimatisation has occurred or environmental conditions have reverted back to their pre-stress state (Kültz 2005) (Figure 1.3; blue boxes). In this way, the CHR, unlike the CSR, is stressor-specific. Because of its stressor-specificity and potentially non-ubiquitous nature, components of the CHR are likely to be more demanding research targets. However, the results of targeting this aspect of the stress response may yield more insightful progress in our understanding of stress physiology than CSR-derived markers alone. Stressor-specific markers have the potential to identify limitations in an organism's ability to acclimatise into, and adapt to, potentially stressful environments. Using deep-sea migration as an example; CSR biomarkers such as HSP70s might be able to provide information on whether a shallow-water organism is experiencing higher levels of macromolecular damage (stress) upon exposure to increasing hydrostatic pressure (HP) and/or lowering temperature, but will not be able to provide information on what, if any, changes in cellular homeostasis must occur in order for the organism to prosper under once-stressful conditions. Potential CHR biomarkers may provide an insight into the acclimatory processes required for organisms to prosper under those once-stressful conditions.

More recently, the overall metabolic response has been shown to provide valuable insights into the effects of stress on organismal physiology (Sokolova 2013), and although the metabolic response is inexorably linked to both the CSR and CHR, it can also be seen as a response in its own right. The metabolic response can also be quantified at a whole organism level by respiratory or cardiac analysis, where changes in these systems are likely to be directly linked to changes at a cellular and transcript level.

Further, stress can also be assessed at a behavioural level (Oliphant et al. 2011; New et al. 2014). Behavioural observations have the advantage that they can be made directly and instantaneously, and are also likely to be linked to changes at a cellular and transcript level.

This project aims to focus not only on the CSR, but also the CHR and metabolic responses to stressor perturbations at the transcript level, and integrate higher order behavioural and physiological measurements also. Understanding stress, or stressor perturbations, is important in determining significance of stressor or multi-stressor scenarios in terms of physiological studies; particularly those centred on contemporary climate change (Frederich and Pörtner 2000; Pörtner and Farrell 2008; Pörtner 2010; Sokolova 2013).

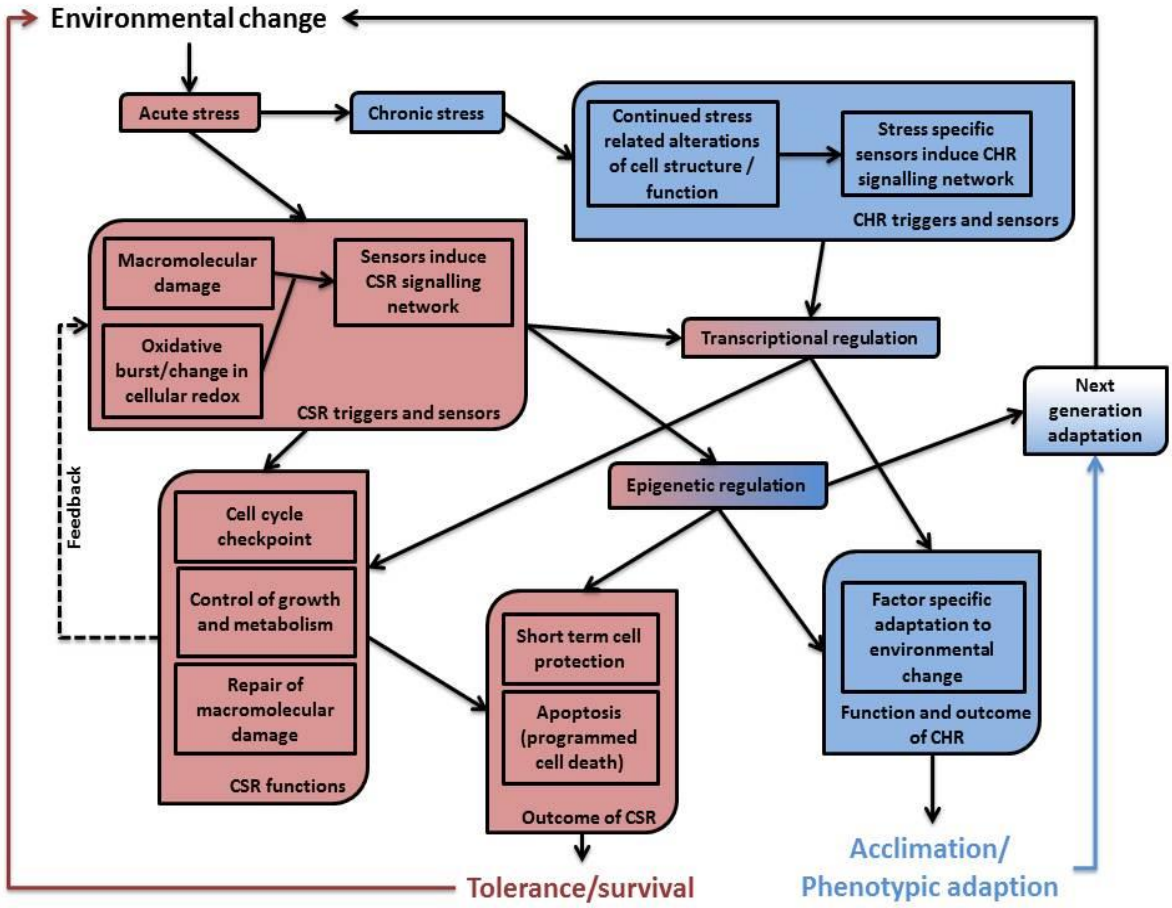


Figure 1.3 Conceptual representation of the two response pathways to stress proposed by Kültz (2005). In red, the cellular stress response (CSR) reacts over short timescales to acute stress to provide either cellular protection, or organismal protection via cellular apoptosis. The CSR confers increased tolerance to stress. In blue, the cellular homeostatic response (CHR) reacts to sustained stress by initiating stress-specific responses that may counteract the effects of the specific stress and acclimation/phenotypic adaptation and the alleviation of the specific stress. Adapted from Kültz (2005)

1.5 Contemporary climate change

The intergovernmental panel on climate change (IPCC) released its fifth assessment in 2013-2014 (IPCC 2013; IPCC 2014) the results of which highlight that the global climate is directionally changing, and that this change is occurring at an unprecedented rate in comparison to previous climate oscillations (IPCC 2014). The effects of these changes are already being observed from a biological perspective (Parmesan and Yohe 2003; Edwards and Richardson 2004; Chen et al. 2011; Somero 2012; Beaugrand et al. 2014) and this project will, in part, address the potential for climate-driven bathymetric range shifts in light of contemporary ocean warming. For this reason, the following section will briefly highlight the most relevant climate trends and predictions from the recent IPCC reports.

The IPCC report highlights that the globally averaged combined land and ocean surface temperature has warmed by 0.85°C between 1880 and 2012 (Figure 1.4), and that, over the longest periods that regional calculations can be made, almost the entire globe has experienced surface warming.

Data shows that the oceans are taking up and storing the majority of the incoming heat energy to the Earth (IPCC 2013). Because of this, significant ocean warming has been observed. Across the globe, ocean warming is greatest in the upper 75 m of the water column, warming by 0.11°C per decade from 1971 to 2010 (Rhein et al. 2013). Where datasets allow a comparison, the global ocean has warmed between 700 m and 2000 m from 1957 to 2009, and also from 3000 m to the sea floor between 1992-2005 (Rhein et al. 2013). The various IPCC model projection scenarios suggest that global average surface temperatures are set to increase up to at least 2100 (IPCC 2013) (Figure 1.5). The global ocean is predicted to continue warming throughout the 21st century, with the strongest warming projected to be in surface tropical and northern hemisphere subtropical regions. Globally averaged warming is projected to be in the region of 0.3°C to 0.6°C at a depth of 1000 m by 2100, with warming most pronounced in the deep Southern Ocean. Warming is also expected at depths below 1000 m over the next century, although the extent of such warming is unclear.

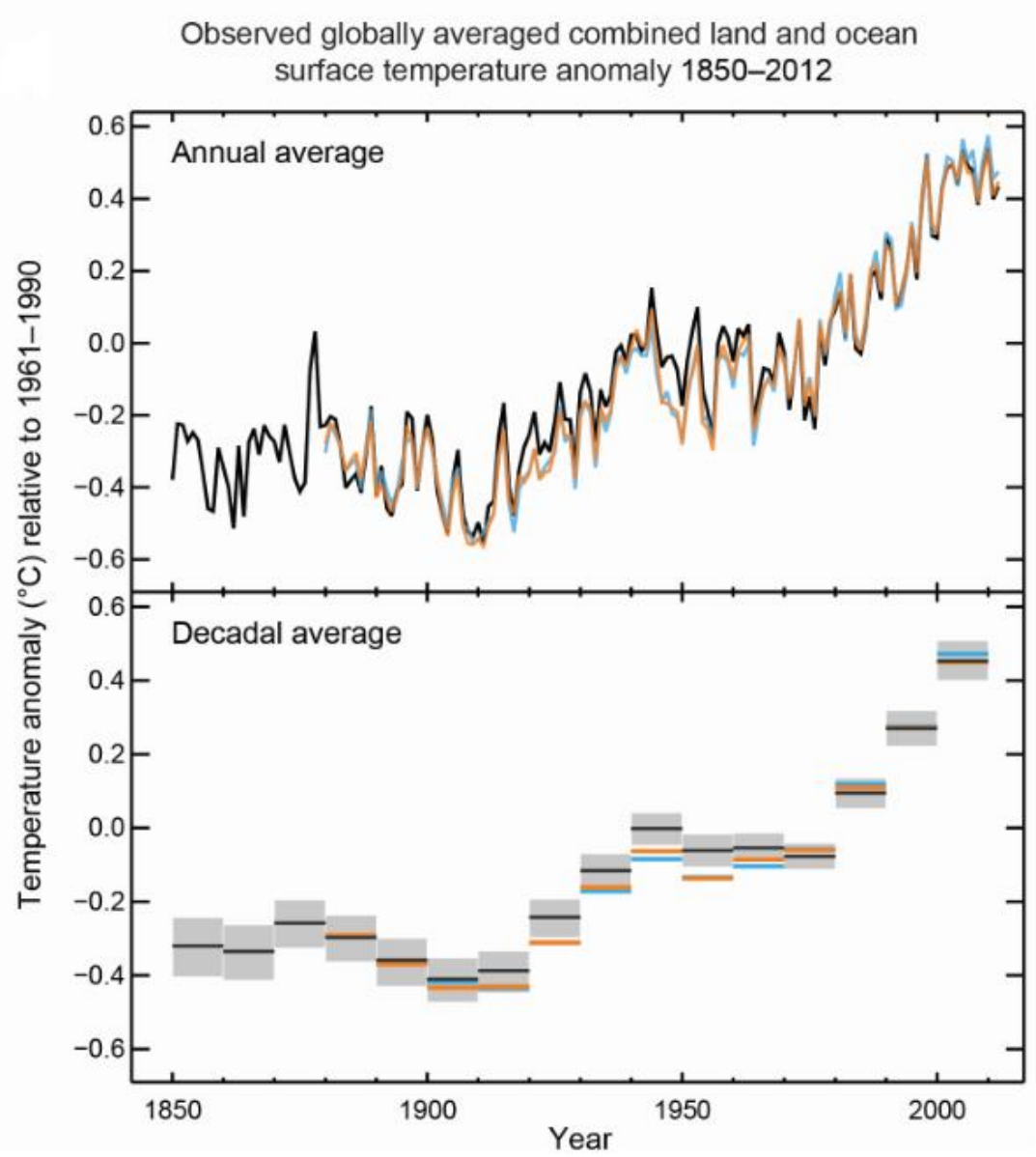


Figure 1.4 The observed global mean combined land and ocean surface temperature anomalies from 1850 to 2012, showing an annual mean in the top panel, and a decadal mean in the bottom panel (uncertainty is shown as the grey area). The coloured lines represent different datasets. Adapted from the 2013 IPCC report from working group I (IPCC 2013)

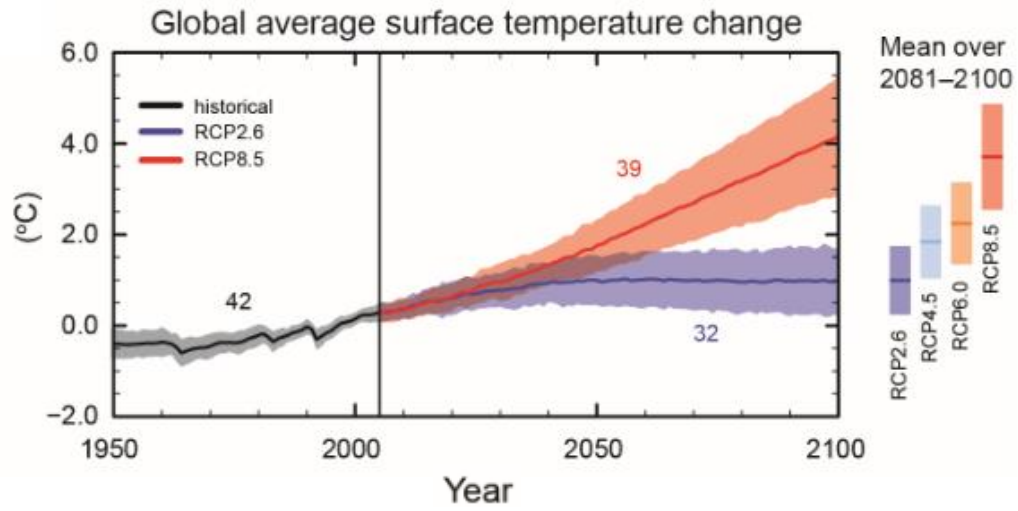


Figure 1.5 The IPCC CMIP5 multi-model simulated time series from 1950 to 2100 showing the projected change in global annual mean surface temperature relative to 1986-2005 from four projected scenarios: RCP2.6, 4.5, 6.0 and 8.5. The coloured numbers represent the number of models used to derive the multi-model mean (IPCC 2013).

1.6 Effects of ocean warming on marine organisms

The climate change trend highlighted in Section 1.5 has led to significant changes in the biology of our oceans (Parmesan and Yohe 2003; Chen et al. 2011; Somero 2012; Beaugrand et al. 2014), and marine species are undergoing migrations, acclimatisation, or adaptation in order to avoid death or extinction (Somero 2012; Kelly and Hoffmann 2013). Although many environmental factors are contributing to these observed changes, temperature is consistently reported as the major driving force (Parmesan and Yohe 2003; Chen et al. 2011; Poloczanska et al. 2013). The warming trend in the surface oceans is generally observed in marine ectotherm distribution patterns as a poleward shift in order to stay within a certain thermal range, and has been well documented over the last half century (Beaugrand et al. 2002; Edwards and Richardson 2004; Perry et al. 2005). More recently, several studies have suggested that some marine ectotherms are undergoing bathymetric range shifts (migration to deeper waters) as an alternative to latitudinal range shifts (Weinberg 2005; Dulvy et al. 2008; Pinksy et al. 2013). Although the deep sea is warming also, it is doing so at a slower rate, and is generally cooler than overlying surface waters (Section 1.5) providing ectotherms with an alternative to poleward range shifts.

Bathymetric range shifts are a little studied, but potentially important response to contemporary climate change. This project aims to explore the capacity of shallow-water marine ectotherms to tolerate environmental factors characteristic of deeper waters in order to better understand the potential for climate-driven bathymetric range shifts. One key environmental factor that changes ubiquitously with depth is hydrostatic pressure (HP), and this project aims, specifically, to explore the effects of increasing HP on shallow-water adapted ectotherms. Beyond contemporary climate-driven bathymetric range shifts, the results of this project may also have implications on our currently limited understanding of the evolution of deep-sea fauna, past and present, as described below (Section 1.7).

1.7 Evolution of life in the deep sea

It is generally accepted that deep-sea fauna originated from shallow-water origins (Crimes 1974), although some theories suggest that life may have originated in deep hydrothermal vent systems (Miller and Bada 1988). One prevalent explanation of shallow-water origination implicates near shore species' innovation followed by migration offshore into unoccupied niches, or at the expense of older faunal communities, as a potential mechanism (Jablonski et al. 1983; Lindner et al. 2008). Climate-driven extinction events (past global warming) and subsequent re-colonisations over many geological eras are thought to have shaped both past and present deep-sea species assemblages (Jacobs and Lindberg 1998; Rogers 2000; Aquino-Souza et al. 2008). The current deep-sea faunal assemblage comprises both young and ancient lineages (Wilson 1999; Raupach et al. 2009; McClain and Hardy 2010). Several studies have elucidated close taxonomic relationships between shallow-water and deep-sea species (Distel et al. 2000; Raupach et al. 2009). One possible route of past and present deep-sea migration is via an isothermal water column which would negate the need for temperature adaptation: a potential barrier to deep-sea migration (Young et al. 1997). Currently, isothermal water columns exist at high latitudes (Wilson and Hessler 1987; Thatje et al. 2005), however, warmer deep-sea conditions during the Mesozoic and early Cenozoic eras may have resulted in isothermal water columns at lower latitudes, presenting a potential deep-sea migratory route for non-stenothermal species, which may have subsequently thermally adapted gradually as temperatures dropped to their present cold state (Tyler et al. 2000; Oliphant et al. 2011).

In further support of the idea that deep-sea migration may occur via an isothermal water column are a number of laboratory-based studies that have shown that many shallow-water

adapted organisms can tolerate HPs outside of their natural distribution (Benitez-Villalobos et al. 2006; Oliphant et al. 2011). Studies have also shown that larval stages are particularly tolerant of HP increases, exceeding that of their related adult stages (Mestre et al. 2009). Such laboratory studies, however, do not explain the absence of adult specimens of these test species, or close relatives, in the present deep sea suggesting that physiological limitations may be acting to restrict the deep sea migration of current shallow-water invertebrates. Our limited knowledge of the physiological processes that must have occurred at the organismal and molecular level to allow adaptation to the high HP, low temperature environment of the current deep sea has hindered our understanding of the origin of life in the deep sea. It is, however, current and future global change events that have given the area new impetus (Section 1.6).

Despite evidence of high HP tolerance in a number of marine invertebrate larval stages, survival of reproductively capable adults at depth is essential for deep-sea colonisation/re-colonisation, or climate-driven bathymetric range shifts. The potential for these may be restricted by physiological limitations in the capacity for HP and temperature tolerance in adult stages of shallow-water ectotherms. HP has been shown to have numerous important effects on organismal physiology, particularly in combination with temperature, which may control the physiological capacity for marine species to shift their depth distributions, these effects are discussed in the section below (Section 1.8).

1.8 Hydrostatic pressure (HP)

HP was first demonstrated as a thermodynamic parameter in 1648 by Blaise Pascal (Demazeau and Rivalain 2011). Since those first observations, high HP has been the subject of growing interest in many fields ranging from physics to geochemistry, and engineering to biology.

Pressure constitutes the single largest continuous gradient on the planet from the outer reaches of the Earth's atmosphere to the centre of the globe. In terms of the global biosphere, HP is of particular relevance: approximately 79% of the volume of the marine biosphere lies below 1000 m and thus experiences HPs of 100 atm (1 atm = 0.101 MPa = 1.01 bar) and above (Somero 1992), and of the total global biosphere, terrestrial and marine, 62% is characterised by HPs above 10 MPa (Boonyaratanakornkit et al. 2002). In the marine environment, HP on the surface water equates to 0.1 MPa and increases linearly

to the deepest oceanic trough, the Marianas Trench, at a depth of 11,000 m and 110 MPa (Pradillon and Gaill 2006). The oceans, which account for around 71% of the Earth's surface, have an average depth of 3800 m. As such, all marine organisms experience the effects of HP, whether it be in the first few centimetres of the water column or vast HPs equivalent to over a thousand atmospheres in the deepest oceanic troughs (MacDonald 1997). Organisms have been found at all depth ranges in the ocean: species that can tolerate both extremely high (Jamieson et al. 2010) and relatively low HPs; such a wide spectrum in HP tolerance is unique to the marine environment. It is thought that all marine organisms are distributed between a high and low depth limit (Tyler and Young 1998; Brown and Thatje 2014). The size of this range is dependent on many variables, and ranges from a few metres to over a kilometre in some cases (Tyler and Young 1998). The marine biosphere therefore provides an ideal setting for studies looking into the effects of HP, particularly those effects that limit shallow water organisms from moving into deeper waters (Brown and Thatje 2015).

A large portion of research conducted on the effects of elevated HP on biology has focussed on prokaryotes, specifically the use of high HP exposures to kill prokaryotes and sterilise food for human consumption (Simonato et al. 2006). Although such research is not directly relevant to the potential for bathymetric range shifts in marine eukaryotic ectotherms, the effects of elevated HP on prokaryotes have been shown to be so fundamental that they are comparable to some of the effects observed in eukaryotes (MacDonald 1984; Somero 1992). The effects of high HP can be seen throughout the prokaryotic genome, affecting all processes involved in volume changes (Vezzi et al. 2005). Piezophiles (high HP-adapted prokaryotes found in the deep sea (Yayanos 1995)), that are able to proliferate in high HP environments are not able to grow under atmospheric pressures, suggesting that they have adapted specifically to high HPs in the course of their evolution (Simonato et al. 2006). Three key mechanisms have been suggested to explain the ability of piezophiles to proliferate under high HPs: firstly, fine tuning of gene expression to compensate for any loss of biological activity associated with elevated HPs (Campanaro et al. 2005); secondly, the expression of genes directly related to elevated HPs (Kato and Qureshi 1999); and finally the adaptation of cellular structures and macromolecules to maintain functionality under elevated HPs (Allen et al. 1999; Chilukuri et al. 2002; Alpas et al. 2003; Pavlovic et al. 2005).

One particularly important discovery in high-HP prokaryotic studies has been observation of synergistic effects of low temperature and high HP on biological processes. At a

temperature of 2°C and a HP of 100 MPa, the effects on prokaryotic membranes are directly comparable to -18°C at 0.1 MPa (Simonato et al. 2006). As temperature and HP changes are two factors that change with depth, the combination of the two may be important in understanding the potential for bathymetric range shifts in marine eukaryotic ectotherms. Such synergistic effects have not been studied in eukaryotes at a cellular or molecular level, so one of the aims of this project is to explore whether the effects seen in prokaryotes are analogous in eukaryotes, and whether they influence the potential for climate-driven bathymetric range shifts.

It is believed that every marine organism has a vertical distribution window outside of which they will experience the detrimental effects of stress (Tyler and Young 1998; Pradillon and Gaill 2006; Brown and Thatje 2014). Vertical distribution profiles have been demonstrated in a number of shallow-water and deep-sea species (Brown and Thatje 2014), however HP is commonly under-represented as a potential factor in the shaping of such distribution profiles (other factors include biological interactions, food supply, temperature, current regimes, sediment characteristics etc. (Levin et al. 2001)).

Relatively few studies have looked at the effects of HP on marine ectotherms. Shallow-water fishes have shown marked decreases in action potential of the vagus nerve in response to HPs (Somero 1992). Studies on decapod crabs found that in the shallow water species *Maja brachydactyla*, resting heart rate showed a marked increase at 150 atm when compared to 1 atm and feeding was never observed at this elevated HP (Thatje and Robinson 2011). It was suggested that survival for any prolonged period at HPs of 150 atm or above was not feasible in this species (Thatje and Robinson 2011). Likewise upon decompression of the hydrothermal vent crab *Bythograea thermydron* disturbances in electrocardiogram readings were seen alongside observations of loss of muscle function and coordination (Mickel and Childress 1982). These examples suggest that HP plays an important role in limiting species' vertical distribution ranges in shallow-water metazoans, and also deep-water metazoans. Just as shallow-water organisms require low ambient HPs to survive it is now clearly understood that are large proportion of deep-sea organisms are obligate barophiles: requiring high HP to survive (Somero 1992). It would appear that the adaptations required for life at different HPs are specialised enough that once adapted to a certain HP, range all HPs outside of that range become inaccessible in terms of physiology (Mickel and Childress 1982).

Research has shown that shallow-water adapted fauna suffer high HP sensitivity in processes that involve membranes. In metazoans, this is commonly expressed through perturbations of neural and muscular functioning. The symptoms that accompany elevated HP episodes are collectively termed high pressure neurological syndrome (HPNS) and are observed as tremors, spasms, and neuropsychiatric disturbances in humans (Bowser-Riley et al. 1984), and behavioural pathologies in experimental animals (Oliphant et al. 2011; Cottin et al. 2012). The symptoms that characterise HPNS have been noted in terrestrial and shallow-water organisms at HPs equivalent of as little as 150 m water depth, to well over 1000m (Brauer and Torok 1984) (Somero 1992). The onset of HPNS-like symptoms has also been shown in deep-sea organisms when exposed to HPs greater than their natural distribution (Brauer et al. 1980). Interestingly, deep-sea adapted organisms have shown HPNS-like symptoms upon exposure to ambient atmospheric pressures (Treude et al. 2002).

The above examples present potential physiological limitations to bathymetric range shifts in shallow-water adapted organisms, and highlight the potential importance of HP in setting depth distribution limits of marine species; both shallow and deep. This project aims to draw upon research conducted on both prokaryotes and eukaryotes involving HP and temperature to explore the effects of changes in both, in isolation and in combination. Using the stress responses discussed in Sections 1.1 to 1.4 as markers of physiological tolerance limits under a variety of HP and temperature exposures, this project aims to develop a shallow-water eukaryotic marine ectotherm into an experimental model for HP and temperature physiology (Section 1.9). Extending our knowledge of the effects of elevated HP and temperature changes at a molecular level will help to determine whether marine ectotherms can migrate bathymetrically in light of ocean surface warming as a viable alternative to latitudinal range shifts.

1.9 Study organisms

Relatively few marine eukaryotes have been used as test species to determine the effects of elevated HP on metazoan biology, as described in Section 1.8. Of these, *Palaemonetes varians* represents the best studied and most accessible study organism, and was thus chosen as the best candidate to conduct further HP and temperature research on. In order to test whether the effects of elevated HP and temperature changes that may be observed in *P. varians* are comparable to other marine ectotherms, and thus allow *P. varians* to be

advocated as an experimental model organism in HP and thermal physiology, similar effects should be shown in other organisms. For this reason, a more eury-baric marine ectotherm, *Lithodes maja*, was chosen as a comparison species as this species is known to have shifted its depth distributions as part of its evolution.

1.9.1 *Palaemonetes varians* (Leach 1814)

1.9.1.1 Pre-text

There are many enduring systematic controversies within the Order Palaemonidae (De Grave and Ashelby 2013). For instance, the first cladistic morphological analysis performed on the sub-family (Palaemoninae) suggested paraphyly at multiple taxonomic levels, including paraphyly within the major genera: *Palaemon, Palaemonetes,* and *Macrobrachium* (Pereira 1997). Further, a phylogenetic study in 2003 suggested that *Palaemon serenus* and *Palaemonetes australis* may be congeneric (Murphy and Austin 2003). In recent years further research has been focussed on caridean shrimp (Tokuda et al. 2006; Bracken et al. 2009; Li et al. 2011), yet no consensus from the level of Order downwards has been reached. In 2013 De Grave and Ashelby published a re-appraisal of the status of selected genera within the subfamily Palaemoninae (De Grave and Ashelby 2013). This re-appraisal included the suggestion that *Palaemonetes* is a "junior synonym" of *Palaemon*, and consequently that *Palaemonetes varians* should be re-classified as *Palaemon varians* (De Grave and Ashelby 2013).

A portion of this thesis was *in press* for publication at the time of this reclassification, and it, and related previous work, is published using the name *Palaemonetes varians*. The reclassification of *Palaemonetes varians* to *Palaemon varians* has little bearing on the scientific content of this thesis and it was thus judged that referring to the species as *Palaemonetes varians* in this thesis has allowed for the maintenance of a simple link to previous important research on the species, and that this was of greater importance to the thesis as a whole. It is, however, recognised that *Palaemon* and *Palaemonetes* are likely to be congeneric. No inferences that rely on previous classifications, or contradict this new classification are made at any point in this thesis.

1.9.1.2 Evolution, physiology, & ecology

Palaemonetes varians (Leach, 1814), or the Atlantic ditch shrimp, is a brackish-water species in the sub-family Palaemoninae, within the order Palaemonidae (De Grave 2009). Palaemonid shrimp have a worldwide distribution in neritic waters within tropical and temperate zones. Palaemoninae species show general adaptations to low salinity environments, have putatively shown freshwater colonisations via brackish waters (Ashelby et al. 2012), and have thus been exploited in research studying the evolutionary processes involved in colonisation of freshwater habitats (Anger 2013; Vogt 2013). Reviewed by Anger (2013), Palaemonidae may have diverged originally from tropical marine habitats, and genera within the clade now exist in tropical, sub-tropical, and temperate zones (Figure 1.6).

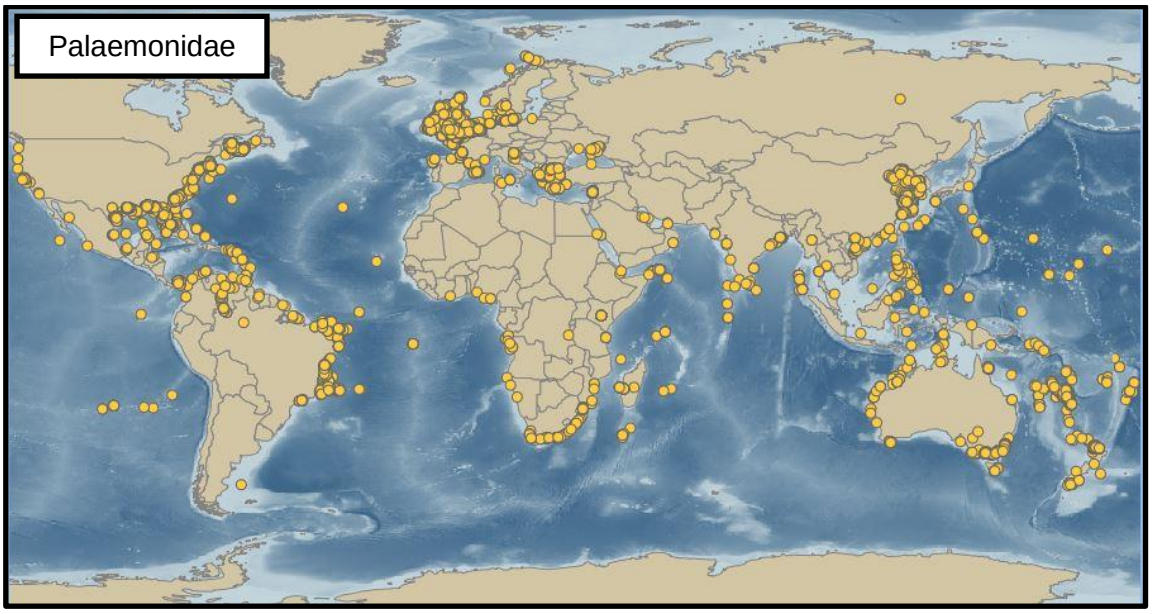


Figure 1.6 The global distribution of the order Palaemonidae, each dot represents a population. No indication of abundance at each sampled point is given, but is available from the freely-available OBIS database: www.iobis.org (OBIS 2015, accessed on 18th February 2015)

P. varians, specifically, is found in variable salinity environments from salinities of 2 up to 45. It is found across the coastlines of Western Europe and down the North East Atlantic coast as far Morocco, also being found in areas of the Mediterranean Sea (Hindley 2001; Dolmen et al. 2004; González-Ortegón and Cuesta 2006) (Figure 1.7). In the UK, *P. varians* is found in salt marsh habitats commonly associated with estuaries, which are abundant on the UK coastline. These habitats are characterised by large fluctuations in temperature, salinity, oxygen concentrations, amongst other factors. Such fluctuations occur on all

temporal scales. Within these habitats, *P. varians* is benthic, and generally associated with plant and algal life (*personal observations*). Although their natural diet has not been well characterised, it is thought that they are opportunistic feeders with a varied diet (Oliphant 2014). They are known to be predated on by a variety of fishes and birds (Oliphant 2014). Breeding is induced by temperature and photoperiod (*personal observations*) and generally occurs from late-spring to mid-summer in salt marsh habitats. Oliphant (2014) reported annual fluctuations in temperature of ~0°C - ~25°C in a ditch at Lymington salt marshes, UK, where *P. varians* is regularly collected (see Section 2.1.1). Lofts (1956) reported fluctuations in temperature of 0°C - 33°C in a salt marsh in Cardiff, Wales UK, where *P. varians* is found. The minimum temperatures recorded in habitats that *P. varians* is reported to exist is close to the experimentally determined lower thermal limit of the species, of ~0°C (Oliphant et al. 2011). The experimentally determined upper thermal limit of *P. varians* also closely matches that of the maximum reported habitat temperature: the established upper thermal limit is ~33°C (Oliphant et al. 2011; Ravaux et al. 2012), although this temperature limit has been shown to vary according to temperature ramping time and acclimation temperature (Ravaux et al. 2012). The first reliable description of the larval development of *P. varians* was published by Gurney (1924), who identified 5 larval stages. In 1979, Fincham validated the development through 5 larval stages and further described all 5 stages as zoeal (Fincham 1979). Fincham also noted large intra-stage morphological variation. Most recently, Oliphant reclassified the five larval stages of *P. varians* into 2 zoeal stages and 3 decapodid stages (For a contemporary description of larval stages see: Oliphant 2014).

Observations that *P. varians* is able to tolerate large variations in environmental conditions has led to it being used in numerous studies focussing on its physiological responses to temperature (Cottin et al. 2010; Oliphant et al. 2011; Cottin et al. 2012; Ravaux et al. 2012; Smith et al. 2013; New et al. 2014; Oliphant et al. 2014), salinity (Lofts 1959; Nugegoda and Rainbow 1989), oxygen concentration (Neilsen and Hagerman 1998), metal toxicity (Rainbow et al. 2006; Gonzalez-Rey et al. 2007; Gonzalez-Rey et al. 2008, Rainbow and Smith 2013), and hydrostatic pressure (HP) (Oliphant et al. 2011; Cottin et al. 2012; Smith et al. 2013; New et al. 2014). *P. varians* has also been compared, in terms of physiology, to deep-sea shrimp (Gonzalez-Rey et al. 2008; Cottin et al. 2010; Oliphant et al. 2011; Cottin et al. 2012; Smith et al. 2013; New et al. 2014). Although not clearly resolved, *P. varians* has been suggested to show phylogenetic relations with deep-sea hydrothermal vent shrimp of

the family Bresiliidae (Tokuda et al. 2006) and more recently, shrimp in the genus *Periclimenes* (Li et al. 2011) which have deep-sea representatives.

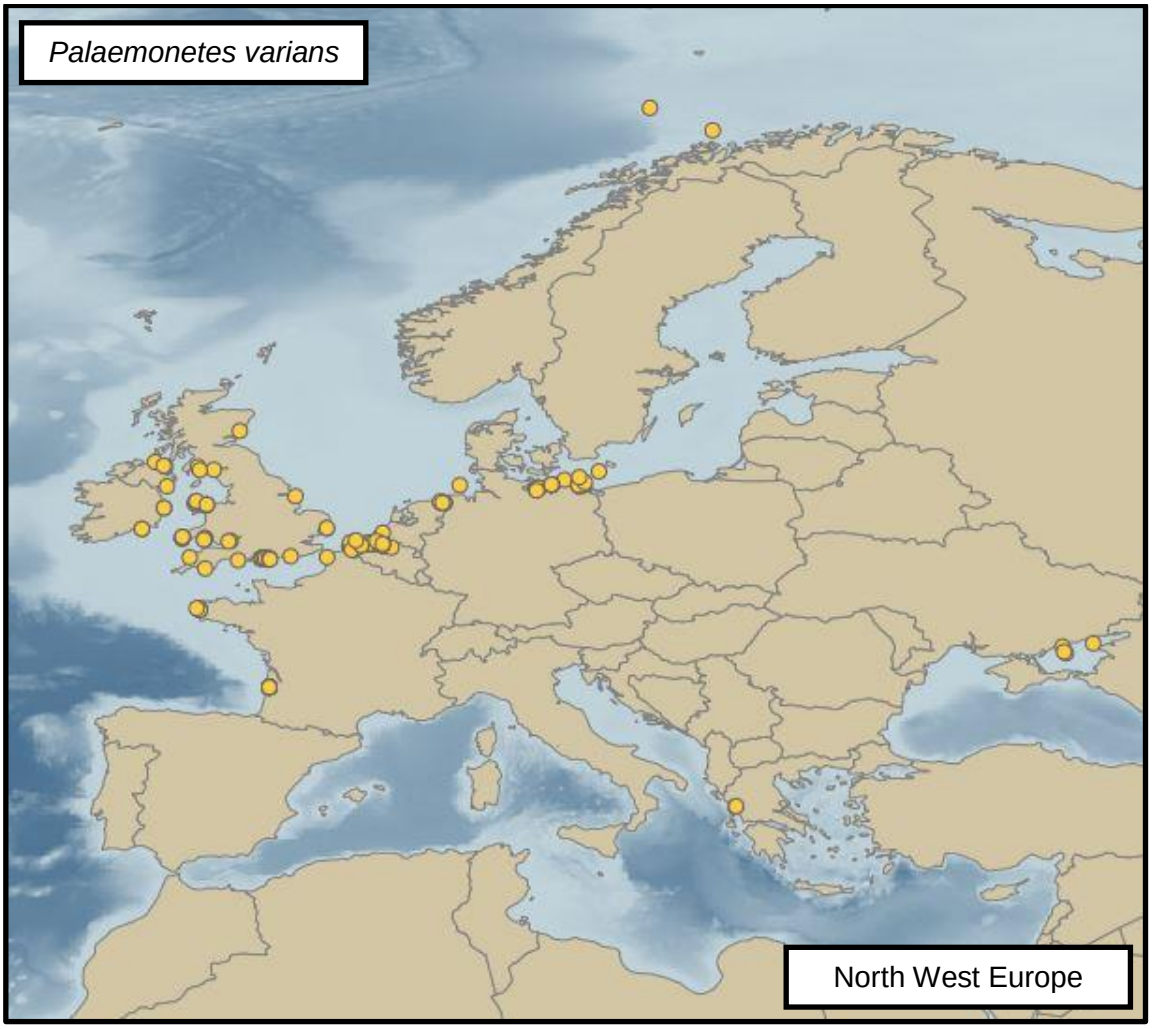


Figure 1.7 The global distribution of the species *Palaemonetes varians*, each dot represents a population. No indication of abundance at each sampled point is given, but is available from the freely-available OBIS database: www.iobis.org (OBIS 2015, accessed on 18th February 2015)

1.9.2 *Lithodes maja* (Linnaeus 1758)

Lithodes maja (Linnaeus 1758), known also as the northern stone crab (White 1857), is an anomuran crab of the family Lithodidae, which has a pan-Pacific and pan-Atlantic distribution (Figure 1.8). The family includes some of the largest predatory crustaceans in the ocean (Lovrich and Vinuesa 1999), and contains species that are the target of large scale fisheries across high-latitude regions (Armistead et al. 2001). The Lithodidae family consists of two sub-families; Haplogastrinae and Lithodinae (Cunningham et al. 1992; Hall and Thatje 2009). Three Lithodine genera: *Paralomis, Lithodes,* and *Neolithodes* are thought to

have colonised the deep sea in the North Pacific and then radiated into other ocean basins. This colonisation may have led to cold-water adaptations which are now characteristic of these three Genera including; facultative or fully lecithotrophic larval development and fewer larval moults (Crain and Mclaughlin 2000; Thatje 2004; Thatje et al. 2005; Morley et al. 2006; Hall and Thatje 2009). *Lithodes maja* is found in the North Atlantic at depths of between 4 and 790 m, and is thought to have arrived in the North Atlantic after dispersing from the North Pacific via the Arctic (Figure 1.9). It occupies both soft and hard substrata. *Lithodid* crab larval development consists of three free-swimming zoeal stages that are planktonic, and a megalopa stage which is also free-swimming. Crab I is the first juvenile stage, and the point at which benthic settlement occurs and feeding begins.

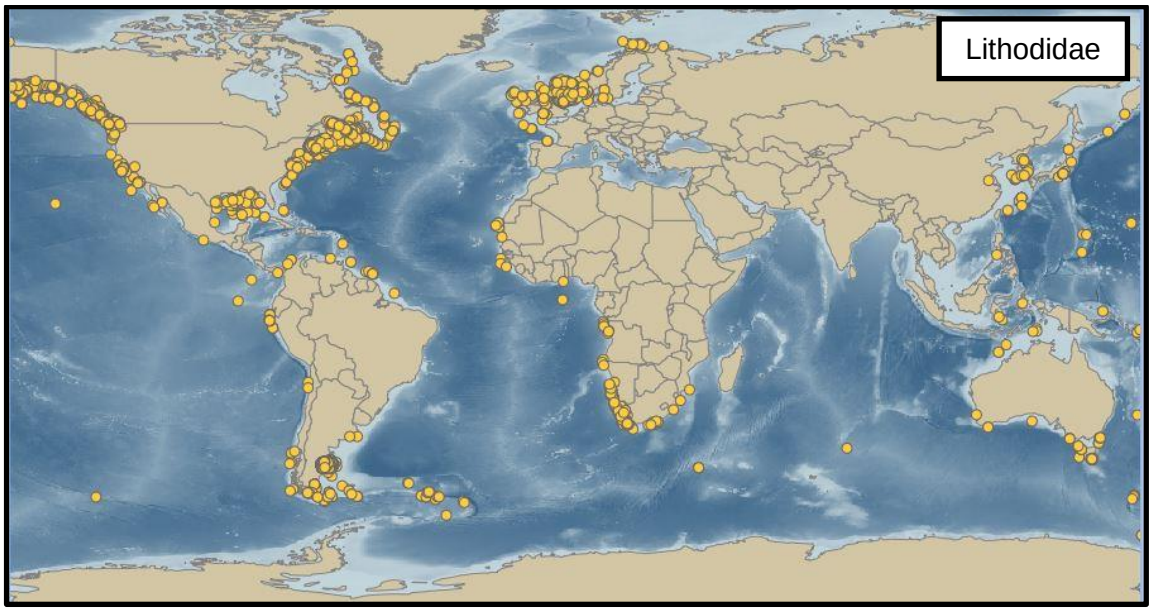


Figure 1.8 The global distribution of the family Lithodidae, each dot represents a population. No indication of abundance at each sampled point is given, but is available from the freely-available OBIS database: www.iobis.org (OBIS 2015, accessed on 18th February 2015)

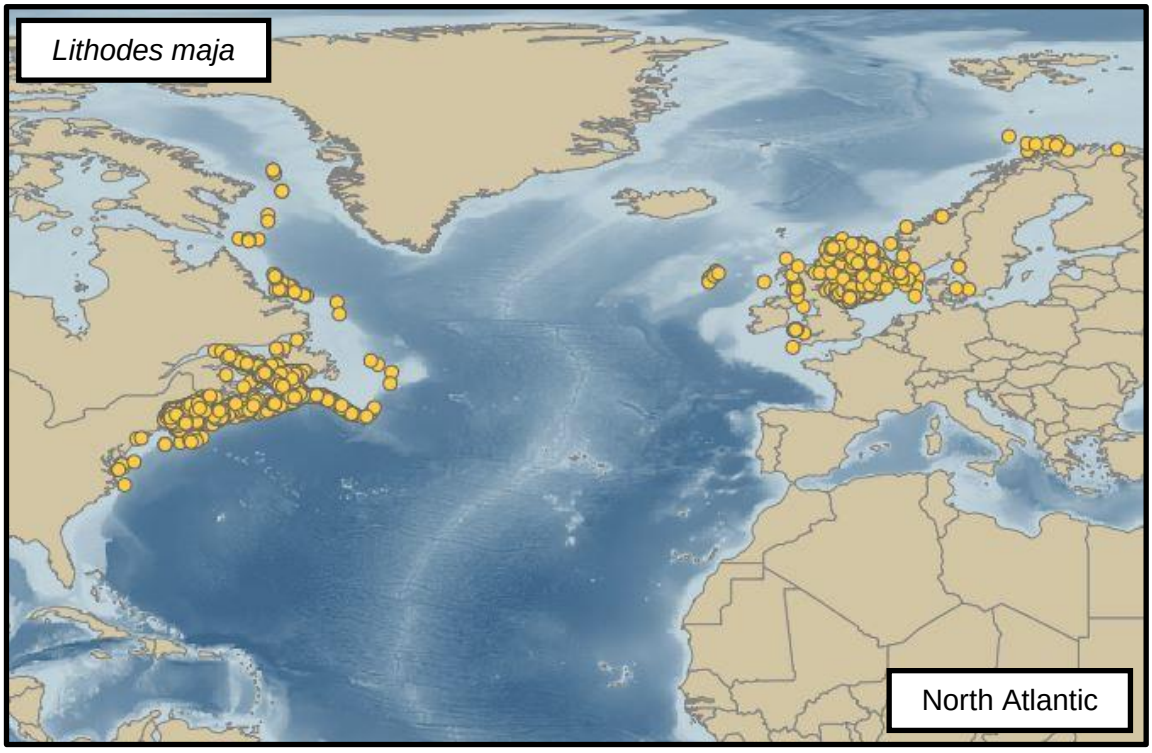


Figure 1.9 The global distribution of the species *Lithodes maja*, each dot represents a population. No indication of abundance at each sampled point is given, but is available from the freely-available OBIS database: www.iobis.org (OBIS 2015, accessed on 18th February 2015)

1.10 Thesis aims, objectives, hypotheses, & structure

1.10.1 Broad thesis aims

This thesis aims to use differential gene expression profiling, alongside other, higher order, measures of physiology, to determine whether the study species, and thus shallow-water ectotherms by proxy, are limited, in terms of physiology, in their ability to tolerate the effects of changes in HP, and also the combined effects of HP and temperature. By determining physiological limitations, inferences can be made as to the acclimatory processes required to allow such species to shift their distribution ranges bathymetrically in light of contemporary, rapid, ocean surface warming, or, indeed, the environmental characteristics that allow for such shifts without any need for acclimatisation processes. Further, physiological limitations may provide a precursory understanding of the evolutionary adaptations that shallow-water taxa may have undergone during the numerous colonisations/re-colonisations that have taken place over the biological history of the deep

sea. Understanding the evolutionary processes involved in these deep-sea migration events is of particular relevance now as evidence is mounting that some marine species are already undergoing bathymetric range shifts in response to warming surface waters.

1.10.2 Thesis objectives

***O*$_1$** – Identify and characterise novel differentially-expressed genes under changing HP and temperature exposures in laboratory-acclimated *Palaemonetes varians* shrimp, and quantify changes in transcriptional regulation alongside well established gene biomarkers.

***O*$_2$** – Simultaneously measure distinct physiological responses (Behavioural responses, CSR, CHR, and metabolic responses) in *Palaemonetes varians* over an acute HP exposure, and also over the subsequent recovery period, to provide an idea of the hierarchy and kinetics of pressure-specific stress responses.

***O*$_3$** – Measure the same physiological markers identified in response to acute HP and temperature exposure over long-term sustained exposures to provide an indication of whether any acclimatory processes are occurring.

***O*$_4$** – Identify, characterise, and quantify the expression of the same gene markers recognised to be pressure-responsive in *Palaemonetes varians* in another marine ectotherm species *(Lithodes maja*) with a differing natural depth distribution to provide an indication of whether stress responses (CSR, CHR, and metabolic responses) can be used to infer more general patterns of physiological limitations in the marine environment.

1.10.3 Broad thesis hypothesis

For each scientific chapter (Chapters 3, 4, 5 and 6), a set of specific hypotheses are provided. However, the work of this project can be encompassed in a single overarching hypothesis:

***H*$_1$** – The effects of elevated HP affect transcriptional-level processes, and invoke stress responses at the cellular and whole organism level in shallow-water adapted marine ectotherms. These effects are exacerbated, or mitigated, by concurrent changes in temperature. As such, specific combinations of HP and temperature are important in delineating depth distribution patterns of marine ectotherms, and act as limiting factors on the potential for bathymetric range shifts, as well as playing an important role in the

acclimatisation/adaptation processes of the current deep-sea faunal assemblage to deep-sea conditions.

1.10.4 Thesis structure

A general description of the materials and methods used throughout this thesis is provided in Chapter 2. Where general methods are used in the scientific chapters (Chapters 3, 4, 5 and 6) reference has been made back to the relevant section in Chapter 2. Only chapter-specific methods are listed in each scientific chapter.

Some of the scientific data reported in this thesis is the result of collaborations between myself and other researchers, and although all the work presented was led by me, some researchers require further acknowledgement than simply a mention in the acknowledgments section. As such, where these collaborations arise, a "pre-text" section has been included prior to the chapter introduction describing the specific contributions of other researchers to that chapter.

A bridging statement has been included at the end of each science chapter in order to further draw attention to the logical progression of the thesis as a whole. These bridging statements will concisely summarise the preceding chapter's findings, as well as its limitations, and highlight the rationale of the following chapters work in the context of the preceding chapters.

The final chapter (Chapter 7) synthesises and conceptualises all the data discussed in this thesis to provide a single coherent model from which future work can proceed. A list of suggested future studies is also provided in this chapter as a guide to areas which will yield significant further progress in the field.

Chapter 2

Materials & Methods

2. MATERIALS & METHODS

2.1 Collection & maintenance of study organisms

2.1.1 *Palaemonetes varians* (Leach 1814)

Palaemonetes varians were net caught at the marshlands adjacent to Eight Acre Pond on the outskirts of Lymington in southern Hampshire (UK) (Figure 2.1). The salt marshes are enclosed within seawalls that are connected to the western arm of the Solent by sluice gates. The collection site is one of a number of drainage ditches that lie alongside the main channels; the specific ditch can be found as the straight line connecting these two co-ordinate points, between; 50°44'19.8"N 1°32'16.9"W and 50°44'22.2"N 1°32'12.9"W. The ditch is around 100 m long, about 1-3 m wide, and 0.5-1 m deep. The temperature and salinity of the collection ditch has been previously monitored over several years (Oliphant 2014). Large fluctuations in temperature (~0°C to ~26°C), and salinity (~1 to ~43) were observed from 2011 to 2013 (Oliphant 2014).

In all cases, adult *P. varians* were randomly net caught with a 6 mm mesh size generic fisherman's landing net, and placed into 10 litre plastic buckets (Fisherbrand, Fisher Scientific, UK) filled with seawater from the ditch in which the shrimp were caught. Water temperature measurements were taken at the time of collection. Less than 100 individuals were placed into each bucket for transit back to the laboratory to ensure that oxygen did not become limiting. Transit back to the laboratory aquaria took no longer than 2-hours in each case.

Once in the laboratory aquarium, the shrimp were initially kept in their buckets, but with the addition of a bubbling airstone to keep the water oxygenated. Open-circulator water baths (Thermo Electron Corporation Haake® W46, Sigma-Aldrich, UK) were then set to the water temperature (accuracy = ± 0.5°C) which was recorded during collection at the field site. The water baths were filled with freshwater, and two 15 litre plastic aquariums containing sea water were placed into each the water bath. Seawater for the plastic aquaria was obtained through the laboratories seawater taps, had a salinity of 32 to 34, and was run through a three filters: with 10 µm, 5 µm, 1 µm mesh sizes. Once the seawater within the

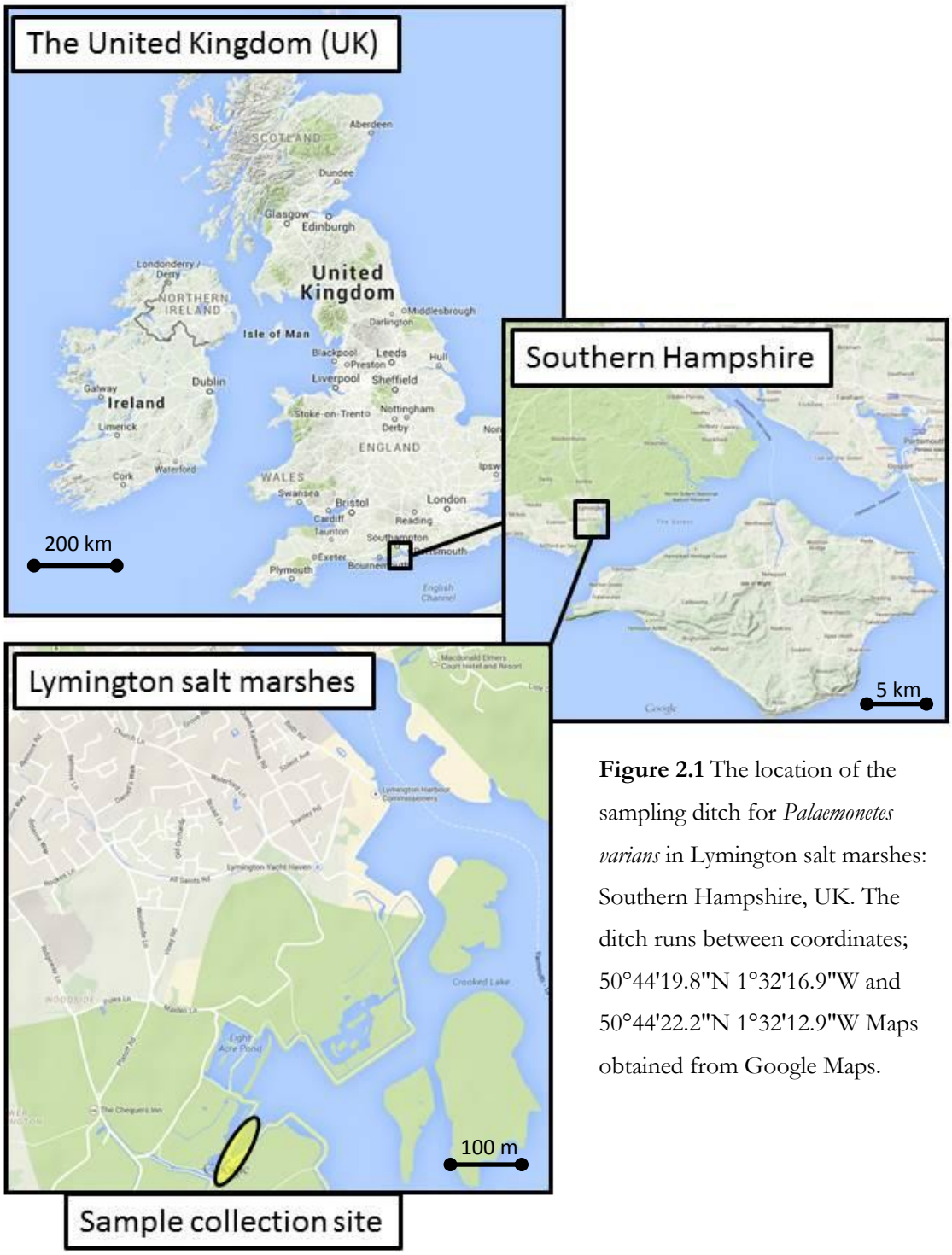


Figure 2.1 The location of the sampling ditch for *Palaemonetes varians* in Lymington salt marshes: Southern Hampshire, UK. The ditch runs between coordinates; 50°44'19.8"N 1°32'16.9"W and 50°44'22.2"N 1°32'12.9"W Maps obtained from Google Maps.

water baths had reached the correct temperature, shrimp were transferred to the plastic aquaria in the water baths. No more than 40 shrimp were kept in a single aquarium.

Prior to experimentation, the shrimp were maintained in the water baths under constant conditions of the temperature they were caught at; a 12:12 light: dark cycle; 32-34 salinity; 1 µm filtered laboratory seawater; and fed TetraMin™ Goldfish flakes (Tetra; United Pet

Group, USA) three times a week. Water changes of 75% were made the day following each feed, with pre-temperature acclimated water of the same characteristics.

2.1.2 *Lithodes maja* (Linnaeus 1758)

Adult specimens of the Northern stone crab *Lithodes maja* were collected by fishermen using baited traps in the Gullmarsfjord area, around 70 km North of Gothenburg (Figure 2.2) (58°20'00.2"N, 11°33'22.7"E). The crabs were caught at depths of approximately 60 m. Before being transported back the laboratory at the National Oceanography Centre, Southampton (NOCS) the animals were maintained for 2-3 weeks in open aquaria at the Sven Loven Centre for Marine Sciences – Kristineberg, Sweden, in seawater drawn in from a deep water intake within the Gullsmarfjord at temperatures of around 6°C. The crabs were exposed to a natural light cycle whilst being held (approx. 14/10 light/dark cycle). The crabs were then transferred to a refrigerated van maintained at 6°C. The crabs were not fed for 3-days prior to transportation, and were transferred in polystyrene boxes lined with water-soaked towels. The crabs were transplanted into laboratory aquaria at the NOCS within 24-hours of leaving the holding tanks in Sweden, and then maintained in recirculating aquaria within a 6°C ±1°C temperature-controlled laboratory under 24-hour darkness, atmospheric pressure, and 32-34 salinity. After the crabs had acclimated to the laboratory conditions, females were matched with males and placed together into aquaria. Mating holds between the males and females were observed, and once each female was determined as carrying eggs they were isolated into individual aquaria.

Larvae were obtained from a single gravid female (pre-moult carapace length – 63.4 mm; post-moult carapace length 70.1 mm). The male crab that fertilised the eggs had a carapace length of 83.2 mm. The female brooded the eggs over a period of 328-days, and hatching began on the 24th February 2013 and continued for 24-days. In total, 2005 larvae were released over the 24-days period. Larvae released each night were collected in 1 mm filters placed in the outflow of each aquarium. Larvae were removed every morning, and thus each larval age was determined to within 24-hours. Larvae were maintained individually in 100 ml rearing cups within the same temperature-controlled laboratory as the adult crabs under constant conditions (6°C ±1°C, 24-hour darkness, atmospheric pressure, 32-34 salinity). Larvae were inspected daily for moults, and 100% water changes were made every 2nd day. Morphological changes and the presence of exuvia, observed under a dissection

microscope, were used to determine ontogenetic stage, according to Anger (1996, 2001). The larvae of *L. maja* have lecithotrophic development, and were not fed during larval development (Anger 1996). Upon reaching the first juvenile stage (Crab I) individuals were fed freshly hatched *Artemia sp.* nauplii *ad libitum* once every 2-days following a 100% water change. In all cases, water changes were made with 1 µm and UV filtered seawater. Crab I individuals were not fed for 3-days prior to experimental exposures.

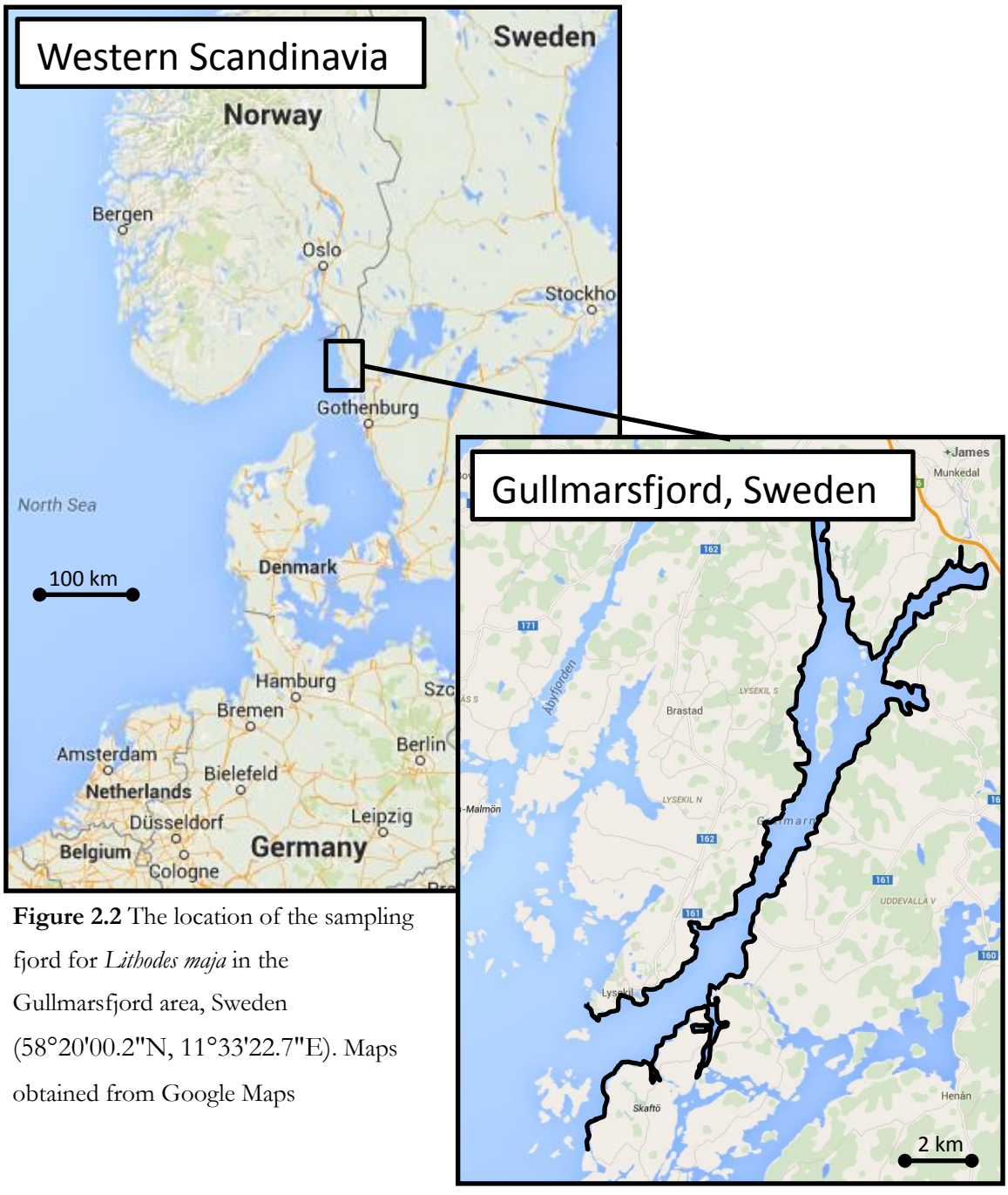


Figure 2.2 The location of the sampling fjord for *Lithodes maja* in the Gullmarsfjord area, Sweden (58°20'00.2"N, 11°33'22.7"E). Maps obtained from Google Maps

2.2 Pressure & temperature manipulation

2.2.1 Small isolated pressure systems

For short-term acute hydrostatic pressure (HP) exposures on small specimens, those able to be kept in a volume of seawater of less than 3 ml for up to 4-hours, small isolated HP vessels (Stauff, Germany) were preferred over larger systems such as the IPOCAMP system (Section 2.2.2) because of ease of use and reliability. This system is able to produce HPs of up to 60 MPa, equivalent to 6000 m of water depth, whereas the IPOCAMP system can only produce HPs of up to 38 MPa. The small isolated pressure vessel system cannot easily be used for experiments where HP is manipulated in a stepwise fashion, such as for HP acclimation and ramping studies, or where HP needs to be varied accurately during an exposure. In order to control temperature, the steel vessels were incubated at the required temperature for 24-hours prior to each experiment, and held at the desired temperature either in temperature control laboratories or large incubators during the experimental period. These vessels were used for the *L. maja* larval experiment (Chapter 6).

The steel HP vessels (Stauff, Germany) (Figure 2.3), when filled with filtered freshwater, can hold up to 8 - 2.8 ml cyrovials (NUNC, UK) (Figure 2.3c). Typically these vials are filled with filtered seawater and the experimental organism, and sealed prior to submergence in fresh water within the steel vessel.

Once sealed, the HP vessels are hand pumped to the desired experimental pressure by a Maximator manual hydraulic pump (Maximator, Germany). The hydraulic hand pump was connected to the pressure vessel by a locking valve (Figure 2.3a). Once attached, the pressure in the system, and thus also in the steel vessel, is determined by the HP gauge on the hydraulic pump itself, rather than the one attached to the steel vessel, although both should show the same value. The steel vessel is then isolated from the hydraulic pump and placed back at the desired temperature. At the end of each experimental exposure, the steel vessel is instantaneously depressurised by opening the locking handle (Figure 2.3b), at which point the specimens within the cyrovials can be removed.

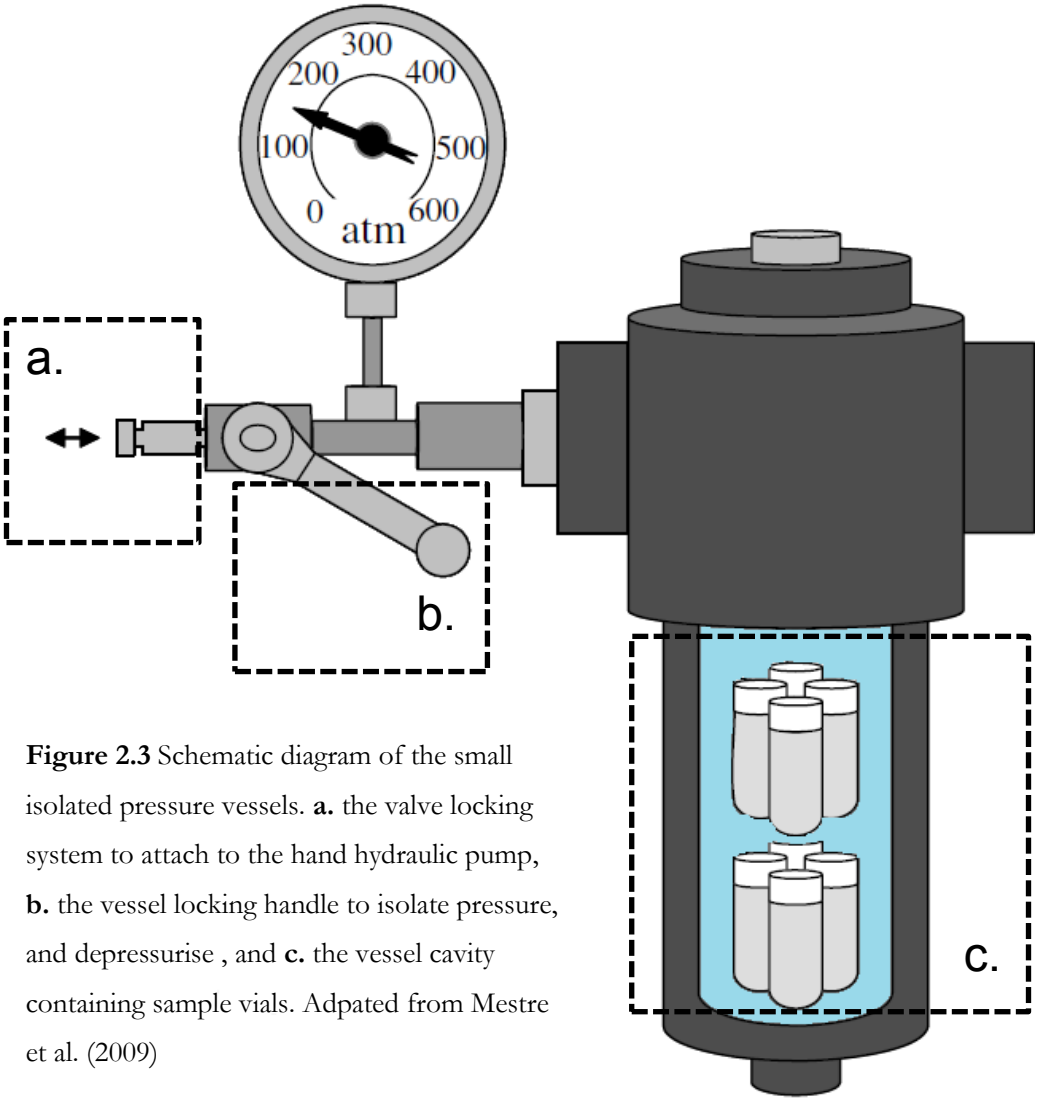


Figure 2.3 Schematic diagram of the small isolated pressure vessels. **a.** the valve locking system to attach to the hand hydraulic pump, **b.** the vessel locking handle to isolate pressure, and depressurise , and **c.** the vessel cavity containing sample vials. Adpated from Mestre et al. (2009)

2.2.2 The IPOCAMP™ system

The IPOCAMP™ (*Incubateur Pressurisé pour l'Observation et la Culture d'Animaux Marins Profonds*) pressurisation system (Autoclave™, France) is a large 19 litre temperature- and HP-controlled hyperbaric chamber with a flow-through water system designed for the simulation and maintenance of organisms under high HP conditions (Shilitto et al. 2014). HP and temperature can both be acutely and accurately controlled; HP between 0.1 and 30 MPa (± 1 MPa), and temperatures between 5°C and 45°C (± 0.1°C). The IPOCAMP system consists of the main stainless steel hyperbaric chamber (Figure 2.4a), a reservoir tank (Figure 2.4b), a high HP pump (Figure 2.4c), and a temperature regulation unit (Figure 2.4d). Freshwater or seawater can be pumped into the hyperbaric chamber from the reservoir tank by the high HP pump. The water passes via the temperature regulation unit,

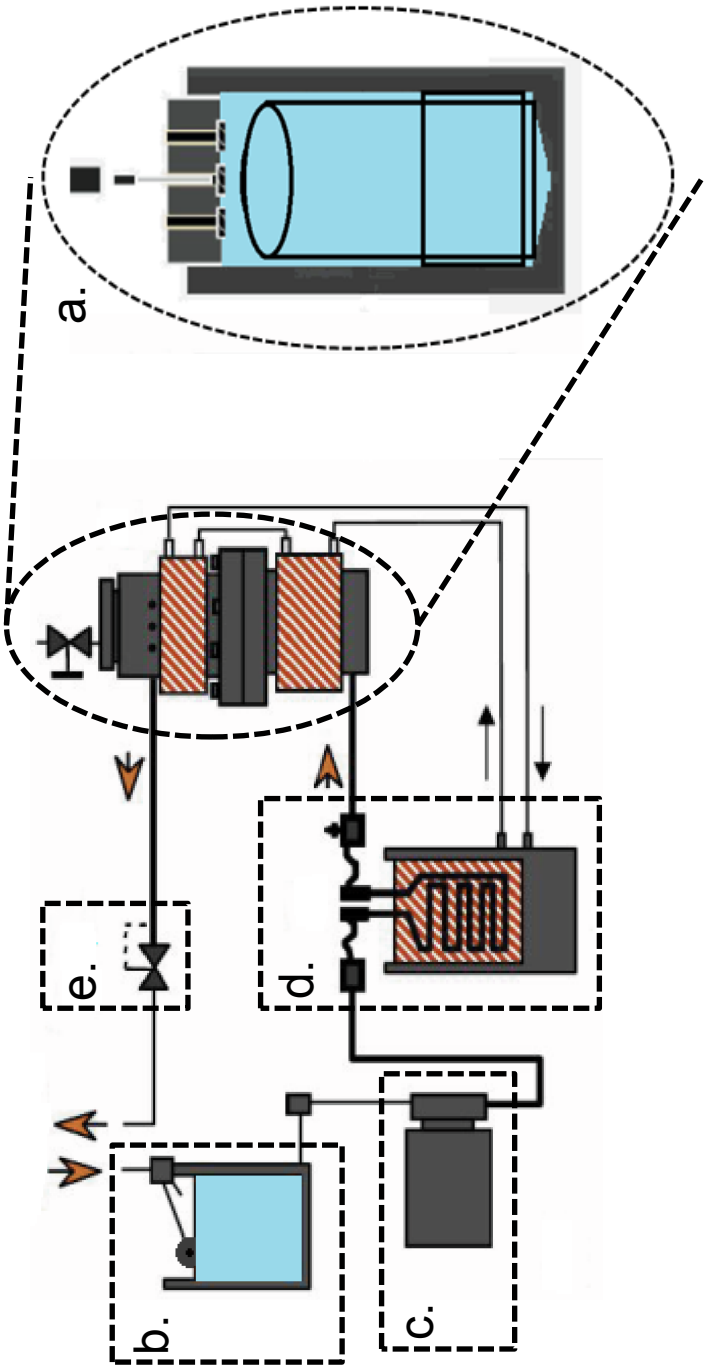


Figure 2.4 Schematic diagram of the IPOCAMP™ system; **a.** the 19 litre pressure chamber with viewing ports; **b.** the 80 litre water reservoir tank; **c.** the high pressure pump; **d.** the temperature regulation system; and **e.** the back-pressure valve. Components are not drawn to scale. Adapted from Shilitto et al. (2014)

where it is heated or cooled to the desired temperature prior to entering the hyperbaric chamber. Water inside the hyperbaric chamber is also maintained at the desired temperature by the temperature regulation unit which also heats or cools the chamber via an insulation sleeve covering it. Flowing water exits the hyperbaric chamber and is returned to atmospheric pressure by a back-pressure valve (Figure 2.4e) before being returned to the

reservoir tank. Flow-rates through the IPOCAMP system can reach 20 litres per hour (Shilitto et al. 2014).

The IPOCAMP system allows for sustained HP exposures, and also the acute control and variation of temperature and HP during exposure. The system also permits behavioural observations to be made during exposure via an endoscopic camera through viewing points in the lid of the chamber (Shilitto et al. 2014). The flow-through system ensures the experimental seawater can kept oxygenated throughout the duration of each exposure. The system is usually filled with >60 litres of seawater. Air stones bubble air through the reservoir seawater, and for multiday exposures, 30% water changes of the total system volume are conducted every 48-hours, from the reservoir tank.

2.3 Respiration rate analysis

The respiration rate of a marine organism, or the rate at which oxygen is obtained and consumed from surrounding waters, can provide a useful indicator of the immediate metabolic rate, and state, of that organism. Respiration rate analysis was used to support evidence of metabolic changes in response to increasing hydrostatic pressure over the period of a 2-hour elevated HP exposure, and over several periods up to 26-hours into recovery.

Small adult male (total length = 4-5cm) *Palaemonetes varians* were sealed into 55 ml falcon tubes (FisherBrand, Thermoscientific Fisher, UK) with filtered seawater (15°C ±0.5°C, 1 µm filtered, 32 salinity). During sealing, care was taken to ensure no air bubbles remained in the tube or the lid. Prior to the experiment, each falcon tube was wrapped in black duct tape to prevent light from entering the tubes, as the experiment required the shrimp to be kept in dark conditions. When sealing each individual shrimp into its own falcon tube, 3-minutes was allowed. Thus, replicates were staggered at 3-minute intervals to ensure the maximum accuracy of respiration rate data, which are highly dependent on isolation periods.

Once sealed, the tubes were kept horizontally at 15°C ±0.5°C in water baths for a period of 2-hours. Preliminary experiments were conducted to ensure that oxygen saturation of the seawater within the experimental tubes did not fall below 60% over a two hour period at 15°C with adult male shrimp of a total length of 4-5 cm. A 2-hour isolation period was deemed appropriate for the size of shrimp, volume of seawater, and the temperature of exposure in order to observe a measurable change in oxygen saturation, yet not allow for a

drop in oxygen saturation of greater than 40%, a point approaching hypoxia which might start further influencing physiology (Oliphant et al. 2011; Morris *unpub. obs.*). Three control tubes containing the experimental seawater, but without a shrimp, were subjected to the same regime as the experimental tubes for each period of analysis. Unfilterable bacteria within the experimental seawater samples will respire over the two hour isolation period contributing to the overall oxygen consumption value. Control tubes allow the results to be adjusted against bacterial respiration.

During the isolation period, the Presens Microx TX3 temperature adjusted oxygen meter and micro-optode (PreSens, Germany) was set up and calibrated with aerated seawater (32-34 salinity, 1 µm filtered) at 15°C as a 100 % oxygen saturation control. Another sample of the same seawater was treated with excess anhydrous sodium sulphite to create a 0 % oxygen saturation control. The probe was also calibrated to the day's atmospheric pressure (mb) via the manufacturers' software, which was obtained via "www.sotonmet.co.uk" (freely accessible) from a meteorological sensor located within 500 m of the laboratory at the National Oceanography Centre, Southampton.

Following the 2-hour isolation period, tubes were gently inverted 3 times to mix the water contained within the tubes and disturb any saturation gradients that may have developed over the 2-hour period within the tube. Once mixed, the tubes were opened and the % oxygen saturation of the water inside the tubes was measured immediately using the calibrated Presens Microx TX3 micro-optode. A value of % oxygen saturation was taken after 1-minute of continuous measurement once the readings had stabilised within the sample tube. Individual shrimp were then removed from the water, carefully blotted dry and then euthanised at -80°C. At a later period, shrimp were removed from -80°C storage, defrosted, blotted dry and weighed to an accuracy of ±0.001 g on fine measurement weighing balance. This measurement was termed a wet tissue mass, and was used to calibrate the oxygen consumption measurements between individuals of different mass.

Oxygen concentration (µmol l^{-1}) for 100 % oxygen saturated seawater at 15°C and 32-34 salinity was calculated according to standard calculations, described by Benson and Krause (1984). The amount of oxygen consumed (µmol l^{-1}) by each shrimp, or control seawater, within a single tube was calculated using equation 2.1. This oxygen consumption measurement was then scaled according to the volume of the tube (equation 2.2), and isolation period (equation 2.3). The adjusted oxygen consumption rate was normalised

against the wet tissue weight of each individual shrimp (mg) yielding a value of oxygen consumption per milligram, per hour (μmol^{-1} mg^{-1} hr^{-1}) (equation 2.4). Finally, this normalised oxygen consumption rate value (μmol mg^{-1} hr^{-1}) was then adjusted against the arithmetic mean of the three control tubes (equation 2.5). This accounted for any oxygen consumption attributed to bacterial respiration within the seawater rather than the shrimp respiration.

Equation 2.1:

$$O_2\ consumption\ (\mu mol\, l^{-1}) = \left(100\,\%\ O_2\ (\mu mol\, l^{-1})\ -\ \left(\frac{tube\,\%\ O_2\ concentration}{100}\right) * 100\,\%\ O_2\ in\ \mu mol\, l^{-1}\right)$$

Equation 2.2:

$$O_2\ consumption\ (\mu mol) = \frac{(O_2\ consumption\ (\mu mol\, l^{-1})\ *\ tube\ volume\ in\ ml)}{1000}$$

Equation 2.3:

$$O_2\ consumption\ rate\ (\mu mol\, hr^{-1})\ =\ \left(\frac{O_2\ consumption\ (\mu mol)}{time\ in\ minutes}\right) *\ 60$$

Equation 2.4:

$$O_2\ consumption\ rate\ (\mu mol\, mg^{-1}\, hr^{-1}) = \frac{O_2\ consumption\ (\mu mol\, hr^{-1})}{wet\ tissue\ weight\ (mg)}$$

Equation 2.5:

$$Control\ corrected\ O_2\ consumption\ rate\ (\mu mol\, mg^{-1}\, hr^{-1}) =\ Arithmetic\ mean\ of\ 3\ control\ tubes\ -\ O_2\ consumption\ rate\ (\mu mol\, mg^{-1}\, hr^{-1})$$

2.4 Behavioural analysis

The IPOCAMP was set running the evening prior to behavioural analysis experiments in order to stabilise at desired temperature of 15°C. The next morning, 10 small adult male shrimp (4-5cm total length), pre-acclimated to 15°C for a period of 10-days, were placed into the IPOCAMP system inside a PVC viewing cage mounted inside the pressure chamber. The IPOCAMP system was sealed and set running at atmospheric pressure (0.1 MPa) for 1-hour prior to behavioural analysis to allow the shrimp time to acclimate to the viewing cage and recover from any handling stress. Behaviour was measured by video recording through a viewing port in the IPOCAMP system lid using an endoscopic camera (Section 2.2.2). Behaviour was then recorded; for 2-hours prior to experimental exposure (baseline measurements) during the 1-minute pressure ramp, 2-hour pressure exposure, and 1-minute decompression; and then for 2-hour periods at 1-hour post-exposure, 6-hours

post-exposure, and 24-hours post-exposure. Behaviour was measured and characterised for 30-seconds at 5-minute intervals over each recording period, and throughout the pressure ramp and decompression stages. Behaviour was characterised into distinct categories, as described by Oliphant et al. (2011), with the addition of a "tail flicking" category. "Motionless" was determined as no visible movement over the 30-second analysis periods. "Active movement" was determined as a directed movement of greater than a single body length over the 30-second time periods. "Tail flicking" was determined as a reflexive contraction of the entire abdomen leading to a fast backward movement, and represents a common behavioural escape response. "Loss of equilibrium" and "Spasms, convulsions, and tremors" were to be noted, but neither type of behaviour was observed during the experiment. Position of the shrimp vertically was also determined at 5-minute intervals. Shrimp were noted as being either in the lower 50% of the chamber for more than 20-seconds of the 30-second time period; in the upper 50% of the chamber for more than 20-seconds of the 30-second time period; or spending less than 20-seconds either solely in the lower or upper 50% of the chamber, and thus deemed to be "transitioning".

2.5 Nucleic acid extraction & preparation

2.5.1 Total RNA extraction

For all molecular analysis contained within this thesis, mRNA was used as the template molecule, and was extracted as portion of a total RNA extraction.

Total RNA was extracted for all samples in each experiment following discreetly modified versions of the standard TRI Reagent® (Sigma-Aldrich, UK). Modifications of the manufacturer's standard protocol were experiment-, organism-, and tissue-specific, and are listed below (Table 2.1).

TRI Reagent®, and the TRI Reagent® protocol are adaptations, made by Sigma-Aldrich (UK), based on the "acid guanidinium thiocyanate phenol chloroform extraction" (or AGPC) method devised by Chomczynski and Sacchi (1987). The AGPC method is one of two techniques widely employed for the isolation of total RNA from tissue samples, the other being a spin-column based system. The major principle of this technique is phase separation by centrifugation, and subsequent cleaning. Guanidinium thiocyanate is one of the key compounds in the AGPC method that acts to denature various macromolecules,

importantly including proteins such as RNase enzymes, in homogenised tissue samples (Chirgwin et al. 1979). Chloroform then allows the separation of phenol and isopropanol into distinct phases: the upper isopropanol layer containing nucleic acids, and the denser phenol and chloroform phase containing proteins and dissolved lipids (Chomczynski and Sacchi 1987).

TRI Reagent® was used preferentially over spin-column based techniques as it may allow for greater total RNA concentration recovery, and is a more cost-effective method. Spin column based techniques may be preferential when total RNA concentration is not a potentially limiting factor, as the standard column based protocols take considerably less time than phases separation methods.

All tissue samples were removed from storage at -80°C and immediately placed into liquid N_2 sealed within 1.8 ml cyrovials (Fisherbrand, Fisher, UK). In each experiment, tissue samples were removed from liquid N_2 and placed immediately into an RNase- and DNase-free 5 ml microcentrifuge tube containing a predetermined volume of TRI reagent®, as listed in Table 2.1. The tissue was then rapidly homogenised using a pre-sterilised handheld Ultra-Turrax® homogenisation system (IKA, Germany), always within 2-minutes of removal from liquid N_2. Depending on the TRI Reagent® volume used, the resulting homogenate was mixed by pipetting and split amongst 1.5 ml RNase- and DNase free microcentrifuge tubes so that the volume of each was 1 ml, any remaining homogenate was discarded at this stage. The samples were left for 15-minutes at room temperature. After 15-minutes, 0.2 ml of molecular grade chloroform (Sigma Aldrich, UK) was then added to each of the tubes containing 1 ml of homogenate, shaken by hand vigorously (not by vortexing) for approximately 15-seconds before being left upright at room temperature for a further 15-minutes.

Samples were then carefully transferred to a pre-chilled refrigerated centrifuge and spun at 12000*g* at 4°C for 15-minutes in order to separate total RNA, total DNA, and protein phases. The uppermost liquid phase, clear in appearance, was carefully transferred by pipetting to another RNase- and DNase-free 1.5 ml microcentrifuge tube containing 0.5 ml of molecular grade isopropanol (Sigma Aldrich, UK). Care was taken not to disturb the DNA inter-phase whilst pipetting in order to prevent DNA contamination. Once transferred, the RNA/isopropanol mixture was mixed gently by inversion for 10-seconds before being left standing upright at room temperature for 10-minutes (unless otherwise stated in Table 2.1)

The RNA/isopropanol mixture was then spun at 12000*g* for 10-minutes at 4°C to produce an RNA pellet at the bottom of the microcentrifuge tube. The resulting supernatant was removed by pipetting. The pellet was then quickly re-suspended in 1 ml of 75% molecular grade ethanol, diluted with DEPC-treated MilliQ™ water (Merck-Millipore, UK), mixed by vortexing, and spun at 7500*g* for 5-minutes at 4°C. Following second pellet formation, the supernatant was carefully removed and the pellet was air dried within the tube, on ice, in a laminar flow hood to minimise sample contamination. Drying time was dependent on pellet size. Pellets were deemed to be dry once they began to become glassy in appearance. Pellets were then re-hydrated in a volume of DEPC-treated MilliQ™ water (Table 2.1)

2.5.1.1 Total RNA analysis

RNA purity, integrity, and quantity are all prerequisites for robust gene expression analysis assays, as highlighted in the MIQE guidelines authored by Bustin et al. (2009), which are discussed in greater detail in Section 2.8.

RNA purity pertains to the level of contaminants, such as chemical residues from the TRI Reagent® or DNA/Protein contamination, present after total RNA extraction. This can be measured using a Nanodrop™ 1000 spectrophotometer (NanoDrop, Thermo Scientific, USA). The Nanodrop™ system provides measurements of absorbance at 260 nm and 280 nm in as little as 1 µl of extracted total RNA in a solution. The ratio between A_{260}: A_{280} can be used to determine RNA purity in a sample (Denisov et al. 2008), with a ratio of 2.0 considered to represent pure total RNA. This spectrophotometric technique provides a simple and quick measure of RNA extraction quality and concentration. However, the method has several limitations, and as a standalone quality control measurement, is now not thought to be suitable for gene expression analysis studies (Fleige and Pfaffl, 2006).

In all experiments, samples with an A_{260}: A_{280} ratio of greater than 1.8, an A_{260}: A_{230} ratio of greater than 2.0, and a RNA concentration greater than 220 ng/µl were further analysed for molecular integrity and a more precise measure of concentration.

Table 2.1 Specific weights and volumes used for total RNA extractions in each experiment alongside any additional modification from standard manufacturer's protocol

Experiment	Species	Life stage	Tissue type	Weight of tissue used (mg)	Volume of TRI reagent™ used for homogenisation (ml)	Additional modifications to standard protocol	Volume of DEPC-treated MilliQ™ used for pellet rehydration (µl)
Stage 1	*P. varians*	Juveniles	Whole organism homogenisation	<0.1	2	Held in isopropanol for 2 hours on ice	11
GeneFishing™	*P. varians*	Juveniles	Whole organism homogenisation	<0.1	2	Held in isopropanol for 2 hours on ice	11
Kinetics	*P. varians*	Adult	Head tissue	0.1 to 0.15	2.2	/	25
	P. varians	Adult	Abdomen tissue	~0.15	3	/	40
Sustained pressure	*P. varians*	Adult	Abdomen muscle tissue	0.1 to 0.15	3	/	30
Lm ontogeny	*L. maja*	Zoea1	Whole organism homogenisation	<0.1	2	/	10
		Megalopa	Whole organism homogenisation	<0.1	2	/	10
		Crab1	Whole organism homogenisation	<0.1	1	Held in isopropanol for 2 hours on ice	10
Lm adult	*L. maja*	Adult	Leg muscle tissue	0.15 to 0.2	3	/	40

RNA integrity is a measure of the degradation of a total RNA sample. A fully integral total RNA sample would consist of full length mRNA and rRNA molecules. Some degree of RNA degradation is inevitable. High levels of RNA degradation can have significant effects on the efficiency of cDNA synthesis and subsequent gene expression analysis (Gingrich et al. 2006), although moderate degradation has been shown not have significant effects on gene expression analysis by quantitative PCR in some cases (Fleige and Pfaffl 2006). Lab-on-chip technologies employ micro-fluidic capillary electrophoresis in order to assess RNA quality and concentration. In brief, total RNA samples combined with an intercalating dye are electrophoretically separated on a micro-chip and fluorescence is measured by laser-induced fluorescence detection. Fluorescence is compared to an RNA standard ladder which allows for the estimation of RNA band size estimates. Ribosomal RNA (rRNA) makes up around 95% of total RNA from a eukaryotic cell or tissue sample. For this reason, RNA integrity measurement systems quantify total RNA degradation based upon 18S and 28S ribosomal RNA subunit fluorescence signals predominantly, but may also assess the "pre-18S" region (Denisov et al. 2008). RNA degradation is indicated by a reduction in 28S:18S fluorescence ratio from the norm: where a 2:1 ratio would be expected, and also elevated levels of fluorescence in the region prior to the 18S band (Mueller et al. 2004).

In each experiment, total RNA samples that passed initial testing by spectrophotometry were analysed for integrity and quantity using an Experion™ automated electrophoresis system (Bio-Rad Laboratories, UK). Standard sensitivity kits for Eukaryotic total RNA (StdSens) were used in all cases. The Bio-Rad Experion™ software applies a complex algorithm to map measurements taken from the electropherogram output for each sample (Figure 2.5), as described in (Denisov et al. 2008) in order to generate a standardised measure of RNA degradation or integrity, termed RNA quality indicator value (RQI value), this obviates the need for visual comparisons between samples. The RQI value is scaled from 1 to 10: with 10 indicating intact RNA, and 1 indicating highly degraded RNA (Figure 2.5).

In all experiments, samples with an RQI value of greater than 7.5, and a concentration higher than 220 ng/µl were considered of high enough quality to be used for gene expression analysis. The generally accepted RQI value for gene expression analysis is 7.5 or higher (Denisov et al. 2008).

2.5.1.2 RNA extraction & analysis troubleshooting

Low total RNA concentrations were initially obtained after using the standard TRI Reagent® protocol for *P. varians* juvenile samples, and *L. maja* crab I samples. As recommended in the TRI Reagent® protocol troubleshooting guide, samples were held on ice after the addition of isopropanol for 2-hours, and marked improvements in total RNA yield were observed in these cases.

During the total RNA extractions for Chapter 3 experiments, RQI values were initially poor (RQI = <7.5). After testing a number of alternative procedures, it was identified that minimising the time taken between the tissue sample being frozen in liquid N_2 to complete homogenisation in TRI Reagent® was key to obtaining high RQI values. Following this, only samples where full homogenisation had been completed within 2-minutes of being removed from liquid N_2 were further extracted.

In both *P. varians* and *L. maja* total RNA extractions, 28S rRNA peaks were either not visible or highly reduced following Experion™ analysis. Although this did not affect the result of the analysis: total RNA concentrations and RQI values are not solely dependent on a distinct 28S peak, it was deemed anomalous as both species will have 28S rRNA present in their cells. Similar observations have been noted by Winnebeck et al. (2010). Studying insects, they proposed a potential breaking point in the 28S rRNA structure that, when broken, results in two hydrogen-bound fragments of a similar size to 18S rRNA, termed 28Sα and 28Sβ (Winnebeck et al. 2010). This would explain the lack of a major 28S rRNA peak in both *L. maja* and *P. varians* samples after Experion™ analysis.

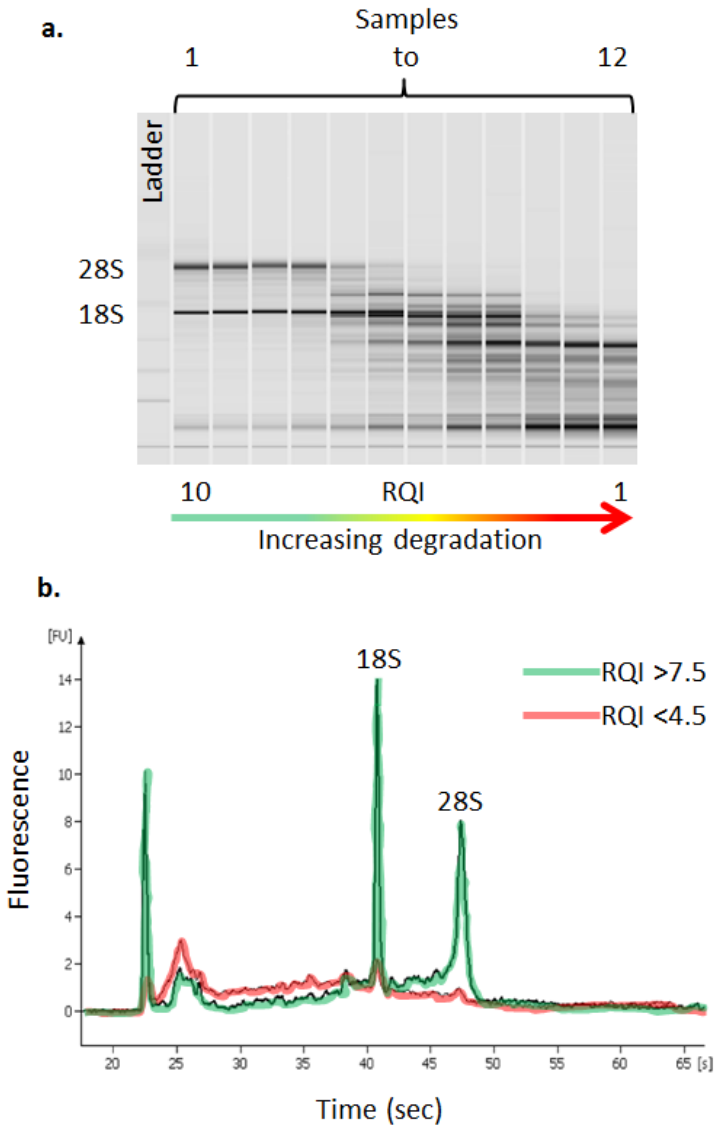


Figure 2.5 Examples of the results output obtained from micro-fluidic capillary electrophoresis across a series of samples of varying degradation. **a.** a virtual gel image generated in 12 samples of varying degradation, standardised against an RNA ladder, adapted from Mueller et al. (2004). **b.** a typical electropherogram image of an intact RNA sample (green line), compared to a degraded RNA sample (red line). The 18S and 28S peaks are labelled, and the peak at 23-seconds represents the samples start marker. Adapted from Denisov et al. (2008)

2.5.2 DNase treatment

Despite all efforts to avoid DNA contamination during total RNA extraction, a small amount of contamination will always be inevitable. Because even a small amount of DNA contamination can produce erroneous amplification effects during qPCR, total RNA was further purified using a DNase treatment method. DNase treatment further reduces the likelihood of genomic DNA (gDNA) contamination, and was also used as the step during which all total RNA concentrations were normalised, which is preferable but not essential for qPCR analysis. The Promega DNase treatment kit (Promega Ltd, UK) was used following the manufacturer's standard protocol. For each sample, in a 0.2 ml RNase-/DNase- free PCR tube, a volume equating to 1.5 µg of total RNA in DEPC-treated MilliQ™ water was added to 1 µl of RQ1 RNase-free DNase 10x buffer, and 1.5 µl RQ1 RNase-free DNase. The volume was then made up to 10 µl in total volume with MilliQ™ water. The mixture was then incubated at 37°C for 30-minutes. Following incubation, 1 µl of RQ1 DNase stop solution was added, and the mixture was heated at 65°C for 10-minutes. The mixture was then immediately placed on ice for 5-minutes before being stored at -80°C until further use.

2.5.3 First strand cDNA synthesis

First-strand cDNA synthesis using oligo(dT)$_{18}$ primers exploits the poly(A) tails present on all mRNA molecules, and allows the transcription of the mRNA portion of the total RNA pool to be transcribed to cDNA. Using SuperScript® III reverse transcriptase (Life Technologies™, UK), and following the manufacturer's standard protocol; 1 µl (10 mM) dNTPs; 1 µl oligo(dT)$_{18\text{-}23}$; 6 µl MilliQ™ water; and 5 µl of DNase-treated total RNA (0.68µg) were added to a RNase- /DNase-free 0.2 ml PCR tube on ice. The mixture was heated to 65°C for 5-minutes, and then immediately returned to ice for 5-minutes. After brief centrifugation; 4 µl 5x first strand buffer; 1 µl 0.1 M DTT; 1 µl RNase OUT; and 1 µl SuperScript® III reverse transcriptase were added, and the mixture was incubated at 50°C for 1-hour, and then heated to 70°C for 15-minutes. The cDNA was then stored at -20°C until further use.

2.6 Identification & isolation of genes of interest

2.6.1 Gene hunting by degenerate PCR

Neither *Palaemonetes varians* nor *Lithodes maja* had extensive nucleotide sequence data available on online gene databases such as the NCBI's GenBank or the EMBL-EBI repository. The gene transcripts or expression sequence tags (ESTs) that were available were common phylogenetic markers such as Cytochrome Oxidase subunit 1 (CO1), which by their nature, are not of interest to studies assessing physiology, or were genes that were not deemed of interest in relation to the experimental exposures conducted in this thesis. Appendix A1.1 lists all the gene transcripts and ESTs that were available on NCBI's GenBank in both species prior to this work.

In order to build a set of "genes of interest" two techniques were used; GeneFishing™ (described in Section 2.6.2) was used to discover random novel differentially-expressed mRNA fragments between experimental exposures; and gene hunting by degenerate PCR (degenPCR) was used to elucidate species-specific sequences of genes that were thought, *a priori*, to be of interest to the experimental exposures conducted, and that were known to be highly conserved and well characterised amongst related species.

The technique of gene hunting by degenPCR involves designing degenerate primers based upon conserved amino acid regions of sequences from phylogenetically related species that have been built into a multiple amino acid alignment. The consensus sequence obtained from multiple alignments was then analysed for highly conserved short sequences within which degenerate primers could be designed for degenPCR. Ideally, degenerate primers include enough degeneracy in their code to account for differences in sequence between the sequences used to build the multiple alignment, and the sequence being hunted in the test species. However, there is a fine balance because although increasing primer degeneracy will increase the likelihood of primer binding, it will also increase the likelihood of non-specific binding to other regions of the mRNA derived cDNA pool. Degenerate PCR was used to isolate gene sequences in Chapters 5 and 6: degenerate primer sequences and strategy is explained in detail in these chapters.

2.6.2 GeneFishing™

Developed by Seegene (Biogene, UK), the GeneFishing™ kit and protocol allows for the discovery and identification of a random subset of differentially expressed mRNA fragments, termed "differentially expressed genes (DEGS)". The kit is designed for total RNA extractions derived from a number of distinct samples: in this case, experimental exposures.

In principle, the protocol involves a specific cDNA synthesis step followed by a standard two-stage PCR. The protocol is built upon primers with three distinct structural sections. These primers are termed arbitrary "annealing control primers" (ACPs) and consist of; a "core sequence" localised to the 3' end; a "universal sequence" localised to the 5' end; and a "regulator sequence" connecting the two, forming a unique tripartite structure (Figure 2.6). It is this tripartite structure that provides reaction specificity beyond that obtained through other techniques of differential display, such as microarray analysis.

The GeneFishing™ protocol was selected for the identification of differentially expressed mRNA fragments in this study as an inexpensive alternative to microarray analysis in which PCR products can be visualised and extracted using standard Ethidium Bromide (EtBr) stained agarose gel electrophoresis. This obviates the need for more specialised visualisation techniques such as polyacrylamide gel electrophoresis (PAGE). Seegene also guarantee that no false positives can be detected by this technique, a common shortcoming in other differential display protocols (Seegene 2003).

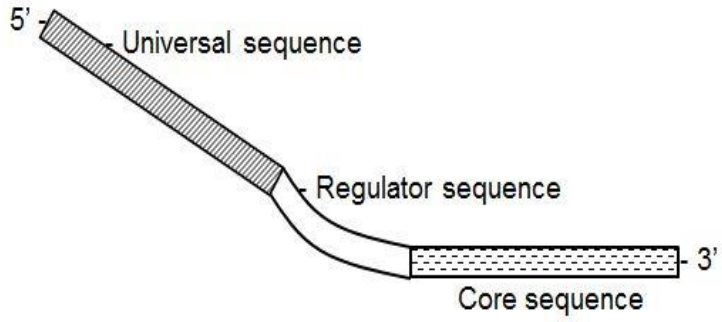


Figure 2.6 The tripartite structure that is central to the GeneFishing™ technique. The primer is termed an "annealing control primer" (ACP)

2.6.2.1 GeneFishing™ reverse transcription

The reverse transcription reaction uses the dT-ACP1 (10 µM) universal primer supplied with the GeneFishing™ kit:

dT-ACP1: 5'-CTG TGA ATG CTG CGA CTA CGA T… $(T)_{18}$ -3'

The dT-ACP1 anneals to the poly(A) tail on all mRNA molecules during the first stage of reverse transcription, and ultimately results in cDNA templates, derived from extracted mRNA, with the universal sequence at the 5'-end of the molecule.

Following the manufacturer's standard protocol, 3 µg of total RNA, 2 µl of dT-ACP1 (10 µM) were mixed gently and made up to 9.5 µl total volume with RNase-free water on ice. The mixture was then incubated for 3-minutes at 80°C. The mixture was then held on ice for 2-minutes and subsequently spun down. The following was then added to the mixture; 4 µl 5x RT buffer; 2 mM dNTP; 0.5 µl RNase inhibitor (40 U μl^{-1}); and 1 µl Superscript® III reverse transcriptase (Life Technologies, UK). Mixed by repeated pipetting, the mixture was then incubated at 50°C for 60-minutes, before a 15-minute deactivation heating step at 70°C. The mixture was then placed on ice for 5-minutes and then spun down to collect the liquid in the tube. Finally, 80 µl of RNase-free water was added to the mixture which was then stored at -20°C prior to further use.

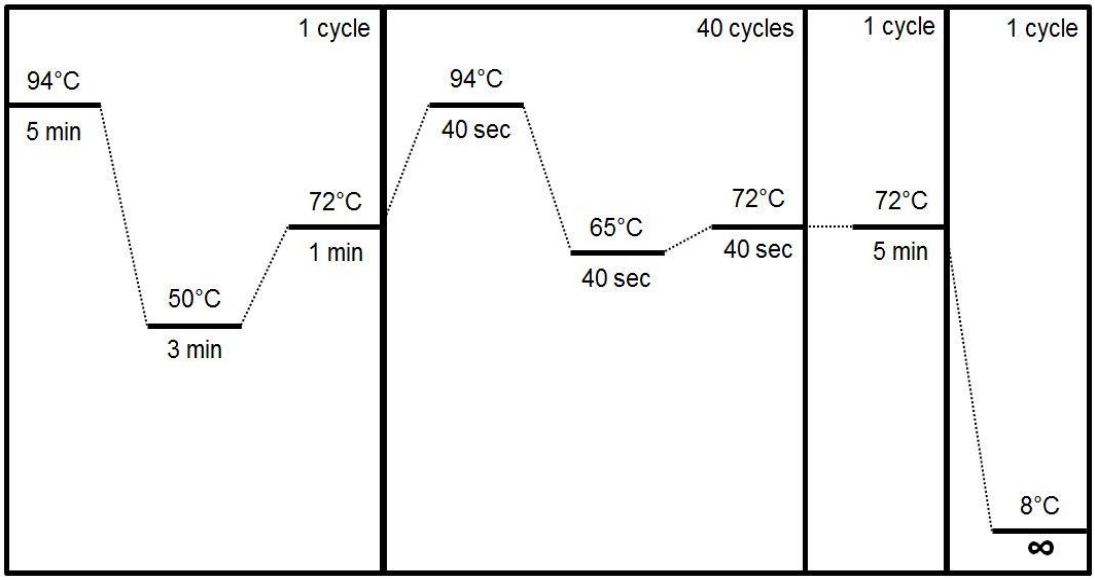


Figure 2.7 Standard GeneFishing™ two-stage PCR reaction profile, with step temperatures and timings, and the number of cycles per segment

2.6.2.2 GeneFishing™ PCR (two-stage PCR)

The following components were added to a DNase-free PCR tube for each sample to be tested: 5 µl of GeneFishing™-specific first-strand cDNA; 2 µl of arbitrary ACP (5 µM); 1 µl of dT-ACP 2 (10 µM); 10 µl 2x SeeAmp ACP Master-mix ; and 2 µl of RNase-/DNase-free water. The tubes were placed into a thermal cycler pre-set to 94°C, and the two-stage PCR reaction was set in progress according to the manufacturer's standard conditions (Figure 2.7).

The two-stage PCR protocol ensures increased specificity of reactions, and the guarantee of no false-positive results. Reaction conditions during the first stage allow only the 3'- end core portion of the ACP primer to anneal to a specific site on the cDNA template. Importantly, annealing of the regulator sequence and 5'-end is not favoured under the first stage PCR conditions. During the second stage of the PCR reaction, the products of successful reactions during the first stage are amplified under high stringency conditions. The conditions during the second stage are such that the 3'-end core portion of the ACP primer can no longer anneal to the original template. Instead, the sequences at the 3'- and 5'- ends of the first stage PCR product can now act as contributors of priming sequences for amplification. This results in amplification of only the target product, with a purported amplification efficiency that is close to the theoretical optimum of a PCR reaction (Seegene 2003).

2.6.2.3 GeneFishing™ PCR product visualisation & analysis

The products obtained from the two-stage PCR reaction were separated by molecular weight, and visualised, using a 1.5% agarose gel made from 1% Tris-acetate-EDTA (TAE) buffer containing 10 mg ml^{-1} Ethidium bromide (EtBr). The products were run alongside 4 µl of Hyperladder™ IV (Bioline Reagents Limited, UK) for reference at 70 volts for 45-minutes (Figure 2.8).

Once products had been electrophoretically separated, the gels were carefully transferred to a Gel Doc™ UV transilluminator (Bio-Rad, UK) where images were taken under UV-light. The clarity and intensity of band fluorescence between samples containing the same arbitrary ACP primers was analysed by eye, and those bands deemed to be of interest, that were also of greater than 200 base pair (bp) in size, were purified by gel extraction using a QIAquick® gel extraction kit (Section 2.6.3) for cloning and sequencing purposes (Sections 2.6.4 – 2.6.6).

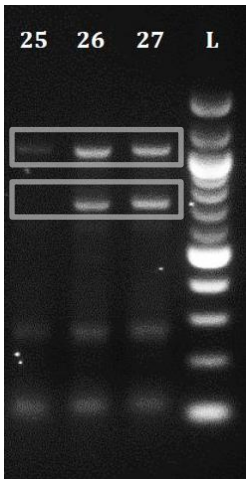


Figure 2.8 An example of the PCR products, separated by gel electrophoresis, obtained from one arbitrary ACP primer (A49) across three distinct experimental treatments (**25**, **26**, and **27**) run against Hyperladder™ IV (**L**) (Bioline Reagents Limited, UK) showing two differentially expressed fragments (within grey boxes) to be purified and cloned for sequencing

2.6.3 Gel Extraction

For both GeneFishing™ and gene-hunting by degenPCR, PCR products were visualised on a 1 - 2% agarose gel made with 1% TAE buffer containing 10 mg ml^{-1} EtBr. Electrophoretically separated gels were visualised using a Gel Doc™ UV transilluminator (Bio-Rad, UK) under UV-light. Fluorescent bands of the correct predicted length were noted, and the corresponding PCR mixture was re-run on a 0.8% agarose gel. This gel was visualised using the same method, and the band was cut out of the gel using a sterile scalpel, with care taken to minimise the amount of non-DNA containing gel. The DNA fragment-containing agarose gel was placed in a DNase- /RNase-free 1.5 ml microcentrifuge tube of predetermined weight (~1 g), and the gel fragment was weighed to a precision of ±0.01 g.

The gel fragment was then dissolved, purified, and eluted using a QIAquick® Gel extraction kit (Qiagen UK) following an optimised version of the manufacturers' standard protocol, as follows:

Once the weight of the excised gel fragment had been determined, 3.5x volume of Buffer QG was added to the gel (350 µl of Buffer QG for every 100 mg of gel weight). The tube was mixed by vortexing and incubated at 50°C and vortexed every 2-minutes until the gel slice had completed dissolved in the solution. Once dissolved, one gel volume of molecular grade isopropanol was added to the sample and mixed by vigorous shaking.

The solution was then applied to a QIAquick spin column held in a 2 ml collection tube, and centrifuged for 90-seconds at 17000*g*. The flow-through was discarded, and 750 µl of Buffer PE was added to the column and centrifuged as before. The flow-through was discarded, and the spin column was placed in a new DNase- /RNase-free 1.5 ml microcentrifuge tube. A volume of 30 µl of nuclease-free water was applied to the centre of the column, let to stand for 2-minutes, and then centrifuged at 17000*g* for 1-minute. Purified DNA solution (1 µl) was then visualised by gel electrophoresis to ensure that the correct predicted length of band had been successfully extracted. The purified DNA solution was then stored at -20 °C until further use.

2.6.4 Cloning

PCR products of interest were extracted from agarose gel and purified using a QIAquick Gel extraction kit (Qiagen, UK) (Section 2.6.3). The resultant DNA was then cloned into an *E. coli* vector using the pGem®-T easy vector system (Promega, UK). Cloning was preferred over a PCR purification technique as it provides significantly more accurate sequencing results in terms of base identity.

First, a ligation reaction was setup according to the standard manufacturer's protocol. In a DNase- /RNase-free PCR tube; 5 µl of 2x Rapid ligation Buffer; 1 µl of pGem®-T easy vector; 3 µl of the gel extracted PCR product at a concentration of ~100 ng/µl; 1 µl of T4 DNA ligase; and 2 µl of nuclease-free water were mixed by pipetting and left for 1-hour at room temperature.

During the 1-hour incubation, agar plates were inoculated with 20 µl of 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal) (Sigma-Aldrich, UK) and 100 µl of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, UK), and left to incubate at 37°C for at least 1-hour.

JM109 *Escherichia coli* competent cells (Promega, UK) were removed carefully from -80°C storage and thawed on ice. Once the ligation had been left for 1-hour, the mixture was briefly centrifuged and 2 µl of the mixture was added to a DNase- /RNase-free 1.5 ml tube. 50 µl of competent cells were added to the ligation mixture and gently mixed by pipetting. The reaction was held on ice for 20-minutes before the tube was placed in a 42°C ± 0.5°C water bath for exactly 45-seconds to heat shock the competent cells. The mixture was then returned to ice for 2-minutes before 950 µl of SOC medium (Invitrogen, UK) was added. The resultant mixture was then incubated at 37°C for 90-minutes with

constant shaking. After the incubation period, 100 µl of the solution was added to the X-Gal and IPTG inoculated agar plates. The plates were then sealed and incubated for 16-hours at 37°C.

Following the 16-hour incubation, the agar plate was assessed for blue and white colonies. Blue-white screening is a standard molecular biology technique for determining whether *E. coli* colonies have taken up DNA insert during the 42°C heat shock. In the presence of X-Gal, colonies containing the DNA insert will appear white in colour, whilst colonies without the insert will appear blue in colour. The JM109 competent cells carry the *lacZa* gene which contains a multiple cloning site. The multiple cloning site can be split by a restriction enzyme to allow DNA inserts to be added. If an insert is added, the gene function is disturbed. The lack of gene function ultimately prevents β-galactosidase from being formed. X-gal is an analog of lactose that is hydrolysed by β-galactosidase into galactose and 5,5'-bromo-4-chloro-4,4'-dichloro-indigo, the second of which has strong blue colouration (Kiernan 2007). Competent cells with a DNA insertion at the multiple cloning site of the *lacZa* gene appear white because they do not form β-galactosidase, whilst those without a DNA insert are able to form β-galactosidase, and the colonies appear blue (Ullmann et al. 1967).

Colonies with a white appearance were putatively deemed to contain the desired DNA insert and were picked from the agar plate using a sterile tooth pick. The tooth pick was then dipped into a PCR mixture containing; 5 µl 5x PCR Buffer; 0.125 µl TAQ polymerase; 0.5 µl of both M13F (TGT AAA ACG ACG GCC AGT) and M13R (AGG AAA CAG CTA TGA CCA T) universal primers; 1 µl of dNTPs; 3 µl $MgCl_2$; and 9.875 µl of nuclease-free water. Once the tooth pick had been dipped into the corresponding PCR mixture, it was smeared on to a new agar plate, sealed, and incubated for 16-hours at 37°C.

A "colony PCR" reaction was conducted under the following conditions (Figure 2.9). The PCR products were separated by molecular weight, and visualised, using a 1.5% agarose gel made from 1% TAE buffer with 10mg ml^{-1} EtBr. The products were run alongside 4 µl of Hyperladder™ IV (Bioline Reagents Limited, UK) for reference at 70 volts for 45-minutes. The gel was then transferred to a Gel Doc™ UV transilluminator (Bio-Rad, UK) where images were taken under UV-light. PCR products containing the desired DNA insert produced a bright band at around 200-bp longer than the predicted insert length. PCR

products without the desired DNA insert produced either no visible bands or a faint band of around 200-bp in length.

Re-grown colonies that gave positive results following colony-PCR were then picked from the agar plate and added to 5 ml of Lysogeny broth (LB) (Sigma-Aldrich, UK) with ampicillin and incubated at 37°C with shaking for 16-hours. Following incubation, the culture solution was prepared for sequencing using a QIAprep Miniprep kit (Qiagen, UK).

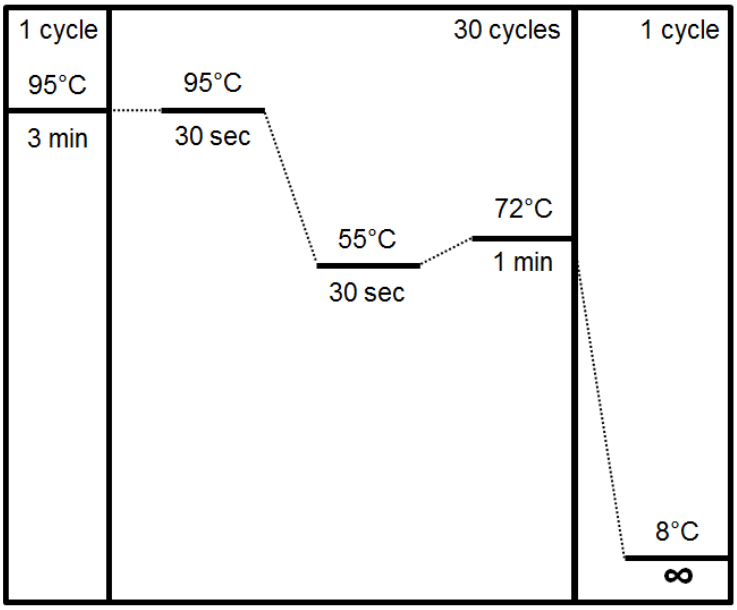


Figure 2.9 Standard colony PCR reaction profile, with step temperatures and timings, and the number of cycles per segment

2.6.5 Plasmid extraction

Cloned cell cultures, incubated for 16-hours with shaking in an LB medium were prepared for sequencing using the QIAprep Spin Miniprep kit (Qiagen, UK). Following an optimised manufacturer's protocol, the cell culture was pelleted by centrifugation. The supernatant was removed and the pellet was re-suspended in 250 µl of the supplied Buffer P1. The mixture was then transferred to a DNase- /RNase-free 1.5 ml microcentrifuge tube. Buffer P2 (250 µl) was added and mixed by inversion. Immediately, 350 µl of Buffer N3 was added and the mixture was mixed by inversion once more. The mixture was then centrifuged for 10-minutes at 14000*g*. The resulting supernatant was then transferred to a QIAprep spin column and centrifuged at 14000*g* for 1-minute. The flow through liquid was

discarded, and 750 µl of PE buffer was added to the spin column and centrifuged again at 14000*g* for 1-minute. The flow-through was discarded, and the spin-column was centrifuged once more at the same settings to remove any remaining buffer solution. Finally, the spin column was placed into a DNase- /RNase-free 1.5 ml microcentrifuge tube, and 30 µl of nuclease-free water was added to the spin column. The tube was then centrifuged at 14000*g* for 1-minute. The elution fluid was assessed for DNA concentration using a Nanodrop™ 1000 spectrophotometer (NanoDrop, Thermo Scientific, USA), and diluted to ~100 ng/µl for sequencing.

2.6.6 Alignment & analysis of sequence data

DNA fragments of interest from GeneFishing™ and Gene-hunting by degenPCR that were cloned, extracted, and cleaned, were sent for sequencing in duplicate (one forward read, and one reverse read) by Source Bioscience (UK) using M13 universal primers and conventional dideoxy-termination sequencing.

Returned sequence data were analysed using Geneious Pro™ R6 software (Biomatters, New Zealand). Chromatographs for each sequence were analysed for reading errors, base ambiguity, and base mismatching. Forward and reverse reads for duplicate sequences were aligned, and multiple sequences from distinct colonies derived from a single insert were also aligned. The sequences were cut according from identified universal M13 primer sequences, vector start and end sequences obtained from the pGem®-T easy vector manual, or from identified GeneFishing™ or Gene-hunting primer sequences. Once a clean consensus sequence had been obtained from forward and reverse reads of multiple colonies, the sequence was identified as described in Section 2.7

2.7 Gene identification

All putatively gene coding mRNA fragments obtained from Genefishing™ or Gene hunting by degenPCR were identified and validated by comparison against known gene sequences from phylogenetically related organisms from the online GenBank and EMBL-EBI gene sequence databases using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1990). In all cases, a nucleotide BLAST was initially conducted using the MegaBLAST algorithm which is designed for highly similar searches; in most case this was sufficient to produce significant related hits (Altschul et al. 1990). If the

MegaBLAST algorithm did not produce significant hits then the discontiguous MegaBLAST algorithm was used which allows for a degree of sequence base-pair mismatching (Altschul et al. 1990).

Further, translated nucleotide sequences, for nucleotide sequences producing significant hits, were compared to online protein databases, using the BLASTx search algorithm (Altschul et al. 1990). Importantly, putative sequence identities were accepted if corresponding nucleotide and protein sequences were identified, and the protein fell within the correct protein family or domain. Putative protein family and domain identities were resolved using Pfam-A and Pfam-B databases using hidden Markov models as part of the BLASTx search tool (Bateman et al. 2004), and the conserved domain database (CCD) (Marchler-Bauer et al. 2011).

Nucleotide, and translated-nucleotide (protein), sequences producing significant hits were then aligned to published sequences using ClustalW and Clustal Omega computer programs that produce pairwise alignments (Higgins and Sharp 1988). Standard user parameters of gap opening penalty, and gap extension penalty were used in all cases (Higgins and Sharp 1988). Multiple alignments were used to identify conserved regions within each obtained putative gene fragment.

Finally, putatively gene coding fragments were analysed for open reading frames (ORFs) using the ExPASy translate tool designed by the Swiss institute of Bioinformatics (SIB) freely available online at "http://www.expasy.org" (Artimo et al. 2012). The mRNA sequence was translated to a protein sequence online and analysed for start and stop codons in each of the three possible frames (frame 1, 2 or 3) and in both directions (5' to 3', and 3' to 5'). Long, uninterrupted ORFs were then compared to online protein sequences by BLASTP (Altschul et al. 1990).

Below is the evidence of identity for each of the genes of interest and reference genes identified by Genefishing™ (Section 2.6.2) or Gene-hunting by degenPCR (Section 2.6.1);

2.7.1 *Palaemonetes varians*

2.7.1.1 *Narg* gene

A 181-bp differentially expressed mRNA fragment was obtained from GeneFishing™ differential display. Specifically, derived from the ACP 5 primer mix in kit 101 (Seegene, supplied by BioGene, UK). The fragment was gel-purified and extracted using a QIAquick

Gel Extraction Kit (Qiagen, UK). The sequence was determined using conventional dideoxy-termination sequencing by Source BioScience (Nottingham, UK);

```
TTTGGTGACT CTTTAAGAAA GTGCCATGAA GTAGATCGAC ANTTCNAGGA GATAATCGAG        60
GACCAATTCG ACTTCCACAC ATACTGTATG AGAAAGATGA CGTTAAGATC TTATGTTGAG       120
CTGCTTAGGT TAGAAGATGT CTTGAGGAAT AATAGGTTTT ATTGGGATGC TGCAAGGACA       180
G                                                                       181
```

The 181-bp differentially-expressed fragment (EMBL-EBI accession number – FR667656) was translated as a putative N-methyl-d-aspartate (NMDA) receptor-regulated protein that shared an 81.8% amino acid identity with the NMDA receptor-regulated protein (*narg* gene) of the mosquito *Culex quinquefasciatus* (XP_001845196.1).

The amino acid sequence was also identified as within a predicted NMDA receptor-regulated protein 1 domain (Pfam-A domain prediction).The derived amino acid sequence contained an extended open reading frame (ORF) in the 5'-3' frame 1 reading direction. Again, this ORF produced significant hits against N-methyl-d-aspartate (NMDA) receptor-regulated protein sequences.

These sequences were aligned alongside our putative fragment using the online Clustal Omega computer program in a pair-wise alignment (Appendix A1.2.1).

2.7.1.2 *Arf* gene

A 314-bp differentially-expressed mRNA fragment was obtained from GeneFishing™ differential display. Specifically, derived from the ACP 20 primer mix in kit 101 (Seegene, UK)

```
ATCCGTCCCC TGTGGANGCA TTACTTCCAG AACACCTCAG CCATAATCTT CGTCGTCGAC        60
AGCAACGACC CTCAGAGACT GGCAGAGGCG AAGGAGGAAC TAGAAATCTT GGACGAGGAT       120
AAAGATCTCG AGAGCTGCCC TCTGCTCATC ATGGCCAACA AGCAGGACCT TCCGCAAGCT       180
GCCTCGCCGT CTTTCATCAC AAACGCCCTC AACCTCCGAA ACCTGAGGCG CACCTGGTTT       240
GTCCAGGGAA CCTGTGCCGT CAATTCCACT GGCATCTACA AGTCCCTTGA CTGGCTGGCC       300
AAGGAAGTTT CGAA                                                        314
```

The 314-bp differentially-expressed mRNA fragment (EMBL-EBI accession number – FR667657) was translated as a putative ADP ribosylation factor that had a 55.8% amino acid identity to the ADP-ribosylation factor 4 (*arf gene*) of the cattle *Bos Taurus* (Q3SZF2.3).

The amino acid sequence was identified as within a predicted ADP ribosylation factor family domain (Pfam-A domain prediction).The derived amino acid sequence contained an extended open reading frame (ORF) in the 5'-3' frame 1 reading direction. Again, this ORF produced significant hits against ADP ribosylation factor protein sequences. These sequences were aligned alongside our putative fragment using the online Clustal Omega computer program in a pair-wise alignment (Appendix A1.2.1).

2.7.1.3 *Cs* gene

A 380-bp mRNA fragment was obtained from Gene-hunting by degenPCR.

```
TGATCACGAG GGTGGTAATG TCTCTGCTCA TACAACACAT CTTGTTGGGT CTGCCTTGTC        60
TGATCCCTAC CTCTCATTTG CTGCTGGTAT GAATGGACTT GCTGGACCAT TGCATGGCTT       120
AGCCAACCAG GAGGTACTCC TTTGGTTAAC CAAGCTACGG TCTGATATTG GCGATGATGT       180
AACAGAAGAT CAACTGAAGG AGTTTATTTG GAAGACCCTG AAATCAGGCC AGGTTGTTCC       240
AGGCTATGGA CATGCTGTCT TGCGTAAAAC TGATCCAAGG TACACTTGTC AGCGAGAGTT       300
TGCTCTCAAG CATCTTCCTG ATGACAAAAT GTTCAAGAAT TGGGTAAGGT GAAGAACCCA       360
TGGCCCAACG TAGACGCCCA                                                  380
```

The 380-bp mRNA fragment (EMBL-EBI accession number – LN713461) was putatively identified as coding for the citrate synthase protein (*cs* gene), and shared a 74% nucleotide identity with the *cs* gene of the sea urchin *Psammechinus miliaris* (FN677804.1). The fragment was identified as within a predicted citrate synthase GltA domain (Pfam-A domain prediction). The derived amino acid sequence contained an extended open reading frame (ORF) in the 5'-3' frame 2 reading direction. Again, this ORF produced significant hits against citrate synthase sequences. These sequences were aligned alongside our putative fragment using the online Clustal Omega computer program in a pair-wise alignment (Appendix A1.2.1).

2.7.1.4 *Ldh* gene

A 503-bp mRNA fragment was obtained from Gene-hunting by degenPCR.

```
TCGCCGTGTT CGCCGATGAT CCAGCCGTGA GTAGAGGATG GTGCTACGTT GAGTTTCTGA        60
GATAAGTGGA ATCTGAATCG AGCAGAGTCC AGATTGGTTC CAGATCCGAT GACACGGTGC       120
TTGGGCAGGC CAGACAATTT CCAGGCCACG TAGGTCAAAA CATCAACAGG GTTGGACACA       180
ACAAGCAGGA TGCAGTTAGG GGAGTGCTTG ACTAAGTTGG GAATGATGCC CTTGAAGATG       240
TCCACGTTTC TCTGGACAAG GGAAAGGCGA GATTCTCCCT CCCTCTGGCG AGCACCAGCA       300
GTCACGATGC AAACTCGAGA ACCTGCAGTC ACGGAGAAGT CCGTGCTAGC CTCGATTTTC       360
ACATTCCTGA GGAAGGTGAG ACCATGCTGG AGGTCCATCA TCTCGCCCCT GAGCTTGTCA       420
GCCATAACGT CAACAAGGGC CAGTTCGGAG CAAATGTGTT GGGTAAGAAG AGAGAAAGCG       480
CAGGCCATAC CCACCTGCCC CCC                                               503
```

The fragment (EMBL-EBI accession number – LN713462) was putatively identified as coding for the lactate dehydrogenase protein (*ldh* gene), and shared a 97% amino acid identity with the *l-ldh* gene of the Hawaiian volcano shrimp *Halocaridina rubra*. The fragment was identified as within a predicted lactate dehydrogenase (L-LDH) domain (Pfam-A domain prediction). The derived amino acid sequence contained an extended open reading frame (ORF) in the 3'-5' frame 1 reading direction. Again, this ORF produced significant hits against lactate dehydrogenase sequences. These sequences were aligned alongside our putative fragment using the online Clustal Omega computer program in a pair-wise alignment (Appendix A1.2.1).

2.7.1.5 *Rps26* gene

A 263-bp constitutively-expressed mRNA fragment was obtained from GeneFishing™ differential display.

```
GGCTATCAAG AAATTCCAGA TCCGTAATAT TGTTGAAGCA GCAGCAGTAA AGGATATTAA        60
TGAGGCCTCA GTGTATCAGG TGTACCAGCT GCCCAAGTTG TACATCAAAC AACATTACTG       120
CGTCTCATGT GCTATCCATT CCAAGGTCGT TAGGAATCGC AGCCGACGTG ACAGGAAGAT       180
TAGAACTCCA CCACCACGTT TCCCAAGGGC TCAGGAGCAA CGTCCACGCA ACCCATAAGC       240
TTGAAATATA GTGGAAATTG AAA                                               263
```

The 263-bp mRNA constitutively-expressed fragment (EMBL-EBI accession number - FR667658) was putatively identified as the ribosomal protein S26 (*rps26* gene), and shared a 75.9% amino acid identity with the *rps26* gene of the starlet anemone *Nematostella vectensis* (XP_001641441.1). The fragment was identified as within a predicted ribosomal s26e family domain (Pfam-A domain prediction). The derived amino acid sequence contained an extended open reading frame (ORF) in the 5'-3' frame 2 reading direction. Again, this ORF produced significant hits against ribosomal protein S26 sequences. These sequences were

aligned alongside our putative fragment using the online Clustal Omega computer program in a pair-wise alignment (Appendix A1.2.1).

2.7.1.6 Genes obtained from published research

In addition to the genes described above, several potential genes of interest in physiological studies had been previously identified in *P. varians*. Cottin et al. (2010) isolated two *hsp70* gene isoforms (FJ875280.1 and FJ875279.1) in *P. varians*, and quantified their expression across a variety of temperature shock treatments. Results suggested that one of the isoforms was stress-inducible, and the other was non-inducible but constitutively expressed (Cottin et al. 2010). These genes were deemed to be of interest in the physiological experiments described in Chapters 3, 4, and 5. Further, expression sequence tags for the *β-actin* gene (FJ654525.1) and the *rpl8* gene (GQ120564.1) were identified in *P. varians* by Cottin et al. (2010) and were used in that study as reference genes of qPCR expression analysis. These two genes were also included in the experiments of Chapters 3, 4, and 5, and tested in each case for their suitability as reference genes for expression analysis.

2.7.2 *Lithodes maja*

2.7.2.1 *Narg* gene

A 1206-bp mRNA fragment was obtained from Gene-hunting by degenPCR.

```
TGCTGGCATG TGTAYGGGCT CCTCCAACGC TCGGACAAGA AGTACGACGA AGCCATCAAG        60
TGTTATCGTA ACGCCTTGAA GTGGGACAAA GATAACCTAC AGATTCTCCG AGATCTGTCC       120
TTGCTACAGA TCCAGATGAG AGATTTGGAT GGCTATAAGG ATACGCGTTA CAAGTTATTT       180
GAGCTGCGGC CGACCCAGAG AGCTTCCTGG ATCGGATACG CCATGTCCTT CCACCTCCTC       240
CATAACTACG ACACTGCCTT GAAGATTTTA GAAGAATTTA GGAAAACAAT TCAGAAAACT       300
ACGTATGATT ATGAATACAG TGAACTATTG CTGTATCAGA ACTTAGTGAT CCGGGAGAGC       360
AAGAACTTAC TGGAAGCCTT GAAGCATCTG GACACCTATG AAGCCAATAT CTGTGATAAG       420
GTCACTCTAC AAGAACTGAG AGGGGAGTTA TTAATGAAGT TGGGTCGACG GGAGGCGGCG       480
TCGGAGGTTT ACCGCGGCCT GATCAAGAGG AACCCCGAGA ACCGCGACTA TTACCTTAAG       540
TTGGAAGACG CCCTCGCCCT TAACACTGTC CACCAAAGAC TACACCTCTA TGATGAACTC       600
AAAGAAAAGT TCCCTCGTGC TCAAGTGCCT AAGCGTCTGC CGCTGAACTA CGCCCACGGA       660
CCCGAGTTAG AATCCCTGCT GGACCCGTAC CTACGAGCGG CGTTACAGAA GGGCGTACCA       720
CCTCTCTTTA CCGACATACA GAGTCTATAC ACCGAGCCCG ACAGAATACT GATCGTCCAA       780
AACCTCATGG AGAGATATCT TAAGAATCTG GAGGAGACGG GACATTTGTG TCAATCAGAT       840
AACGAGTACA AGGAACCCGC GTCTGTTCTG TTCGTGCTGA TGTTCTTGGC CCAACATCAC       900
AGCTATCTGG GAGACACGGA GAAAGCTCTA TCCCTCATAG AGCGAGCCTT AGAGCACACA       960
CCCACGCTAA TAGAGCTTTA TATTGTTAAA GGAAAAATAT TGAAGCACGC GGGTGATTAT      1020
GTGGGAGCGT ACGAGGCGTT GCAGGAAGCC CAGGCGTTAG ACACGGCCGA TCGCTACGTT      1080
AACTCCAAAG CCGCCTCCTA CCTAATCAAA GCCAACCTAG TCAGACAGGC CGAGGAAATG      1140
TGCTCCAAGT TTACCAGAGA AGGAGTGTCG GCGATGGAGA ACCTGAACGA AATGCAATGC      1200
ATGTGG                                                                 1206
```

The 1206-bp mRNA fragment (EMBL-EBI accession number – LN713465) was translated as a putative N-methyl-d-aspartate (NMDA) receptor-regulated protein 1 that shared a 62% amino acid identity with the termite *Zootermopsis nevadensis* (E9IP68). The fragment was identified as within a predicted NMDA receptor-regulated protein 1 (Pfam-A domain prediction). The derived amino acid sequence contained an extended open reading frame (ORF) in the 5'-3' frame 1 reading direction. Again, this ORF produced significant hits against NMDA receptor regulated protein 1 sequences. These sequences were aligned alongside our putative fragment using the online Clustal Omega computer program in a pair-wise alignment (Appendix A1.2.2).

2.7.2.2 *Cs* gene

A 423-bp mRNA fragment was obtained from Gene-hunting by degenPCR.

```
TGGGCGTCTA CGTTGGGCCA GGGGTTCTTT ACCTTGCCCA GTTCGGTCAG GATGGGTGGC      60
ACAACGTTGT ATAGTTTGGC AACCAGCTTG AACAGTTTGT CGTCAGGGAG ATGCTTAAGG     120
GCAAACTCTC GCTGGCAGGT GTATCTGGGG TCAGTCTTCC TCAGCACAGC GTGACCATAG     180
CCGGGCACCA CCTGGCCGGA CTTGAGGGTC TGCCAGATAA ACTCCTTCAG CTGATCCTCA     240
GTCACTTCAT CGCCAATCTC TGAACGCAGC CTGGTCACCC ACATTAGCAC CTCCTGGTTA     300
GCCAAGCCGT GGAGAGGACC AGCCAGACCG TTCATGCCAG CAGCGAAGCT GAGGTAAGGG     360
TCGGAGAGAG CCGACCCAAC CAAATGTGTC GTATGGGCTG ATACGTTDCC ACCCTCATGA     420
TCA                                                                  423
```

The 423-bp mRNA fragment (EMBL-EBI accession number – LN713466) was translated as a putative citrate synthase protein that 86% amino acid identity with the beetle *Dendroctonus ponderosae* (J3JV43). The fragment was identified as within a citrate synthase GltA domain (Pfam-A domain prediction). The derived amino acid sequence contained an extended open reading frame (ORF) in the 3'-5' frame 2 reading direction. Again, this ORF produced significant hits against citrate synthase protein sequences. These sequences were aligned alongside our putative fragment using the online Clustal Omega computer program in a pair-wise alignment (Appendix A1.2.2).

2.7.2.3 *Hsp70a* gene

A 485-bp mRNA fragment was obtained from Gene-hunting by degenPCR.

```
AGCTATGCAT CCAACGCGTT GGGAGCTCTC CCATATGGTC GACCTGCAGG CGGCCGCGAA        60
TTCACTAGTG ATTCCCCGAT CTTTGGTGGC TTGACGCTGG GAGTCGTTGA AGTAGGCTGG       120
GACGGTGATC ACAGCATCTT TGACTATGGA CCCAAGGTAG GCCTCTGCTG TTTCCTTCAT       180
CTTGATTAGC ACCATGGATG AGATTTCCTC TGGGTAAAAT GATTTCTTCT CTCCCTTGTA       240
CTCTACTTGA ATCTTTGGTT TTGTGCTGTC ATTAATTACA TCGAAAGGCC AGTGCTTCAT       300
GTCACTCTGA ACGTGGTGGT CCTCAAATTT GCGGCCAATC AGTCTCTTGG CATCAAAGAC       360
AGTGTTGTTG GGGTTCATGG CTACTTGGTT CTTGGCAGCA TCTCCAATGA GACGCTCTGT       420
GTCTGTGAAG GCCACATAGG AAGGAGTGGT GCGGTTTCCC TGATCGTTGG CATGATGATC       480
TCCAC                                                                   485
```

The 485-bp mRNA fragment (EMBL-EBI accession number – LN713468) was translated as a putative heat shock 70kDa protein shared a 93% amino acid identity with the crab *Goniopsis cruentata* (M4PVJ8). The fragment was identified as within an hsp70 DnaK domain (Pfam-A domain prediction). The derived amino acid sequence contained an extended open reading frame (ORF) in the 3'-5' frame 3 reading direction. Again, this ORF produced significant hits against heat shock 70kDa protein sequences. These sequences were aligned alongside our putative fragment using the online Clustal Omega computer program in a pair-wise alignment (Appendix A1.2.2).

2.7.2.4 *Hsp70b* gene

A second 485-bp mRNA fragment was obtained from Gene-hunting by degenPCR.

```
AGCTATGCAT CCAACGCGTT GGGAGCTCTC CCATATGGTC GACCTGCAGG CGGCCGCGAA        60
TTCACTAGTG ATTCCCCGGT CTTTAGTTGC CTGTCGCTGG GAGTCATTAA AATATGCTGG       120
CACTGTGACC ACAGCATCCT TCACAGATAT GCCTAAGTAC GCCTCGGCCG TTTCCTTCAT       180
CTTTATAAGC ACCATGGATG AAATCTCTTC GGGAAAGAAC TTTTTATTCT CTCCCTTATA       240
CTCCACAACG ATCTTTGGCT TGCCACCTTC ATTGACGACA GTGAATGGCC AGTGCTTCAT       300
GCCACCTTGC ACCGTTGTGT CTTCGAACTT GCGGCCGATA AGTCTCTTGG CATCAAAGAC       360
AGTGTTGTTG GGATTCATGG CCACCTGGTT CTTGGCGGCG TCCCCAATGA GACGCTCGGT       420
GTCGGTGAAG GCCACATAGG AGGGCGTGGT GCGGTTACCC TGATCATTGG CATGATGATC       480
TCCAC                                                                   485
```

The 485-bp mRNA fragment (EMBL-EBI accession number – LN713469) was translated as a putative heat shock 70kDa protein shared an 88% amino acid identity with the crab *Cancer pagurus* (B7ZEC1). The fragment was identified as within an hsp70 DnaK domain (Pfam-A domain prediction). The derived amino acid sequence contained an extended open reading frame (ORF) in the 3'-5' frame 3 reading direction. Again, this ORF produced significant hits against heat shock 70kDa protein sequences. These sequences

were aligned alongside our putative fragment using the online Clustal Omega computer program in a pair-wise alignment (Appendix A1.2.2).

2.7.2.5 Identification of *hsp70* isoforms

The nucleotide and translated amino acid sequences for both putative HSP70 isoforms (*hsp70a* and *hsp70b*) were aligned and showed distinctiveness in both nucleotide and protein sequences (Appendix A1.3). Their identity as distinct isoforms of the HSP70 gene was further confirmed by relative expression analysis, where each isoform showed differential expression patterns, as described in Chapter 6.

2.7.2.6 *Eef1α* gene

A 947-bp mRNA fragment was obtained from Gene-hunting by degenPCR.

```
GGGGAGTTTG AAGCTGGTAT CTCCAAAAAC GGACAGACCC GTGAACACGT ACTGCTCTGC      60
TTCACCCTTG GTGTAAAGCA GCTCATCGTG GCTGTCAACA AGATGGACAG CACAGAACCA     120
AAATTCTCAG AGGCCAGGTT CCAGGAAATT AAGAAGGAAT TGACTACTTA TGTAAAGAAG     180
GTTGGCTACA ACCCTACCAT TGTGCCAATC CTTCCCATCT CTGGCTTCAA TGGAGACAAC     240
ATGCTGGAGA AGTCTGATCA CATGCCCTGG TGGACAAAGG CAAAGATTGA ACGCAAGAGC     300
GGCTCCTACG AGTTCACCAC ACTCTTCGAG GCCCTGGACA ACATCGAGCC CCCTAGCAGA     360
CCTGTTGACA AGGCCCTCCG TCTTCCTCTC CAGGATGTGT ACAAGATCGG TGGTATTGGA     420
ACAGTGCCCG TGGGTCGTGT TGAGACTGGT ATTCTGAAGC CCGGCATGGT GGTGAACTTT     480
GCCCCTACTG GCCCCACCAC TGAGGTAAAG TCTGTAGAGA TGCACCACGA AGCTCTGACC     540
GAGGCATGCC CTGGTGACAA CGTTGGCTTC AACGTGAAGA ACGTGGCAGT CAAGGATCTG     600
AAGCGTGGTT TCGTTGCCTC TGACTCTAAG AACGATCCCG CCAGGGAGGC CGGGGACTTC     660
ACCGCCCAGG TCATTGTGCT CAACCACCCT GGCCAGATCC AGCCTGGTTA CTCTCCGGTG     720
CTGGATTGCC ACACTGCTCA CATTGCCTGC AAGTTTGCCG AGCTAATCCA GAAGATCGAC     780
AGGCGTACTG GTAAGGAGAT TGAGGCCAAC CCCAAGGTTA TTAAGTCTGG AGACTCTTGT     840
ATCGTCAAGA TGATTCCCAG CAAGCCTATG TGTGTGGAGA CCTTCCAGAA GTACGCTCCC     900
CTCGGAAGGT TTGCTGTGCG TGACATGAAG CAGACTGTGG CAGTAGG                   947
```

The 947-bp mRNA fragment (EMBL-EBI accession number – LN713464) was translated as a putative elongation factor 1-alpha (EEF1α) that shared a 96% amino acid identity with the crab *Macrophthalmus japonicas* (B9ZZQ1). The fragment was identified as within the elongation factor 1-alpha TEF1 domain (Pfam-A domain prediction). The derived amino acid sequence contained an extended open reading frame (ORF) in the 5'-3' frame 1 reading direction. Again, this ORF produced significant hits against elongation factor 1-alpha sequences. These sequences were aligned alongside our putative fragment using the online Clustal Omega computer program in a pair-wise alignment (Appendix A1.2.2).

2.7.2.7 *Rpl8* gene

A 449-bp mRNA fragment was obtained from Gene-hunting by degenPCR.

```
GTGCATTTCA GTGACCCCTA CCGCTACAAG ACCCGGAAGG AGCTCTTCCT GGCTGCTGAG        60
GGCATGTACA CCGGACAGTT CATCTACTGT GGGAAGAAGG CCAACCTGGA TGTGGGCAAC       120
GTAATGCCCA TTGGCACCCT GCCTGAGGGT ACCGTCATCT GCAACCTTGA GGAGAAGACC       180
GGTGACCGTG GCCGTATTGC TCGTGGCTCC GGGAACTATG CTCAAGTAAT TGCCCACAAC       240
CCGGAGACAA AGAAGACCCG TGTGAAGCTG CCGTCCGGTG CCAAGAAGGT CCTTCCCTCA       300
GCCAACCGTG CAATGATTGG TATTGTTGCT GGTGGCGGCC GTATTGACAA ACCCATCCTG       360
AAGGCTGGTC GTGCGTACCA CAAGTACCGC GTGAAGCGTA ACAGCTGGCC CAAGGTGCGT       420
GGTGTGGCCA TGAACCCCGT RGAGCATCC                                         449
```

The 449-bp mRNA fragment (EMBL-EBI accession number – LN713464) was translated as a putative ribosomal protein L8 that shared a 94% amino acid identity with the shrimp *Litopenaeus vannamei* (Q2I3E8). The fragment was identified as within RPL domains (Pfam-A domain prediction). The derived amino acid sequence contained an extended open reading frame (ORF) in the 5'-3' frame 1 reading direction. Again, this ORF produced significant hits against ribosomal protein L8 sequences. These sequences were aligned alongside our putative fragment using the online Clustal Omega computer program in a pair-wise alignment (Appendix A1.2.2).

2.8 Quantitative PCR (qPCR)

2.8.1 qPCR instruments

2.8.1.1 Rotor-gene 3000

The Corbett Rotor-Gene 3000 (Corbett Life Sciences, Qiagen, UK) is a multi-filter, real-time thermo-cycling analyser. The Rotor-Gene 3000 system was used for the experiments described in Chapters 3 & 5. Uniquely, instead of the common 96-well system, the Rotor-Gene 3000 has a 72-well rotor that spins at 500 rpm. This rotor is housed inside an air-locked temperature chamber. The advantage of this spinning rotor system is that there is no inter-well temperature variation, a common shortfall in 96-well plate systems. qPCR assays run on the Rotor-Gene 3000 were initially analysed using the Rotor-Gene Q series software version 2.0.2.

2.8.1.2 Agilent MX Pro 3005

The Agilent MX Pro 3005 is a 96-well plate compatible multi-filter, real-time thermo-cycling analyser. The MX Pro 3005 system was used for experiments described in Chapters 4 & 6. This system was used during a period of maintenance of the Rotor-Gene 3000 instrument. Plate style thermo-cyclers come with the inherent problem that heat from the heating/cooling element is not distributed exactly equally between each of the 96-wells, unlike rotor based systems where measurements of different tubes are taken in the same location. Although this inter-well temperature variation is likely to be very small, qPCR is a highly sensitive technique, and slight differences in reaction conditions can yield significant differences in reaction efficiency, and ultimately the overall result. Because of this, ROX® reference dye (Life Technologies, UK) was added to each reaction to account for any inter-well variation as a result of differential reaction conditions (1 µl in every 25 µl reaction). qPCR assays run on the MX Pro 3005 system were initially analysed using the MxPro ET qPCR software.

2.8.2 qPCR detection chemistry

Non-specific signal detection chemistry was used in all experiments described in this thesis. SYBR®-Green I intercalating dye is a commonly used dye in qPCR experiments, developed by Molecular Probes Inc. (Thermo Scientific Fisher, UK). The dye binds preferentially to double-stranded DNA, rather than single-stranded DNA (Zipper et al. 2004). The resulting combination of dye and DNA absorbs blue light and emits green light at λ_{max} = ~520nm. The change in fluorescence intensity of SYBR®-Green I during a PCR reaction is proportional to the DNA concentration of the mixture. Non-specific DNA-binding dyes, such as SYBR®-Green I, are comparatively simpler to use than specific, probe-based dyes and are a significantly cheaper option (Bustin and Nolan 2004). The only major limitation of non-specific dyes in comparison to specific dyes is that it is not possible to differentiate between the target DNA of interest and non-specific double-stranded DNA products such as genomic DNA (gDNA) contamination, and the formation of primer-dimers. Both gDNA contamination and primer-dimer formation can be minimised by *a priori* primer optimisation (Section 2.8.5), melt curve analysis (Section 2.8.4), and DNase-treatment (Section 2.5.2).

2.8.3 qPCR primer design

qPCR specific primers were designed from sequences obtained from GeneFishing™ and Gene-hunting by degenPCR. qPCR primers must be highly specific to the mRNA that is being targeted, must also facilitate a highly efficient (exponential) PCR reaction, and must minimise primer-dimer formation. qPCR specific primers were designed using Primer Express® software (Applied Biosystems, UK) according to the manufacturers' standard guidelines. Primers were designed according to the following rules; a minimum length of nine bases, and a maximum length of 40 bases; a melting temperature of between 58°C and 60°C; a guanine-cytosine content of between 30-80%; a maximum of two out of five G/C bases in the last five bases at the 3' end; and an amplicon length of between 80-150 bases. The m-fold web server (http://mfold.rna.albany.edu) was also used to determine whether primer sequences were designed over areas of mRNA secondary structures including hairpins, or cross-dimers. Secondary structures can significantly negatively affect qPCR reaction efficiency. Areas of high likelihood of secondary structure in the mRNA fragment of interest were avoided during primer design. Once designed, primer sequences were synthesised by Eurofins MWG Synthesis GmBH (Germany). Lyophilised primers were rehydrated in nuclease-free water to a concentration of 100 µM, and then further diluted to 5 µM and stored a -20 °C.

2.8.4 Melt curve analysis

Designed primer pairs were initially tested using a target sample at 1 ng/µl cDNA in a 25 µl reaction with 300 nM of both forward and reverse primers. These samples were run in duplicate (technical replicates). The same reactions were set up without the target cDNA, as no template control (NTC) reactions. Following the full standard qPCR reaction (Section 2.8.6), a melt curve analysis was performed consisting of melting all qPCR derived products at 95°C, annealing them at 60°C, and then exposing them to increasing temperature in a step-wise fashion from, 72°C to 95°C, to determine the point of dissociation of all double stranded products in each reaction mix. This dissociation point is seen as the change in fluorescence of the SYBR®-Green I intercalating dye as the double-stranded DNA begins to dissociate. By plotting the negative first derivative (-df/dt) of the standard melting curve (fluorescence over temperature), the appearance of a single peak can be used to determine that a single distinct amplicon is being produced by the reaction. By measuring the dissociation of double-stranded DNA against increasing temperature,

amplicon length, G/C content, and complementarity can be inferred. For melt curve analysis, a single peak at a single temperature across all samples of the same target is sufficient to infer primer specificity.

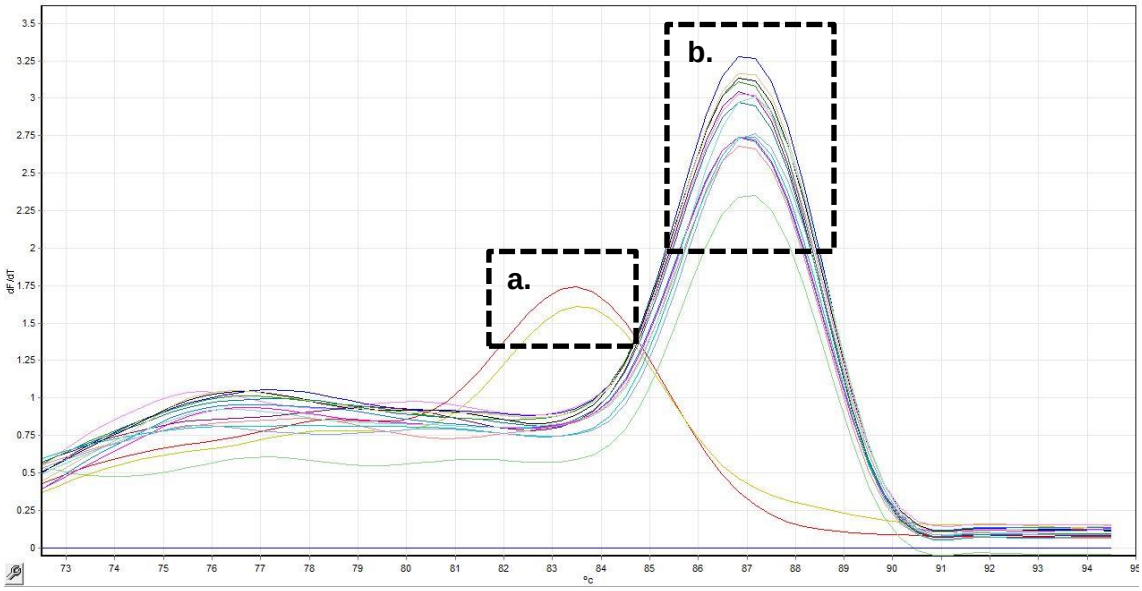


Figure 2.10 Example melt curve produced by the Rotor-Gene™ 3000 software showing the negative first derivative (-df/dt) of the standard "fluorescence over temperature" melt curve. **a.** shows the melt curve of a no template control (NTC) run in duplicate: the peak representing primer dimer formation; **b.** shows a single distinct melting peak for a set of samples using optimised *gapdh* primers.

2.8.5 qPCR primer optimisation

Primer concentrations in the qPCR mixture significantly affect the efficiency of the reaction. In order to optimise reaction efficiency, determining optimal primer concentration is vital. However, reaction efficiency is not the only consideration when determining primer concentration, primer-dimer formation and non-specific binding at high concentration is also of importance. In order to quantify the best forward and reverse primer concentration for each gene of interest (GOI), a primer concentration matrix run was performed. This run consisted of running a single target sample at single concentration, but a varying FWD and REV primer concentrations, as according to Table 2.2.

Table 2.2 Primer concentration matrix to determine optimal concentrations of both forward and reverse qPCR primers

	Forward primer conc. (nM)		
Reverse primer conc. (nM)	**50**	**300**	**900**
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

The best primer concentration mix was determined for each GOI and reference gene according to the maximum obtained Cq value difference between corresponding positive template samples and no template control (NTC) samples. Full primer optimisation results, including primer concentration values, and primer sequences are shown in Table 2.3 & Table 2.4.

The next stage in primer optimisation is to test the sensitivity and linear dynamic range of efficient performance of the primer pairs. An 8-fold dilution series was performed at a starting concentration of 2 ng/µl of cDNA template. This dilution series was run in a standard qPCR reaction with the optimised primer concentrations for each GOI and reference gene. These samples were run in duplicate (technical replicates), and a single NTC (duplicated) was included for reference. The obtained Cq values were plotted against log (concentration) to give a standard curve. Dilutions that had Cq values within 3.3 cycles of the NTC were discarded. Amplification efficiency (*E)* of the reaction was determined according to the slope of a linear regression line of the standard curve (Pfaffl, 2004), as described in equation 2.6;

Equation 2.6:

$$E = \left(10 - 1^{/slope}\right) - 1$$

Amplification efficiencies of between 0.9 - 1.1 are generally accepted as highly efficient in qPCR studies (Pfaffl 2004; Bustin et al. 2009; 2010). All primer-pairs used in this thesis were optimised to within a 0.9 – 1.1 efficiency range, as shown in Table 2.3 & Table 2.4. The correlation co-efficient (R^2) of the linear regression was also assessed in order to determine the goodness-of-fit of the model. R^2 values of greater than 0.97 were deemed to be of good enough fit to accurately represent the data for amplification efficiency

determination (Bustin et al. 2009) and are shown for each GOI and reference gene in Table 2.3 & Table 2.4. Standard curves for all primer pairs are shown in Appendix A1.4.

2.8.6 Relative expression analysis

Once primer pairs for all GOIs and putative reference genes had been optimised according to Bustin et al. (2010), qPCR runs were designed to cover all genes and samples, in a sample-maximisation style strategy (Hellemans et al. 2007). Sample-maximisation involves designing runs were the emphasis is placed on maximising the number of samples for a single gene in a run. This is opposed to the gene-maximisation strategy where the number of genes rather than samples is maximised in a single run. Sample maximisation reduces the potential for primer pair contamination within a single run, and also facilitates simple reaction setups using master mixes, thus reducing the potential for human error. For experiments where all samples do not fit into a single run, and inter-run calibrator (IRC) is used. The IRC can be any cDNA similar to the samples, and is the same cDNA source for each run, which is later normalised against temporal variation between runs. For each qPCR run, an initial master mix was set up consisting of multiples of individual duplicate reactions. The reaction and reagents were kept on ice at all times, and all stages of the reaction were setup in a laminar-flow cabinet to avoid airborne contamination. Nuclease-free water was added to 2x PrecisionPlus qPCR master mix with premixed SYBR® Green I dye (PrimerDesign, UK). Primer pairs were then added to the master mix, and the reaction was mixed thoroughly by pipetting. Finally, sample cDNA was added to each of the duplicate master mixes, which was subsequently split into individual wells ready for analysis. All qPCR reactions were subjected to standard conditions (Figure 2.11).

Once each qPCR run was complete, the normalised fluorescence of each sample was plotted against its corresponding cycle number. A threshold line was then drawn just above the highest normalised fluorescence of any sample within the first 5 cycles, where changes in normalised fluorescence represent signal noise rather than an increase in double stranded DNA. At the point where each samples fluorescent signal crosses the threshold line, after the single noise region, the cycle number of that point is taken and called the quantification cycle or "Cq". The Cq value of each sample is determined as the arithmetic mean of the two Cq values of its technical replicate, and it is this value that is used for further analysis (Figure 2.12).

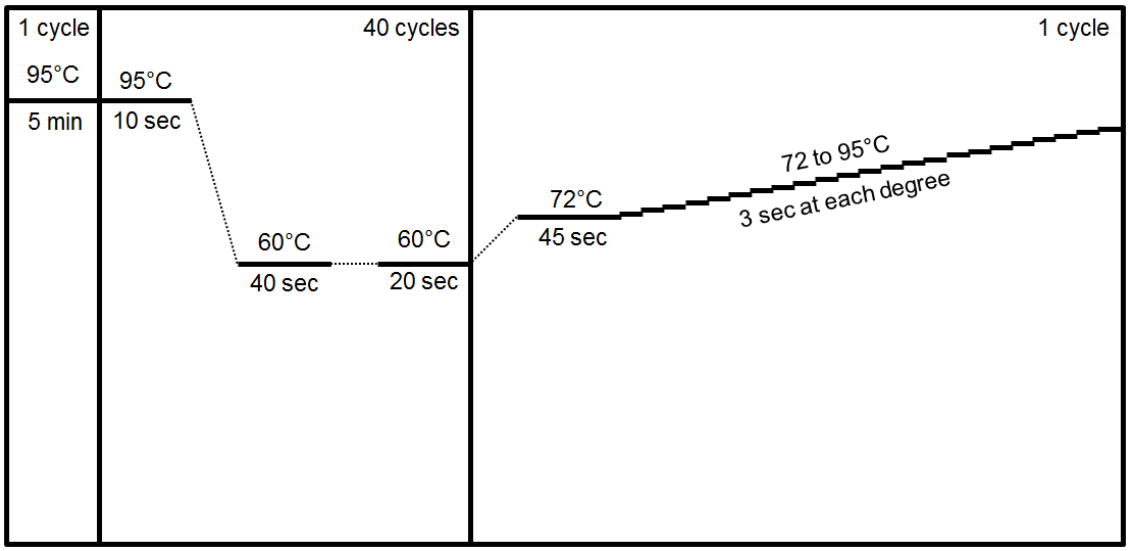


Figure 2.11 Standard qPCR reaction profile, with step temperatures and timings, and the number of cycles per segment

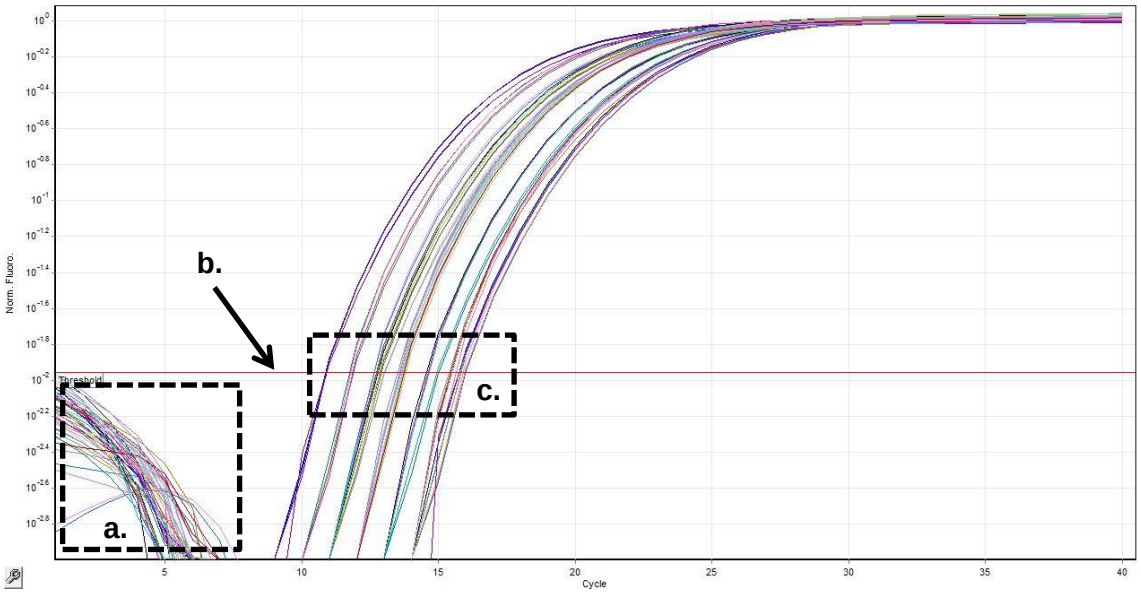


Figure 2.12 Example fluorescence curve from the *gapdh* gene produced by the Rotor-Gene™ 3000 software showing normalised fluorescence over reaction cycle number: from 1 to 40. **a.** shows the region of noise where fluorescence fluctuates at the start of the reaction independently of double strand DNA concentration. **b.** shows the threshold line drawn just above the region of noise from which Cq values are obtained. **c.** individual samples showing an exponential increase in fluorescence associated with an exponential increase in cDNA, Cq values are taken at the point each sample line crosses the threshold line

A number of initial quality control checks were conducted. Firstly, NTC Cq values were assessed, any positive sample falling within 3.3 Cq values of a corresponding NTC was excluded from further analysis, according to Hellemans et al. (2007), at such a similarity in concentration it would be hard to differentiate a true gene expression signal from primer dimer or contamination signals. Secondly, all samples for each gene within a single run were assessed for amplicon melting point by melt curve analysis. All samples should yield the same melt curve profile, and have a single distinct peak. Any sample that showed a non-uniform melt curve was excluded from further analysis. Finally, the arithmetic mean and standard deviations of duplicate technical replicates were calculated for all remaining samples. Any sample whose duplicate technical replicates were more than 0.33 standard deviation point parts was excluded from further analysis, according to Hellemans et al. (2007), as pipetting error was deemed significant in the final result.

Assessed mean Cq values were exported into qBase+™ software (Biogazelle, Belgium) for further analysis.

2.8.7 qBase+™ software

qBase+™ software (Biogazelle, UK) is a program designed to provide an easy model for determining the relative expression of GOIs from raw qPCR data, namely Cq values. The software employs an updated version of the widely used delta delta Cq method first devised by Livak and Schmittgen (2001) and later improved by Pfaffl (2007). A detailed list of calculations and error propagation equations is published by Hellemans et al. (2007).

The basic principle of the model in the qBase+™ software is that the known difference in Cq value between two samples is transformed into a relative quantity by the exponential function, with reaction efficiency (Section 2.8.5 – Equation 2.6) as its base (Hellemans et al. 2007). This relative quantity is then normalised to the geometric means of greater than one putative reference gene. Reference gene stability is assessed by geNorm analysis within the qBase+™ program (described below). This will give a normalised relative quantity (NRQ) value for each sample. If samples from a single gene are split over numerous runs then the IRC samples from each run will be used to calibrate all NRQs to the same scale to form calibrated normalised relative quantity (cNRQ) values. Finally, the qBase+™ software allows sample groups (biological replicates from a single treatment) to be scaled to other groups. In this way, treatment groups can be scaled to control groups and the NRQ values

can be quoted as relative fold changes (RFC). Relative fold change is quoted for all gene expression values in this thesis, and is scaled to the corresponding control group for each experiment unless otherwise stated.

2.8.8 geNorm™ software

Relative gene expression analysis relies on stably expressed reference genes in order to differentiate between variation in cDNA concentration between samples, and variation in cDNA expression level. Such reference genes can be chosen *a priori* through knowledge of expression under the experimental conditions or from previously conducted studies, but must be tested and quantified for each new experiment. The generally accepted gold standard for normalisation (Bustin et al. 2009) suggests that maximising the number of reference genes used in the NRQ calculation is preferred. This again, however, is experiment specific. geNorm™ analysis allows any number of putative reference genes to be assessed against GOI expression and will calculate the stability (as an M value) and the coefficient of variation (CV value) for each reference gene in isolation, and then all reference genes in all combinations, and provides the best normalisation strategy from the putative reference genes submitted in the form of the combination or single gene producing the lowest M value and CV%. Generally, M values of lower than 0.5, CVs of less than 25% are considered highly stable, and therefore a good reference strategy (Hellemans et al. 2007). Specific normalisation strategies are listed for each experiment in their corresponding chapters.

2.8.9 Statistical analysis

NRQ values produced by qBase+™ software, were initially analysed using functionality built in to the program. One-way analysis of variance (ANOVA) was used alongside *post-hoc* Tukey-Kramer tests to determine significant differences between experimental treatments (Hellemans et al. 2007). However, this functionality was limited in its application, and in some cases raw NRQ values were exported into R statistical software (R core team, 2013) and analysed further. Specific statistical analyses are listed for each experiment in their corresponding chapters.

Table 2.3 List of; primer sequences, optimised primer concentrations, primer pair efficiency (E), the dynamic range over which efficiency was achieved, and the coefficient of determination (R^2) for all genes used to study *Palaemonetes varians*

Gene ID	Primer	Final conc. (nM)	Sequence	Primer efficiency (E)	Dynamic linear range of E (orders of magnitude)	R^2
Narg	Fwd	300	AGA AAG TGC CAT GAA GTA GAT CGA	1	5	0.99
	Rev	300	AAC CTA AGC AGC TCA ACA TAA GAT CTT			
Arf	Fwd	300	GGC CAA CAA GCA GGA CCT T	1	5	0.99
	Rev	900	TTG AGG GCG TTT GTG AGT AA			
Gapdh	Fwd	300	AAG GGC GCC GAG GTT GTT GCT GTA A	0.91	6.5	0.99
	Rev	300	GCA CCA GCC TTG CTC CAT GGA ATG T			
Hsp70 f1	Fwd	50	CCA GCC GTC ACC ATC CAG GTG T	1.04	5	0.98
	Rev	50	GCG GTC GAT GTC CTC CTT GCT G			
Hsp70 f2	Fwd	300	TTC CTG AGG AGG ATC GCA AA	0.9	6	0.98
	Rev	900	CAT TCC ACC AGG AGG AGC AC			
Cs	Fwd	300	CCT TGT CTG ATC CCT ACC TCT CA	0.99	3.5	0.99
	Rev	300	CAT GCA ATG GTC CAG CAA GT			
Ldh	Fwd	300	TGG GAA TGA TGC CCT TGA A	0.92	4	0.98
	Rev	900	GAA TCT CGC CTT TCC CTT GTC			
β-actin	Fwd	300	ACC AGT GCT GAA CGT GAG ATC G	0.9	6	0.98
	Rev	300	TCT CGT GGA CAC CAG CAG ATT C			
Rps26	Fwd	300	CGA CGT GAC AGG AAG ATT AGA ACT C	0.9	6	0.99
	Rev	900	ATG GGT TGC GTG GAC GTT			
Rpl8	Fwd	900	TCC CGG TCG TGG TGC ACC TAT T	0.92	6	0.99
	Rev	900	GAC GGC CTC GGT CAC CAG TCT TT			

Table 2.4 List of; primer sequences, optimised final primer concentrations, primer pair efficiency (E), the dynamic range over which efficiency was achieved, and the coefficient of determination (R^2) for all genes used to study *Lithodes maja*

Gene ID	Primer	Final conc. (nM)	Sequence	Primer efficiency (E)	Dynamic linear range of E (orders of magnitude)	R^2
Narg	Fwd	300	ACC GCG GCC TGA TCA A	0.97	3	0.97
	Rev	300	CGA GGG CGT CTT CCA ACT TA			
Cs	Fwd	300	TCA GTC ACT TCA TCG CCA ATC T	0.99	4.5	0.98
	Rev	900	TGG CTA ACC AGG AGG TGC TAA			
Hsp70a	Fwd	300	TCT TTG GTT TTG TGC TGT CAT TAA TT	0.99	5	0.99
	Rev	300	GAG ACT GAT TGG CCG CAA AT			
Hsp70b	Fwd	300	TTC ATG GCC ACC TCG TTC TT	0.99	4	0.99
	Rev	300	CCC TCC TAT GTG GCC TTC AC			
Eef1a	Fwd	300	CCT GGT GGA CAA AGG CAA AG	1	4	1
	Rev	300	GCC TCG AGG AGT GTG GTG AAC			
Rpl8	Fwd	300	ACC GTC ATC TGC AAC CTT GAG	0.96	6	0.99
	Rev	300	CCG GAC CCA CGA GCA AT			

Chapter 3

Elucidating molecular responses to pressure & temperature

3. ACUTE COMBINED PRESSURE AND TEMPERATURE EXPOSURES ON A SHALLOW-WATER CRUSTACEAN: NOVEL INSIGHTS IN TO THE STRESS RESPONSE & HIGH PRESSURE NEUROLOGICAL SYNDROME

[Published as: **Morris JP**, Thatje S, Ravaux J, Shillito B, Fernando D, Hauton C (2015) Acute combined pressure and temperature exposures on a shallow-water crustacean: Novel insights into the stress response and high pressure neurological syndrome. Comparative Biochemical Physiology: Part A 181:9-17]

3.1 Abstract

Little is known about the ecological and physiological processes governing depth distribution limits in species. Temperature and hydrostatic pressure (HP) are considered to be two dominant factors. Research has shown that some marine ectotherms are shifting their bathymetric distributions in response to rapid anthropogenic ocean surface warming. Shallow-water species unable to undergo latitudinal range shifts may depend on bathymetric range shifts to seek refuge from warming surface waters. As a first step in constraining the molecular basis of HP tolerance, or intolerance, in shallow water crustaceans, differential gene expression was examined in response to acute HP and temperature exposures in juveniles of the shallow-water shrimp *Palaemonetes varians*. Significant increases in the transcription of genes coding for an NMDA receptor-regulated protein, an ADP ribosylation factor, β-actin, two heat shock protein 70kDa isoforms (HSP70), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were found in response to elevated HP. NMDA receptors have been implicated in pathways of excitotoxic damage to neurons and the onset of high pressure neurological syndrome (HPNS) in mammals. These data indicate that the sub-lethal effects of acute barotrauma are associated with transcriptional disturbances within the nervous tissue of crustaceans, and cellular macromolecular damage. Such transcriptional changes

lead to the onset of symptoms similar to that described as HPNS in mammals, and may act as a limit to shallow water organisms' prolonged survival at depth.

3.2 Pre-text

Initial preliminary GeneFishing™ experiments were conducted at varying temperature and HP exposures in the same species and life stage: juvenile *Palaemonetes varians*. The study was conducted by Daniel Fernando as part of a MSc. research project (Fernando 2010). Fernando (2010) isolated and extracted initial expression sequence tags for the *narg* gene, the *arf* gene, and the *rps26* gene, and assessed their differential expression by conventional PCR and band fluorescence intensity analysis. The results presented by Fernando (2010) became the basis for the initial study of this PhD thesis. The work carried out by Fernando (2010) was repeated and revalidated, and then a new and different experimental setup was designed, and more appropriate/powerful methods were employed to produce a more robust analysis (Morris et al. 2015).

3.3 Introduction

Laboratory-based studies have shown that many shallow-water adapted organisms can tolerate hydrostatic pressures outside of their natural distribution (Brown and Thatje 2013). Studies have also shown that early ontogenetic stages are particularly tolerant of hydrostatic pressure (HP) increases, exceeding that of their related post-larval stages (Tyler and Young 1998; Mestre et al. 2009). Such laboratory studies, however, do not explain the absence of post-larval specimens of these species, or close relatives, in the present deep-sea. This suggests that physiological and/or ecological limitations may be acting to restrict depth ranges into the deep sea by extant shallow-water invertebrates. Research has shown that marine ectotherms are shifting their latitudinal (Southward et al. 1995; Pörtner and Farrell 2008) and bathymetric distributions (Perry et al. 2005; Weinberg 2005; Dulvy et al. 2008) in response to global changes such as surface-ocean warming. As global surface waters continue to warm, and to a lesser extent deeper waters also (Balmaseda et al. 2013), shallow-water adapted species close to their thermal limits may be forced into bathymetric range shifts. This process may, in particular, affect faunas along continental margins (Levin and Sibuet 2012), and subsequently abyssal faunas (Jablonski et al. 1983). Despite evidence of high HP tolerance in a number of marine invertebrates, particularly larval stages (Tyler and Young 1998; Mestre et al. 2009), survival of later ontogenetic stages at depth is

essential for bathymetric range shifts and may be restricted by physiological limitations in their capacity for HP and temperature tolerance.

HP and temperature are two key stressors that may act as physiological limits on the ability of shallow-water organisms to tolerate deep-sea conditions. As thermodynamic variables, they affect biochemical equilibria and rates of biological processes in similar ways (Brauer and Torok 1984). Low temperature and high HP both result in reduced fluidity of bio-membranes, principally caused by an increased or tightened packing of fatty acyl chains (Simonato et al. 2006). This tightly packed, and highly ordered, configuration restricts molecular motion. Low temperatures and high HPs have both been shown to decrease membrane fluidity in shallow-water adapted organisms (Bartlett et al. 1995). Additionally, observed reductions in oxygen consumption of organisms subjected to acute HP exposures may reflect a drop in metabolism because of compromised membrane and enzyme functionality (Brown and Thatje 2011). Conversely, high temperature may act to ameliorate, or reduce, the effects of high HP by the same thermodynamic principles. Consequently, physiological limits to HP and temperature tolerance may be closely entwined and affected by shifts in either factor. An increase in ocean surface water temperature, and subsequent deep-water temperature increase (IPCC 2007), may actually facilitate bathymetric range shifts of shallow-water organisms by ameliorating any physiological limitations imposed by increasing HP.

The symptoms that accompany elevated HP episodes are collectively termed high pressure neurological syndrome (HPNS) and are observed as tremors; spasms, and neuropsychiatric disturbances in humans (Bowser-Riley et al. 1984). HPNS has been primarily used to describe the acute effects of increasing HP in mammals. However, HPNS-like symptoms have been noted also in terrestrial, shallow-water, and deep-sea invertebrates in response to HPs outside their natural distributions (Brauer et al. 1980; Brauer and Torok 1984; Bartlett et al. 1995). Despite observations of spasms, convulsions, and tremors in marine invertebrates (Oliphant et al. 2011) little is known about the physiological or molecular mechanisms that trigger such perturbations.

In order to establish the limits to physiological plasticity in response to temperature and HP stress, studies of transcriptional regulation have been employed to measure sub-lethal changes in physiology. One of the most widely studied molecular markers in marine invertebrates is that of the 70kDa heat shock protein family (HSP70) (Ravaux et al. 2007;

Cottin et al. 2010; Cottin et al. 2012; Ravaux et al. 2012). However, generalised stress markers such as HSP70 and other members of the universal cellular stress response (CSR) can be highly reactive to a multitude of potential stressors (Feder and Hofmann 1999; Morris et al. 2013) and, as such, in studies involving two or more stressors, the relative contribution of individual stressors to the overall observable stress response would be unquantifiable. Previous studies have looked at the effects of HP and temperature on behaviour (Oliphant et al. 2011; New et al. 2014). However, as yet, no study has looked specifically at the synergistic or antagonistic effects of simultaneous changes in HP and temperature at a transcriptional level. Herein, we report the first results on the molecular physiological response to combined HP and temperature stress using a shallow-water invertebrate; the caridean ditch shrimp *Palaemonetes varians* (Leach 1814). This species has previously been used in several studies concerning thermal and HP biology (Oliphant et al. 2011; Ravaux et al. 2012), has a eurythermal physiology and a distribution throughout the North Atlantic salt marshes, and is closely related to a number of species with deep-water habitat ranges (e.g. *Periclimenes* sp.) (Li 2008). This study presents results quantifying the physiological impact of acute HP and temperature stress at a transcriptional level using four novel HP-specific stress markers, discovered by a technique of differential display, alongside 2 more conventional markers of the CSR. The results provide an insight into the physiological limits on bathymetric ranges imposed by HP and temperature.

3.4 Specific chapter hypotheses

$H_{3.1}$ – The effects of elevated HP perturb cellular physiology to the extent that observable changes in transcriptional regulation of characterised biomarker genes can be quantified.

$H_{3.2}$ – Any observed changes in transcriptional regulation in response to elevated HP are further altered by changing temperature by the principle of synergistic or antagonistic thermodynamic effects.

3.5 Materials & methods

3.5.1 Maintenance & rearing of *Palaemonetes varians*

Adult male and non-ovigerous adult female *Palaemonetes varians* were net caught from Lymington salt marshes, UK on the 25th November 2011 (Section 2.1.1). The shrimp were

acclimated in aerated filtered seawater (salinity: 32 – 34) (1µm filter) at 15°C ± 0.5°C with an 18/6 light/dark cycle. The observed average water temperature is 15°C at the collection site during the time of reproduction (June/July) for this species. The majority of females collected already had early stage gonad development. The shrimp were fed with fish flakes (TetraMin™) 3 times a week, and water changes were made the day following each feeding. Once females became ovigerous they were transferred to individual 1 litre beakers and exposed to a 12/12 light/dark cycle. Feeding regimes remained unchanged but 100% water changes (salinity: 32 – 34) were made daily with UV-treated seawater.

Larvae were released approximately 2-months after adult females were noted as ovigerous. Once released, each larva was kept individually in 100 ml plastic beakers at 15°C ± 0.5°C in laboratory incubators. Water changes were made daily with UV-treated seawater, and larvae were given *ad libitum Artemia* spp. nauplii following the first moult. Larval development was monitored through the 5 reported zoeal stages to juvenile (Oliphant et al. 2013). Larvae reached the juvenile stage between 26- to 29-days after larval release. After 2 juvenile moults the shrimp were considered of sufficient size to obtain consistent RNA yields from each individual, obviating the need to pool tissue samples for extraction. Individuals were then subjected to experimental treatments of: 0.1 MPa (atmospheric control pressure), 5 MPa, and 10 MPa pressures at: 15°C (control temperature), 20°C, 10°C, and 5°C respectively. Ten individuals were used for each treatment. The HPs and temperatures chosen were within the critical limits of *P. varians* as shown in previous studies (Oliphant et al. 2011) as this study aimed to quantify the sub-lethal effects of acute HP and temperature exposures at a transcriptional level. In order to study the effects of temperature on HP tolerance, it was necessary to be able to control and account for the entire thermal history of each individual organism to negate the potential effects of cross-tolerance and stress-hardening on gene transcription. For this reason, ovigerous adults and subsequent larvae were maintained in a laboratory-controlled environment prior to experimentation.

3.5.2 Temperature & hydrostatic pressure exposures

The IPOCAMP was used for combined temperature and HP controlled exposures (Section 2.2.2). Prior to each experimental regime, the temperature in the IPOCAMP system was set at 20°C, 15°C, 10°C, or 5°C, respectively. Individual juvenile shrimp were placed in a sealed 20ml falcon tube with 1µm filtered seawater at the temperature at which they were

reared. Preliminary experiments found that oxygen concentration was not a limiting factor over the experimental period in the sealed tubes. The tubes were held in a 1 litre plastic bucket and submerged in fresh water within the IPOCAMP system. Once sealed, the IPOCAMP system was pressurised from ambient to the experimental HP over a period of one minute. The shrimp were then maintained at experimental temperature and HP for 2-hours, after which the HP was brought back to ambient over a period of 1-minute. The shrimp were removed from the IPOCAMP system snap frozen in liquid nitrogen within 5-minutes of the experimental treatment. Once frozen, the shrimp were stored at -80°C until further use.

3.5.3 Selection of genes of interest & endogenous reference genes

In order to identify specific genes that were differentially expressed in response to HP and temperature stress, a technique of differential display was employed using Seegene GeneFishing™ DEG premix kits (BioGene, UK) as according to Section 2.6.2.2. The kit allows for the discovery and identification of a random subset of differentially expressed mRNA fragments between experimental treatments (Figure 3.1). In a preliminary study, samples were taken from; a) 15°C, 0.1 MPa control treatment; b) 15°C, 10 MPa treatment; and c) 10°C, 10 MPa bar treatments. Annealing control primers (ACPs) supplied in the GeneFishing™ kit yielded 3 distinct constitutively expressed fragments and 9 distinct differentially expressed fragments, as well as 6 indistinct, un-extractable bands (Table 1.1). The 12 distinct fragments were gel-purified and extracted using a QIAquick Gel Extraction Kit (Qiagen, UK) (Section 2.6.3) and nucleotide sequences were obtained using conventional dideoxy-termination sequencing by Source BioScience (Nottingham, UK) (Section 2.6.6).

The sequences returned were compared to published sequences lodged with the EMBL EBI Databank by BLAST search (Altschul et al. 1990). Of the 12 fragments extracted, 1 constitutively expressed fragment, and 3 differentially expressed fragments produced significant BLAST hits (Altschul et al. 1990) (Section 2.7). Sequences producing significant hits were compared to published sequences using ClustalW alignment (Higgins and Sharp 1988) (Sections 2.7 & A1.2.1).

Table 3.1 Results from preliminary Genefishing™ (Biogene, UK) experiment showing which of the supplied annealing control primers (ACP) provided usable sequences

ACP	Expression across treatments	Fragment cloned?	Sequence length (bp)	Top BLAST result	Accession no. of top BLAST result
1	No band	n/a			
2	No band	n/a			
3	Differential	Yes	401	No significant hits	
4	Differential (2)	Yes	323	No significant hits	
5	Differential	Yes	247	**NMDAR-regulated protein**	C0H926_SALSA
6	No band	n/a			
7	No band	n/a			
8	Differential	Yes	638	No significant hits	
9	No band	n/a			
10	No band	n/a			
11	Constitutive	Yes	450	No significant hits	
12	Constitutive (3)	Yes	400	**40S Ribosomal protein S26**	Q962Q4_SPOFR
13	Differential	No*			
15	No band	n/a			
16	Differential	Yes	233	No significant hits	
17	No band	n/a			
18	Differential	No*			
19	Constitutive	Yes	255	No significant hits	
20	Differential	Yes	329	**ADP-ribosylation factor**	NP_001025648
49	Differential (3)	Yes	1100	**GAPDH**	HM157285

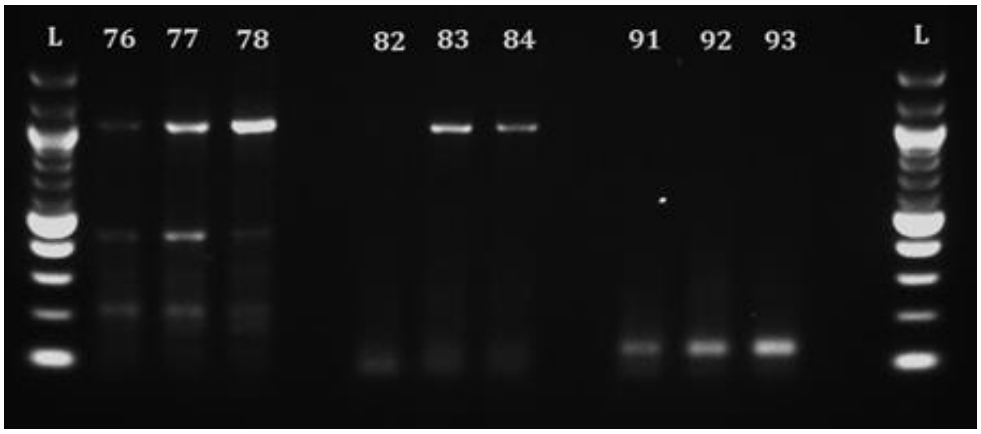


Figure 3.1 Example of the GeneFishing™ products separated by gel electrophoresis and compared against a standard DNA 100-bp ladder (L). Grouped into three exposures with three samples in each; 76, 77 & 78 represent the products of ACP primer A30 across three different HP and temperature conditions. Samples 82, 83, & 84; and 91, 92, & 93 represent ACP primers A31 and A32 respectively. Bands showing differential fluorescence intensity putatively represent differentially expressed mRNA fragments across the test exposures

3.5.4 Total RNA extraction, DNase treatment, & first strand cDNA synthesis

Total RNA was extracted as described in Section 2.5.1. Total RNA purity and integrity was assessed according to Section 2.5.1.1. A volume containing 1.5 µg of total RNA was DNase treated using Promega RQ1 RNase-free DNase (Promega Corporation, Hants, UK), as described in Section 2.5.2. DNase-treated total RNA was reverse-transcribed to cDNA, as described in Section 2.5.3.

3.5.5 Quantitative polymerase chain reaction (qPCR)

Specific primers for qPCR were designed using Primer Express® software (Applied Biosystems, Cheshire, UK) for 3 genes, including 1 putative reference gene, *rps26,* obtained from gene-fishing™ (Table 2.3). Primer sequence data for the additional 5 genes, including another putative reference gene, *rpl8*, were obtained from Cottin et al. (2010) (Table 2.3).

Two HSP70 isoforms and GAPDH primer sets from Cottin et al. (2010) were included in the study to provide a non-specific stress marker comparison to the putative stress-specific markers (DEGs) found through Genefishing™. *β-actin* was tested in a pilot study as a

putative reference gene but was found to be HP sensitive and was thus included as a gene of interest in this study.

All primer-sets tested generated a single and discrete peak by melt curve analysis (Section 2.8.4). Each primer set was then optimised following the MIQE guidelines (Bustin et al. 2009), as described in Sections 2.8 & A1.4.

All qPCR reactions were performed on the Rotor-Gene™ 3000 (Qiagen, UK) (Section 2.8.1.1). Each 25 µl reaction contained 12.5 µl of Precision 2x qPCR Master Mix (Primer-Design, UK) with SYBR green (Section 2.8.2), and 1 µl of template cDNA (34 ng). qPCR conditions are described in Section 2.8.6. Each reaction was run in duplicate (technical replicate). After each run a melt curve analysis was performed in order to demonstrate the specificity of the qPCR products (Section 2.8.4).

Candidate reference genes were tested by geNorm analysis using qBase+™ software (Biogazelle, Belgium) in a pilot study (Section 2.8.8). The combination of the *rps26* and *rpl8* genes provided the best normalisation strategy for this study. Normalised relative quantities (NRQs) were calculated using qBase+ software. NRQs were then scaled to the control treatment in each comparison and converted to relative fold changes (RFC) (Section 2.8.7). Statistical significance was identified at $p < 0.05$ as determined by generalised linear model (GLM) and post-hoc Tukey-HSD test using R statistical software (R core team 2013) and the "multcomp" package (Horthorn et al. 2008).

3.6 Results

The relative expression levels of six genes of interest showed significant differences with both changes in temperature and HP. At 15°C, the relative expression levels of the *narg* gene (NMDAR-regulated protein) (Figure 3.2a), the *arf* gene (ADP ribosylation factor) (Figure 3.3a), *gapdh* gene (GAPDH) (Figure 3.4a), *β-actin* gene (β-Actin) (Figure 3.5a), and the *hsp70 f1* gene (HSP70 isoform 1) (Figure 3.5a) showed significant increases between the atmospheric control treatment (0.1 MPa) and the high HP treatment (10 MPa). Further, the *narg*, *gapdh*, and *β-actin* genes showed significant increases in relative expression levels between the 0.1 MPa and 5 MPa treatments (Figures 3.2a, 3.4a and 3.5a). The effects of HP on the gene expression of all six genes were reduced at the increased incubation temperature of 20°C (Figures 3.2b to 3.6b), showing no significant changes in expression between any of the HP treatments.

At 10°C, the relative expression levels of the *narg*, *gapdh*, *β-actin*, and *hsp70 f2* genes increased significantly between 0.1 MPa and 10 MPa treatments (Figures 3.2c, 3.4c, 3.5c and 3.6c). The *narg* and *hsp70 f2* genes also showed significant increases in relative expression levels between the 0.1 MPa and 5 MPa treatments. The *arf* and *hsp70 f1* genes showed no significant changes in relative quantities between 0.1 MPa and 10 MPa treatments (Figures 3.3c and 3.6c).

At 5°C, *narg* and *arf* genes showed significant increases in relative expression levels between 0.1 MPa and 5 MPa treatments, significant decreases in relative expression levels between 5 MPa and 10 MPa treatments, and no change in relative expression levels between 0.1 MPa and 10 MPa treatments (Figure 3.2d and 3.3d). The *gapdh*, *hsp70 f1*, and *hsp70 f2* genes showed significant decreases in relative expression levels between the 0.1 MPa and 10 MPa treatments and the *hsp70 f2* gene showed significant decrease in relative expression levels between 0.1 MPa and 5 MPa treatments (Figures 3.4d and 3.6d).

Further statistical analysis by GLM showed significant interactions between temperature and 10 MPa HP treatments on the relative expression levels of the *narg* gene, the *gapdh* gene, the *hsp70 f1*, and *hsp70 f2* genes ($p<0.01$). Relative expression levels were compared between the control temperature, 15°C, and experimental temperatures; 20°C, 10°C, and 5°C at 0.1 MPa and 10 MPa HP treatments (Figure 3.7). The *narg*, *gapdh*, *hsp70 f1*, and *hsp70 f2* genes all showed significant increases in relative expression levels at 10°C, and significant decreases in relative expression levels at 20°C in comparison to the 15°C control temperature (Figure 3.7). Further, the *narg*, *arf*, *gapdh*, *β-actin*, and *hsp70 f2* genes showed significant decreases in relative expression levels at 5°C compared to the 15°C control temperature. Across temperatures at 0.1 MPa, significant changes in relative expression were seen in the; *gapdh* gene, *hsp70 f1* gene, and *hsp70 f2* gene. The *gapdh* gene showed no change in relative expression between 15°C and 20°C, but significant increases in relative expression at both 10°C and 5°C in comparison to 15°C. The *hsp70 f1* also showed significant increases in expression at 10°C and 5°C, and no change at 20°C relative to 15°C. The *hsp70 f2* gene showed a significant decrease in expression at 20°C, no change in expression at 10°C, and a significant increase in expression at 5°C in comparison to 15°C.

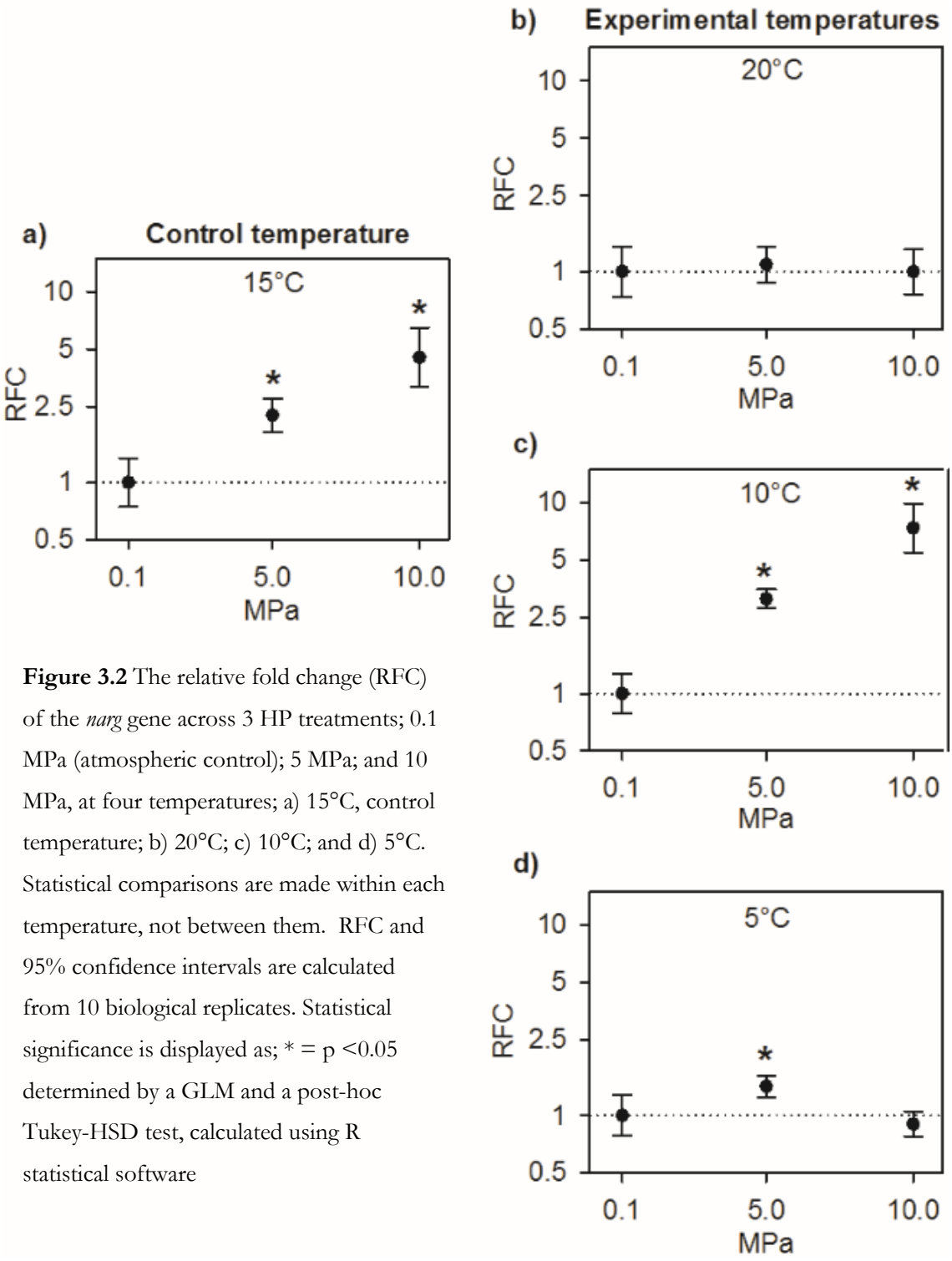


Figure 3.2 The relative fold change (RFC) of the *narg* gene across 3 HP treatments; 0.1 MPa (atmospheric control); 5 MPa; and 10 MPa, at four temperatures; a) 15°C, control temperature; b) 20°C; c) 10°C; and d) 5°C. Statistical comparisons are made within each temperature, not between them. RFC and 95% confidence intervals are calculated from 10 biological replicates. Statistical significance is displayed as; * = $p < 0.05$ determined by a GLM and a post-hoc Tukey-HSD test, calculated using R statistical software

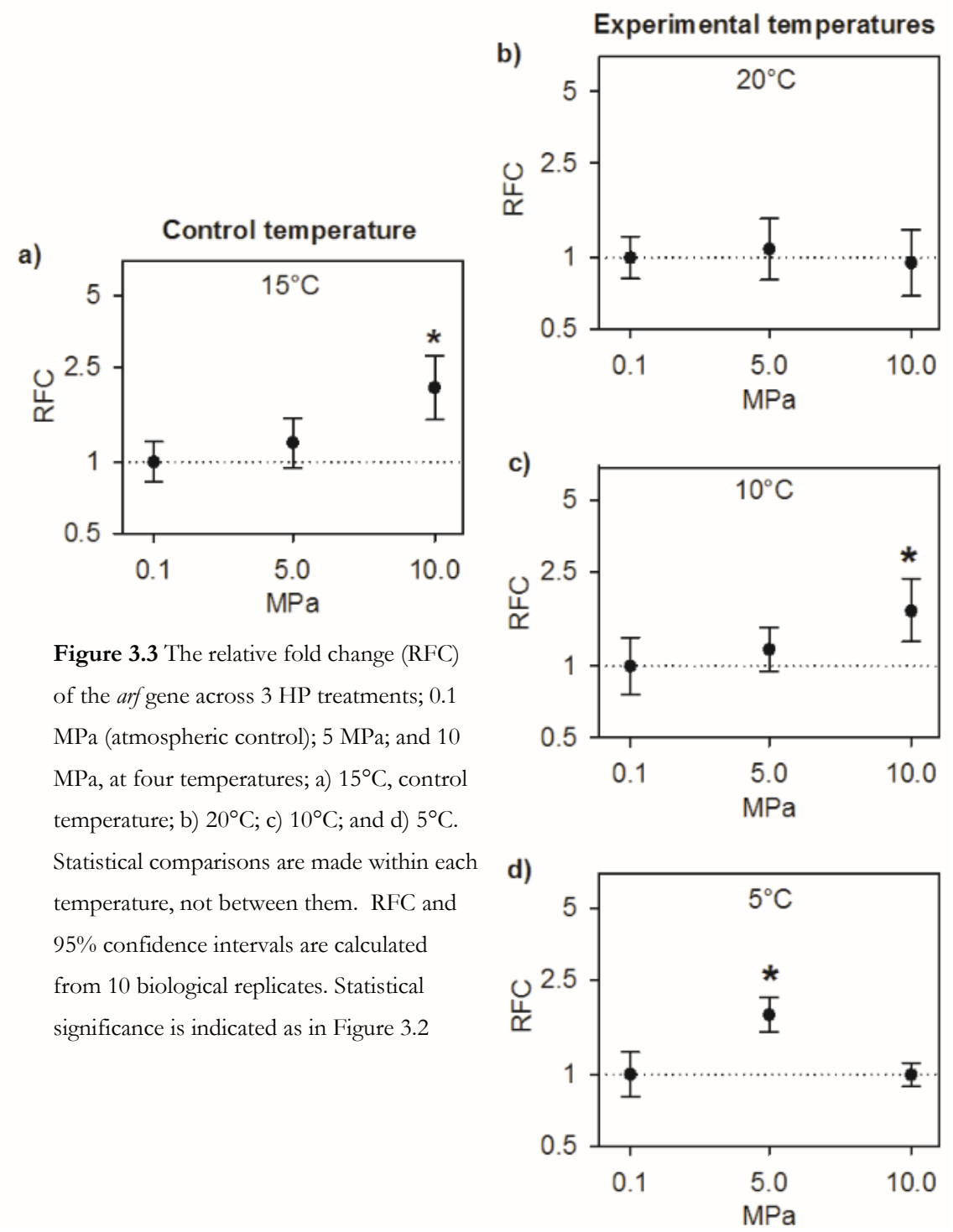


Figure 3.3 The relative fold change (RFC) of the *arf* gene across 3 HP treatments; 0.1 MPa (atmospheric control); 5 MPa; and 10 MPa, at four temperatures; a) 15°C, control temperature; b) 20°C; c) 10°C; and d) 5°C. Statistical comparisons are made within each temperature, not between them. RFC and 95% confidence intervals are calculated from 10 biological replicates. Statistical significance is indicated as in Figure 3.2

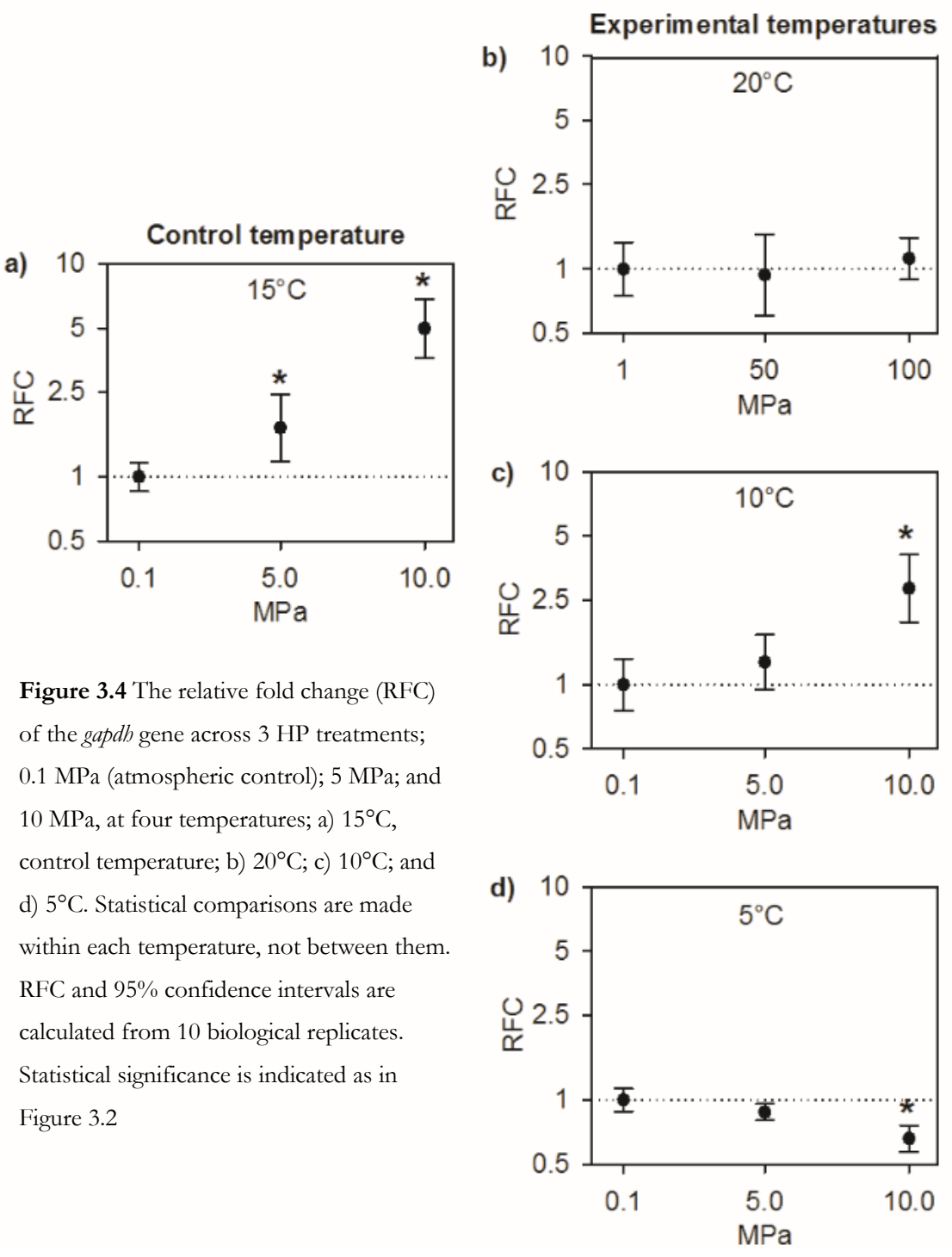


Figure 3.4 The relative fold change (RFC) of the *gapdh* gene across 3 HP treatments; 0.1 MPa (atmospheric control); 5 MPa; and 10 MPa, at four temperatures; a) 15°C, control temperature; b) 20°C; c) 10°C; and d) 5°C. Statistical comparisons are made within each temperature, not between them. RFC and 95% confidence intervals are calculated from 10 biological replicates. Statistical significance is indicated as in Figure 3.2

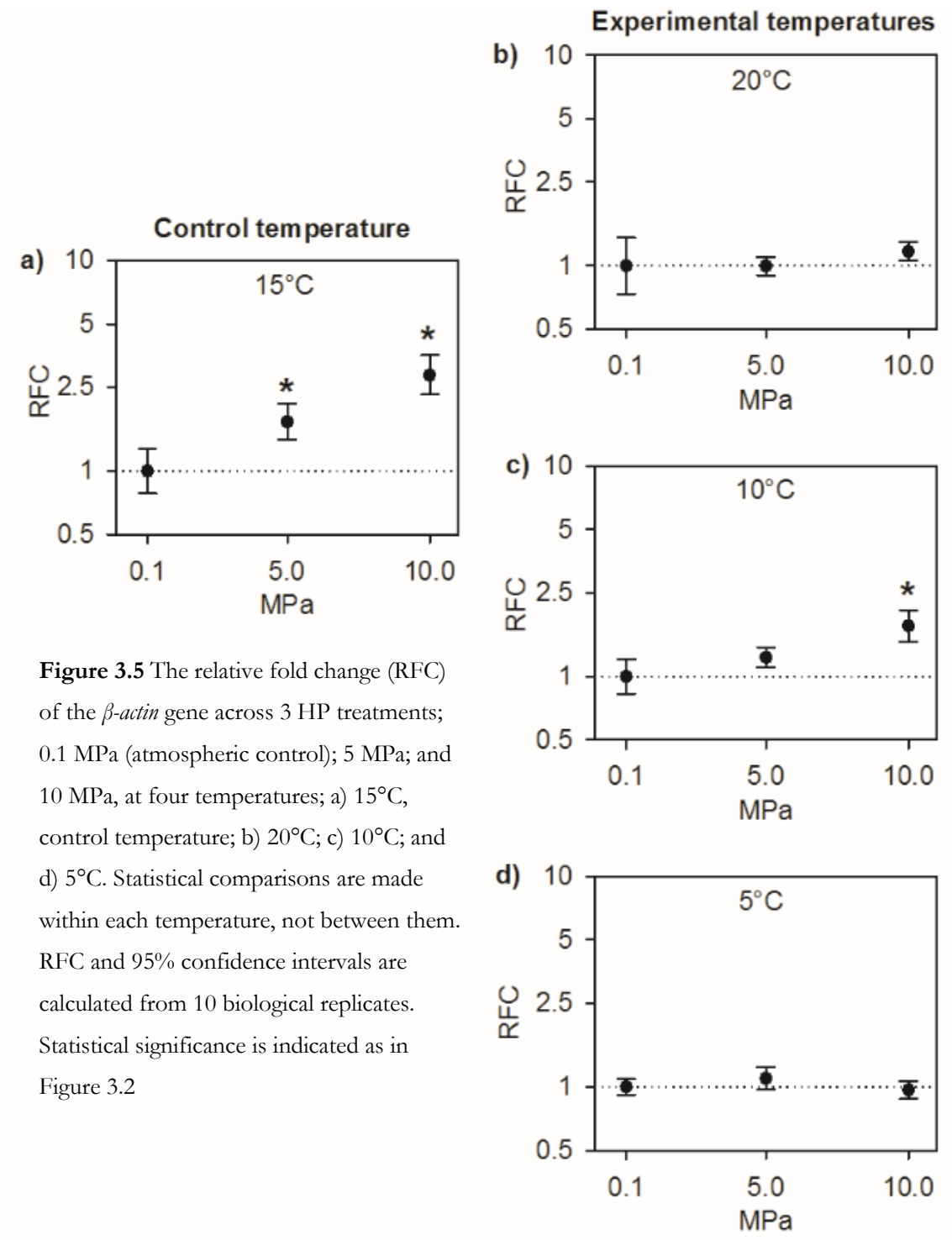


Figure 3.5 The relative fold change (RFC) of the *β-actin* gene across 3 HP treatments; 0.1 MPa (atmospheric control); 5 MPa; and 10 MPa, at four temperatures; a) 15°C, control temperature; b) 20°C; c) 10°C; and d) 5°C. Statistical comparisons are made within each temperature, not between them. RFC and 95% confidence intervals are calculated from 10 biological replicates. Statistical significance is indicated as in Figure 3.2

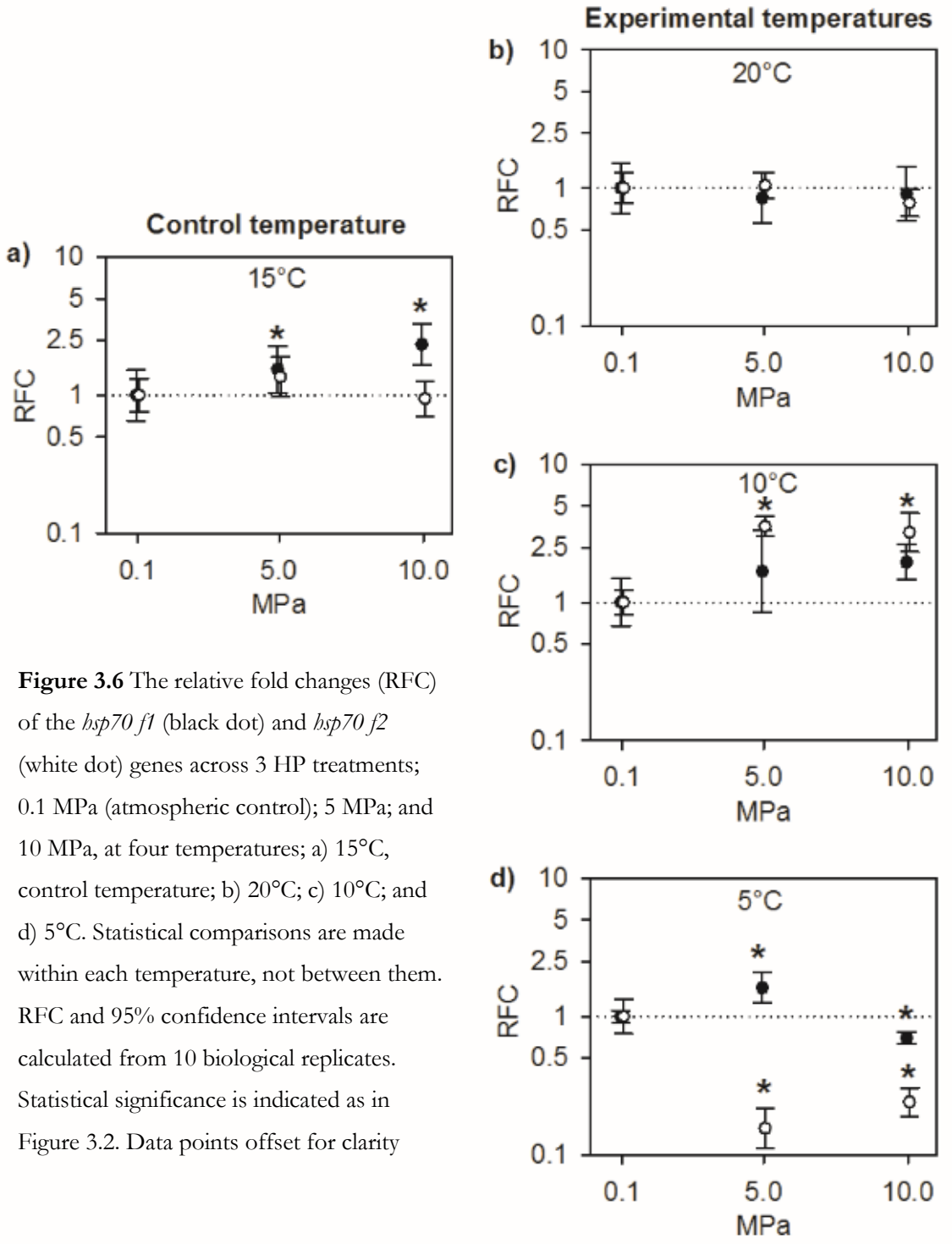
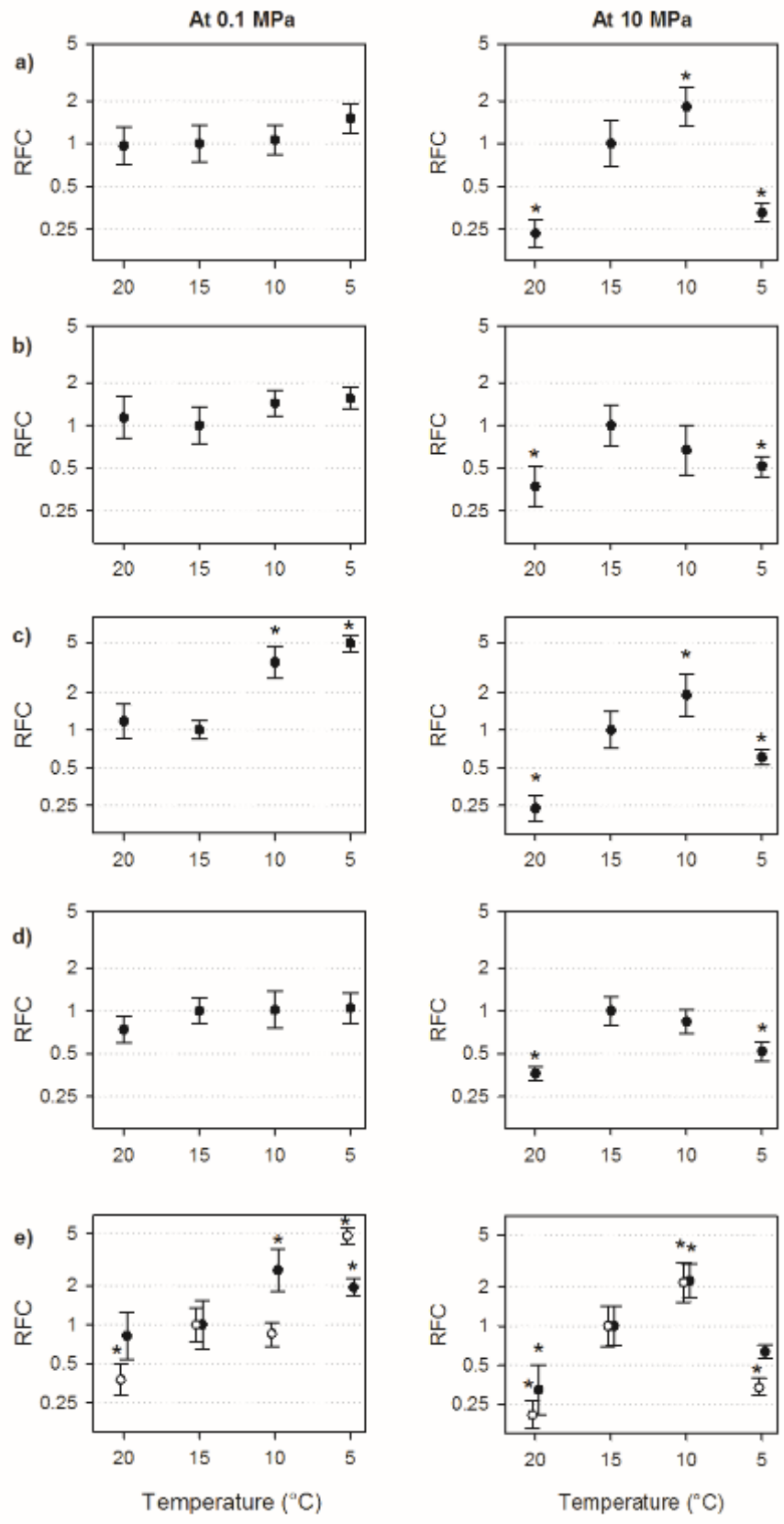


Figure 3.6 The relative fold changes (RFC) of the *hsp70 f1* (black dot) and *hsp70 f2* (white dot) genes across 3 HP treatments; 0.1 MPa (atmospheric control); 5 MPa; and 10 MPa, at four temperatures; a) 15°C, control temperature; b) 20°C; c) 10°C; and d) 5°C. Statistical comparisons are made within each temperature, not between them. RFC and 95% confidence intervals are calculated from 10 biological replicates. Statistical significance is indicated as in Figure 3.2. Data points offset for clarity



Legend overleaf

Figure 3.7 The relative fold change (RFC) of; a) the *narg* gene; b) the *arf* gene; c) the *gapdh* gene; d) the *β-actin* gene; and e) the *hsp70 f1* (black dots) and *hsp70 f2* (white dots) genes across 4 temperatures at 0.1 MPa, and 10 MPa pressure. Statistical comparisons are made between the 15°C control temperature and the; 20°C, 10°C, and 5°C experimental temperatures. RFC and 95% confidence intervals are calculated from 10 biological replicates. Statistical significance is indicated as in Figure 3.2

3.7 Discussion

In metazoans, HP intolerance is commonly expressed through perturbations of neural and muscular functioning. The symptoms that accompany elevated HP episodes are collectively termed as high pressure neurological syndrome (HPNS). A number of studies conducted on shallow-water crustaceans have shown the adverse effects of high HP in terms of both behaviour and physiology. In the Atlantic ditch shrimp *Palaemonetes varians* "loss of equilibrium" (LOE) was seen at approximately 11 MPa at 5°C, with the threshold increasing to 21 MPa at 30°C. LOE was classified as when a shrimp rested in an unnatural position i.e. upside-down or on their side for more than 2 seconds (Oliphant et al. 2011). Oliphant et al. (2011) noted that LOE was accompanied by spasmodic motions, "vibrations of the pleopods, and/or sudden contractions of the abdomen". Spasmodic behaviour was not quantified due to the complexity of such movements (Oliphant et al. 2011), but re-analysis of the original experimental videos consistently showed spasms and tremors during periods of recorded LOE (Morris, *unpub. obs.*). Despite evidence of perturbations characteristic of HPNS in marine invertebrates in response to acute increases in HP, the term 'HPNS' has generally been restricted to mammalian use (Darbin et al. 2000), and little is known about the molecular mechanisms that trigger such symptoms in marine invertebrates.

In this study, significant increases in the transcription of 5 genes (the *narg*, *gapdh*, *hsp70 f1*, *β-actin*, and *arf* genes) were recorded in juveniles of the ditch shrimp *Palaemonetes varians* upon exposure to an increase in HP from 0.1 MPa to 10 MPa at 15°C. These genes are involved in: CNS functioning, cellular metabolism, the cellular stress response, and cell damage repair, respectively. Further, significant increases in the expression of these 5 genes, as well as a second HSP70 isoform, the *hsp70 f2* gene, were shown upon exposure to an increase in HP from 0.1 MPa to 10 MPa alongside a drop in temperature from 15°C to 10°C. These

data provide the first gene transcription data supporting the onset of HPNS in a marine invertebrate in response to acute elevated HP exposures, and correlate well with previous behavioural and physiological studies on crustaceans that report the onset of symptoms that characterise HPNS with increasing HP (Brauer et al. 1980; Oliphant et al. 2011; Cottin et al. 2012).

N-methyl-d-aspartate receptors (NMDARs) have been identified as having a variety of key functions in the central nervous system (CNS) of many metazoans. The majority of NMDAR research has been conducted on mammals. Despite this, a number of studies have investigated these receptors in crustaceans, and have shown that NMDARs have a similar set of pivotal functions in the CNS as NMDARs present in mammals (Gallus et al. 2010; Hepp et al. 2013). NMDARs play an important role in cell survival versus cell death scenarios during trauma, ischemia, and neuronal development (de Rivero Vaccari et al. 2007). NMDARs have also been recognised as playing an important role in the onset of HPNS (Rostain et al. 1986; Millan et al. 1989). Symptoms ranging from agitation to serious seizures have been noted during periods of NMDAR over-activity (Dingledine et al. 1999; Darbin et al. 2000). NMDARs are found in intracellular membranes and are known to be highly calcium permeable (Mor and Grossman 2006). It is, therefore, hypothesised that high HP, known to cause NMDAR over-activity, leads to an influx of Ca^{2+} into the cell that triggers enzymes involved in apoptosis such as calpains, proteases, and lipases (Szydlowska and Tymianski 2010). The *narg* gene has been characterised as an NMDAR-regulated protein showing sequence homology also with genes encoding N-alpha-acetyltransferase enzyme. The N-alpha-acetyltransferase (NatA) enzyme has been implicated in the N-terminal acetylation of proteins in mammals yet little is known about the genes specific function or importance (Gautschi et al. 2003). A high proportion of characterised eukaryotic proteins are thought to be N-terminally acetylated, and NatA may be involved in the vital process of cell-cycle progression (Caesar et al. 2006). Mammalian studies have shown that the *narg* gene is down-regulated during normal NMDAR function, and is consequently up-regulated during periods of non-normal NMDAR functioning such as elevated-HP-induced NMDAR over-activity (Sugiura et al. 2001). I provide evidence that exposure to HPs of 5 MPa and 10 MPa led to a significant increase in the expression of the *narg* gene coding for an NMDAR-regulated protein. Up-regulation of the *narg* gene may, therefore, be used to infer elevated-HP-induced NMDAR over-activity in the shrimp. This, in turn, may be linked to a change in intracellular calcium ion concentration triggering enzymes that promote apoptosis and neuronal cell death. We advocate that an increase in

HP of 10 MPa at a constant temperature of 15°C led to HP sensitivities associated with HPNS, and is supported by the observation of significant increases in the expression of four other genes; the *gapdh* gene, *β-actin* gene, *hsp70 f1* gene, and the *arf* gene.

ADP-Ribosylation factors (*arf* gene) are associated with poly (ADP-Ribose) polymerase-1 (PARP-1), and have been previously characterised in the shrimp *Marsupenaeus japonicus* (Ma et al. 2010). Poly-ADP-ribosylation has been shown to increase in response to DNA damage in neurons that may be caused by over-activity of NMDARs (Gilliams-Francis et al. 2003) and the subsequent increase of intracellular calcium (Lynch and Guttmann, 2002). The interconnected relationship between NMDAR-induced excitotoxicity and the activity of PARP-1 and ADP ribosylation factors has previously been reported (Yu et al. 2003). Herein, we demonstrate that a 10 MPa increase in HP at 15°C led to a significant increase in the expression of the *arf* gene in *P. varians* suggesting an increase in DNA damage in neurons.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a highly conserved enzyme that is involved in cellular metabolism: namely, it catalyses the 6th step of glycolysis (Lodish et al. 2000). It also forms part of the minimal stress proteome (MSP) and cellular stress response (CSR) (Kültz, 2005). Increases in the transcription of the *gapdh* gene seen at 10 MPa at 15°C complement the increase transcription seen for the *narg* gene and *arf* gene, inferring that increasing HP at 15°C may result in HP-induced cellular stress and a subsequent increase in cellular metabolism, required to facilitate the metabolically costly CSR (Kültz 2005).

Heat shock proteins of ~70 kDa weight (HSP70s) are widely used as non-specific markers of cellular stress (Morris et al., 2013). An increase in expression of genes coding for HSP70 isoforms can indicate an increase in intracellular macromolecular damage. HSP70 genes have been shown to be responsive to the combined effects of HP and temperature (Simonato et al. 2006; Cottin et al. 2010; Cottin et al. 2012). Previously, the *hsp70 f1* gene used in this study has been demonstrated to be inducible in response to heat stress, whereas the *hsp70 f2* gene was not stress responsive and assumed to be constitutively expressed (Cottin et al. 2010). In this study, the increased expression of the *hsp70 f1* gene, alongside no change in the expression of the *hsp70 f2* gene, with increasing HP at 15°C, adds weight to the hypothesis of the onset of HP sensitivity and increasing stress levels inferred by *narg* gene up-regulation. It has to be emphasised however, that the analysis of

HSP70 expression holds potential limitations (Morris et al. 2013). HSP70 proteins are deeply engrained in a variety of cellular processes under stressful and non-stressful scenarios, inferring a single role (i.e. HP stress response) from increased transcription may be erroneous (Morris et al. 2013). For these reasons, the observed changes in transcription of the *hsp70 f1* and *hsp70 f2* genes must be viewed with caution and, as such, increases in expression of either HSP70 isoform are deemed only to show a potential increase in macromolecular damage, caused by an increase in the overall stress exposure, and not attributed singly to changes in either temperature or HP.

Actin is a globular protein found in all eukaryotic cells that is essential for a diverse range of cellular functions. Birds and mammals have six actin gene isoforms of which β-actin is one of two cytoplasmic forms (Perrin and Ervasti 2010). Significant increases in the expression of the *β-actin* gene were found with a HP increase of 5 MPa and 10 MPa at 15°C. In humans, a mutation leading to loss of function in the gene coding for *β-actin* was found to be linked to the onset of dystonia (Procaccio et al. 2006), a movement disorder characterized by involuntary muscle contractions, similar in symptom to that of observed HPNS in marine invertebrates (Oliphant et al. 2011; Morris, *unpub. obs.*). The actins of deep-sea fish have been shown to possess adaptive capacities to elevated HP, showing great resistance to polymer dissociation under elevated HP (Swezey and Somero 1982). Clearly, there is a link between elevated HP scenarios, a loss of structure and function of non-HP-adapted actins, and an increase in the transcription of the gene coding for the *β-actin* protein.

Previous studies have shown that increases in ambient temperature can ameliorate the negative physiological effects of exposure to high HP. Likewise, decreases in ambient temperature can exacerbate the physiological effects of high HP (Pradillon and Gaill 2006). At a HP of 10 MPa, the "preferred" temperature range of a variety of shallow-water gammarid amphipods increased by up to 4°C compared to ambient HP (Kinney et al. 1981). Oliphant et al. (2011) found that temperature had considerable effect on the HP tolerance of adult *P. varians*; at 5°C the onset of LOE in 50% of the shrimp occurred at around 10 MPa, in comparison the onset of LOE in 50% of the shrimp only occurred at 21 MPa at 30°C. Another study on the effects of HP and temperature in shallow-water echinoids showed that low temperatures exacerbated the effects of HP (Benitez-Villalobos et al. 2006). Within this context, Airriess and Childress (1994) concluded that increased temperatures mollified the disruption of membrane systems by elevated HP in the hydrothermal vent crab *Bythograea thermydron* (Airriess and Childress. 1994). These

observations support the present study, particularly with regards to 15°C comparisons to 20°C and 10°C, respectively. Significant up-regulation of the *narg*, *β-actin*, *arf*, *gapdh* and *hsp70 f1* genes, with a 10 MPa increase in HP at 15°C, appear to be completely ameliorated by an increase in temperature to 20°C, where no significant over-expression was observed in any of the five genes of interest. Consequently, care must be taken when suggesting that a lack of differential expression is the result of antagonistic effects of increasing HP and temperature rather than the suppressive effects of critical stress. As a standalone comparison, it would not be possible to infer antagonistic effects; however, results from the 10°C treatment show a higher relative mean expression in *narg* at both 5 MPa and 10 MPa than at 15°C. Thus, our results suggest that increasing temperature at elevated HPs may not only ameliorate the effects of HP, but also decreasing temperature may exacerbate the effects of increased HP. These results are also reflected in the expression of the two other biomarkers central to the CSR: GAPDH and HSP70, as well as the expression of β-actin. Interestingly, at 10°C it is the *hsp70 f2* gene that appears to be the stress responsive isoform rather than the *hsp70 f1* isoform under this stress scenario. The *hsp70 f2* gene has previously been described as a non-inducible constitutive isoform in response to heat stress (Cottin et al. 2010). This result highlights the variability of HSP70 isoform responsiveness under differing stress states (Morris et al. 2013).

At 5°C there is a significant up-regulation of the *narg* gene at 5 MPa, but not at 10 MPa. A 10 MPa increase in HP combined with a 10°C drop in temperature might represent the threshold at which stress reaches a critical level in the cell and organism. It is known that once stress reaches a critical level, where cellular or organismal survival is at risk, cell cycle control shuts down along with normal transcriptional regulation (Kültz 2005). Critical stress levels and the shutdown of cell cycle control are reflected in a suppression of cellular metabolism and the abrogation of CSR mechanisms, such as heat shock protein up-regulation. At 5°C, both *gapdh* and *hsp70 f1* genes show no change in expression at 5 MPa and significant down-regulation at 10 MPa, and the *hsp70 f2* gene showed significant down-regulation at both 5 and 10 MPa. These results further support the idea that at 5°C, and at HPs above 5 MPa, stress approaches critical tolerance levels in juvenile *P. varians*. Further, across-temperature comparisons at 0.1 MPa and 10 MPa show that the expression of CSR markers was significantly higher at 5°C than at 15°C under atmospheric HP (0.1 MPa), consistent with a higher but not critical level of stress. At 10 MPa, however, the expression

of these markers was significantly lower at 5°C than at 15°C following an increase at 10°C. I suggest this pattern is best explained by critical stress thresholds being reached.

Across-temperature comparisons at 0.1MPa show that the; *narg*, *arf*, and *β-actin* genes were not responsive to the temperature changes conducted in this study. However, under the high HP treatments significant changes in expression between temperatures are seen. Further, the three CSR markers: the *hsp70 f1, hsp70 f2,* and *gapdh* genes show characteristic changes in expression at atmospheric HP, as the temperature moves away from the organism's acclimated temperature. This pattern is altered with the addition of HP. These results provide evidence of the synergistic/antagonistic effects of temperature and HP at a molecular level, and highlight the utility of stressor-specific gene markers, such as the *narg* gene, in multi-stressor studies.

3.8 Conclusions

The results from this study indicate that upon exposure to increases in HP; increases in cellular metabolism, the induction of the CSR, and disruptions in the neurophysiological functioning of the shallow-water shrimp *Palaemonetes varians* become apparent. These disruptions appear to be exacerbated by decreasing temperature, and ameliorated by increasing temperature. These results provide an indication of the variety of cellular pathways that are affected by changes in HP and temperature, and provide a foundation for further developing the biomarkers in longer-term more ecologically relevant experimental exposures. Our results suggest that today's generally stratified ocean, with water temperatures decreasing with increasing depth, represents the least penetrable scenario for bathymetric range shifts in temperate-zone shallow-water organisms. This is due, in part, to the synergistic effects of increasing HP and decreasing temperature on cellular and whole organism physiology. Conversely, an isothermal water column may provide a more penetrable scenario for shallow-water organisms undergoing a temperature-driven bathymetric range shift. This study shows the complete negation of physiological tolerance limits imposed by increasing HP, equivalent to 1000m water depth, by a temperature increase of 5°C. Therefore, the most penetrable scenario for temperate-zone shallow-water organisms may comprise warming waters with depth, which allow for the exploitation of the antagonistic effects of increasing temperature and HP by mitigating the negative physiological effects imposed solely by increasing HP.

Bridging statement

Chapter 3 has elucidated and tested several new cellular biomarkers for use in studies concerning HP and temperature stress, and quantified their expression alongside more conventional and widely used markers. Acute HP intolerance was observed as changes in gene expression in juvenile *Palaemonetes varians*, and presented alongside previous behavioural studies on adults of the same species. HP stress was shown to be altered by co-varying temperature. For the first time, transcriptional-level evidence of the synergistic and antagonistic of HP and temperature, as according to thermodynamic principles, were quantified. Although acute two-hour exposure to high HP is clearly not ecologically relevant, in terms of a first step towards characterising transcriptional-level physiological disturbances, it was deemed appropriate. Longer-term exposures are to be explored in Chapters 5 and 6. A major constraint on the work carried out for Chapter 3 was the reliance on previously published whole-organism physiology studies, and an inability to directly link changes at a transcriptional level to higher order changes, such as respiration or behaviour. Another point of concern was the use of whole organism homogenisations for obtaining total RNA and mRNA pools, as it would be expected that certain tissues and organs exhibit differential expression under a single stress scenario. Such differential expression might act to mask any key tissue-specific changes in gene expression that may have important effects on the organisms' physiology. Finally, questions were raised as to whether the sampling point post-exposure has an effect on the gene expression profile and ultimately the way physiological state is viewed. The experiments of Chapter 4 were designed in order to study these three major constraints, and provide a more solid platform for which to begin more ecologically relevant and longer-term experiments, as described in Chapters 5 & 6.

Chapter 4

The kinetics & hierarchy of the stress response to pressure

4. CHARACTERISING MULTI-LEVEL EFFECTS OF AN ACUTE PRESSURE EXPOSURE ON A SHALLOW-WATER INVERTEBRATE: INSIGHTS INTO THE KINETICS & HIERARCHY OF THE STRESS RESPONSE

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4.1 Abstract

Abiotic and biotic stressors shape our biological world, playing pivotal roles in species' distribution patterns. Hydrostatic pressure (HP) is an important, ubiquitous, environmental variable of particular relevance in the marine environment. Yet it is widely overlooked despite recent evidence that some marine ectotherms may be demonstrating climate-driven bathymetric range shifts. Studies have shown wide ranging effects of increased HP, from the molecular level, through to the behavioural level. Still, no study has simultaneously examined these multiple levels of organisation in a single experiment in order to understand the kinetics, hierarchy, and interconnected nature of such responses during an acute exposure, and over a subsequent recovery period. Herein, we quantify the transcription of a set of previously characterised genes over, and following, an acute HP exposure in adults of the shrimp *Palaemonetes varians*. Further, we perform respiratory rate and behavioural analysis over the same period. The effects of elevated HP disturb gene expression, respiration, and behaviour during, and following, exposure. These effects occur over differing periods of time. Although acute exposures to high HP are not directly ecologically relevant, characterising generalised responses to elevated HP is a vital pre-cursor to longer–term, acclimation-based HP studies. Results provide a novel insight into what we term the overall stress response (OSR) to elevated HP; a concept that we suggest to be applicable to other environmental stressors. I highlight the importance of

considering more than a single aspect of the stress response in physiological studies, particularly in an era where multi-stressor studies are proliferating.

4.2 Introduction

Stress, and the way organisms respond to stressors, is undoubtedly one of the most important concepts in biology, influencing an organism's physiology at all levels of organisation from molecular to whole organism, as well as their ecology (Bjilsma and Loeschcke 1997; Feder 1999). The ability of organisms to respond to stress plays a pivotal role in a species' potential to migrate, acclimatise, or adapt to new or changing environments, and thus the processes of evolution (Somero 2010). Of the wide variety of potential stressors, biotic and abiotic, there is a set of universal responses, which at the cellular level are known as the cellular stress response (CSR) (Kültz 2005). The highly conserved nature of the CSR has meant that genes and encoded proteins such as heat shock proteins and redox regulatory enzymes have been widely utilised as markers of stress in physiology (Nakasone et al. 1998; Feder and Hofmann 1999). There are, however, other aspects of an organism's stress response that can be stressor-, or scenario-specific. The cellular homeostatic response (CHR) is one such mechanism, which uses stressor-specific sensors to assess changes in specific environmental variables (Tomanek 2011). These mechanisms are likely to be the cellular basis of an organism's acclimatory or acclimatisation response to changing environmental parameters. The cellular homeostatic-type response is not transient like the CSR and will remain, unless environmental conditions change or other more energy efficient mechanisms take over (Kültz 2005). However, not all responses to stress manifest solely at the cellular level; stress responses can be manifest as behavioural modification. Escape responses are a typical example of a behavioural stress response. The archetypal escape response involves an increase in motor activity in order to "escape" from either a biotic stressor such as proximity to a predator, or an abiotic stressor such as a rapid increase in environmental temperature. Behavioural responses are distinct from cellular/molecular level responses, yet both influence each other. A behavioural response such as abdomen flicking will increase metabolic demand thus increasing the transcription of metabolic markers. Likewise, induction of the CSR involves up-regulation of genes involved in metabolism (Kültz 2005). Further, rapid muscle contraction, such as abdomen flicking, has been shown to induce a heat shock response (HSR) (Paulsen et al. 2007). It is clear, then, that in order to understand the complete stress response repertoire of an organism, signatures of all major aspects of the response, and

their relation to one another, need be considered. Henceforth, the complete stress response of an organism, comprising the CSR, CHR, behavioural response, and metabolic response will be termed the overall stress response (OSR).

Hydrostatic pressure (HP) represents the single largest continuous environmental gradient on the planet (MacDonald 1997). As such, all biological processes are affected by HP at varying degrees (Pradillon and Gaill 2006). In the marine environment it has been argued that all organisms have upper and lower bathymetric limits (Tyler and Young 1998). These limits are delineated by a large variety of abiotic and biotic factors, of which HP may be one of the most important (Brown and Thatje 2014). HP is a thermodynamic variable as well as a biological stressor, and thus affects all levels of biological organisation from population structuring to molecular interactions (Boonyaratanakornkit et al. 2002; Brown and Thatje 2014). Exposure to changes in HP has been shown to elicit a number of varied stress responses in a range of marine organisms (Brauer and Torok 1984; Forward and Wellins 1989; Bartlett 2002; Oliphant et al. 2011; Cottin et al. 2012; Smith and Thatje 2012). Studying the effects of increased HP is important in order to gain a better understanding on the potential for marine organisms to respond to rapid anthropogenic-induced ocean warming. Such responses have already been shown to include bathymetric range shifts; the movement of ectotherms to deeper, relatively cooler, waters to remain within their thermal envelope (Perry et al. 2005; Weinberg 2005; Dulvy et al. 2008).

Previous research has investigated the ability of shallow-water invertebrates to tolerate large changes in temperature and HP in combination (Cottin et al. 2012; Morris et al. 2015; Chapter 3). Yet, in order to truly understand the effects of these two important and co-varying factors, it is important to understand the effects of each stressor in isolation. The aim of this study was to quantify the effects of elevated HP during exposure and during recovery across organisational levels changes in gene transcription to overt behavioural responses. These results will help better understand the effects of HP as component of multi-stressor scenarios, and to identify the hierarchy of those effects. The chosen study organism for this research was the Atlantic ditch shrimp *Palaemonetes varians* (Decapoda: Caridea) (Leach 1814). *P. varians* has a eurythermal and euryhaline physiology, and is distributed across North Atlantic brine marshes. It is closely related to a number of species that inhabit deep waters (*Periclimenes* sp.) (Li et al. 2011) but has a strictly shallow-water distribution (<10 m). This species has been used in several complimentary studies in recent

years and represents a future potential experimental model organism in HP and thermal physiology (Oliphant et al. 2011; Cottin et al. 2012; Ravaux et al. 2012). A set temperature of 15°C was chosen as it is within the optimal temperature envelope of the species, and was shown previously to elicit sub-lethal responses in combination with elevated HP (Oliphant et al. 2011; Morris et al. 2015; Chapter 3).

For the molecular aspect of this study, genes were chosen that previously had been shown to exhibit differential expression in response to acute temperature and HP exposures (Cottin et al. 2010; Cottin et al. 2012; Morris et al. 2015; Chapter 3). The *gapdh* gene and the two *hsp70* isoforms form part of the universal CSR (Kültz 2005). The *narg* gene has been suggested as a proxy for the onset of HP-specific stress associated with neuronal pathologies characterised by high pressure neurological syndrome (HPNS), and may represent a component of the CHR (Morris et al. 2015; Chapter 3). The *β-actin* gene has been previously shown to be responsive to HP increases, which, although not entirely resolved, may be associated with loss of conformation and function of actin proteins under increased HP scenarios (Somero 1992; Procaccio et al. 2006). These genes were chosen as they represent different aspects of the OSR. Previous studies quantifying the expression of these genes have only studied their expression at a single time point during, or following, a stress exposure (Cottin et al. 2012; Morris et al. 2015; Chapter 3). The novelty of this study lies in tracing changes in transcription of each of these genes at various time points following an acute stress exposure. To complement the gene transcription analysis, this study also examines metabolism via respiration rate analysis, and analysis of various behavioural characteristics during the experimental exposure, and at various time points into the recovery period. Whole organism metabolism and overt behavioural characteristics are also important aspects of the proposed OSR, and represent changes in phenotype that further validate the observed changes in gene expression. Data presented provide a novel insight into the kinetics and hierarchy of responses to HP stress, and are discussed in the context of generally applicable mechanisms of stress response and recovery.

4.3 Specific chapter hypotheses

$H_{4.1}$ – Previously identified gene biomarkers of HP intolerance in juvenile *Palaemonetes varians* (Chapter 3) are also transcriptionally regulated in mature adults in response to acute elevated HP.

$H_{4.2}$ – Changes in transcriptional regulation of gene biomarkers are observed concurrently with changes in oxygen consumption and overt behavioural characteristics.

$H_{4.3}$ – Observed changes in gene expression, respiratory rate, or behaviour in response to elevated HP occur at different points during the exposure period, and endure for differing time periods over the recovery period. These differential response times may be indicative of a hierarchical system of responses.

$H_{4.4}$ – The expression of previously identified gene biomarkers is tissue specific, and thus differential expression will be observed between muscle-rich abdomen tissue and organ-rich head tissue. These differential expression patterns may indicate tissue-types that are particularly sensitive to HP increases.

4.4 Materials & methods

4.4.1 Maintenance of *Palaemonetes varians*

Adult male *Palaemonetes varians* (total length = 4-6 cm) were net caught from Lymington salt marshes, UK on the 2nd October 2013 (Section 2.1.1). The shrimp were acclimated to 15°C ± 0.5°C from the temperature they were caught at, at a rate of 1°C per day. They were kept under a 12/12 light/dark cycle in aerated filtered (1 μm) seawater (salinity: 32-34). The shrimp were fed with fish flakes *ad libitum* (TetraMin™) 3 times a week, and water changes were made the day following each feeding. The shrimp were kept under these conditions for at least 10-days prior to exposures.

4.4.2 Experimental HP exposure

The IPOCAMP system (Shillito et al. 2014) was used to conduct acute two-hour, 10 MPa HP exposures (Section 2.2.2). Prior to the treatment, the IPOCAMP system was filled with aerated filtered seawater (salinity: 32 – 34) and acclimated to 15°C ± 0.1°C. The HP of the system was ramped from ambient (0.1 MPa) to 10 MPa at 10 MPa min^{-1}. The system was then held at 10 MPa and 15°C for 2-hours followed by a 1-minute depressurisation back to ambient.

4.4.3 Gene expression analysis

Following the experimental exposure, the shrimp were removed and placed back into their original aquarium at 15°C, and kept in the dark. Ten shrimp were randomly sampled at: 0-hours (within 5-minutes of the HP exposure), 1-hour, 3-hours, 6-hours, 8-hours, and 24-hours post treatment. Ten shrimp were also randomly sampled prior to the experimental treatment for a baseline control group comparison. These shrimp were placed under the same conditions in the IPOCAMP system, but without any change in HP. Shrimp were decapitated: single clean cuts were made vertically from the dorsal extension of the carapace, separating the carapace and the 1st abdominal segment. Both portions were placed in individual 1.5 ml RNase and DNase-free micro-centrifuge tubes and snap frozen instantly. Samples were kept at -80°C until further use.

Tissue samples were transferred into 2.2 ml (Head tissue) 3 ml (Abdomen tissue) of TRI-Reagent™ (Sigma-Aldrich) and homogenised. Total RNA was extracted as described in Section 2.5.1. Total RNA purity and integrity were assessed according to Section 2.5.1.1. Total RNA was DNase-treated as described in Section 2.5.2. DNase-treated total RNA was reverse-transcribed according to Section 2.5.3.

Specific primers for qPCR for all genes were obtained from Section 2.8 - Table 2.3. All primer-sets tested generated a single and discrete peak by melt curve analysis (Section 2.8.4). qPCR assays were optimised, as set out by the MIQE guidelines (Bustin et al. 2009), and listed in Sections 2.8 & A1.4. All qPCR reactions were performed on a Stratagene MxPro 3005 (Agilent, UK) (Section 2.8.1.2). Each 25 µl reaction contained 12.5 µl of Precision 2x qPCR Master mix (Primer-Design, UK) with SYBR green and ROX reference dyes (Sections 2.8.2 and 2.8.1.2), and 1 µl of template cDNA. qPCR conditions were as described in Section 2.8.6. Samples were run in duplicate (technical replicate), and melt curve analysis was performed after each run in order to demonstrate the specificity of the qPCR products (Section 2.8.4).

Candidate reference genes were tested by geNorm analysis using qBase+ software (Section 2.8.8). Of 4 candidate reference genes, the combination of the *rps26* gene and the *rpl8* gene was deemed to provide the best normalisation strategy for this study, both showing stable expression across experimental conditions. Normalised relative quantities (NRQs) were scaled to the baseline control treatment and presented as relative fold changes (RFC) (Section 2.8.7). Statistical significance was identified at $p < 0.05$ as determined by GLM and

post-hoc Tukey-HSD test using R statistical software (R core team 2013) and the "multcomp" package (Horthorn et al. 2008). For the anatomical section comparison, a nested GLM was performed followed by a Tukey-HSD test, and statistical significance was determined with Bonferroni correction.

4.4.4 Respiration rate analysis

Respiration analysis was conducted in identical experimental HP exposures to quantify the effects of elevated HP on whole organism metabolism, and validate the changes in the transcription of metabolism related genes. Five shrimp were placed in sealed individual 55 ml falcon tubes immediately prior to the experimental exposure and placed horizontally into the IPOCAMP system (Sections 2.2.2 & 2.3). Three control tubes containing no shrimp were also sealed to control for bacterial respiration (Section 2.3). The volume of the tube was determined in preliminary experiments, and allowed for measurable changes in oxygen saturation to be observed. The tubes were removed from the IPOCAMP system immediately following the experimental exposure. Oxygen saturation measurements were taken less than 5-minutes following exposure. The shrimp were then returned to 100% air saturated seawater. Further 2-hour respiration measurements were taken at 3-hour post-exposure, 8-hours post-exposure, and 26-hours post-exposure. Baseline respiration measurements were also taken by the same method using 5 shrimp and 3 control tubes not subjected to elevated HP exposure. Oxygen saturation was determined using an oxygen micro-optode connected to a Microx TX3 array as described in Section 2.3. Oxygen consumption (MO_2, μmol O_2 mg^{-1} h^{-1}) was calculated, against controls, following standard methods for determining oxygen concentration in air-saturated seawater (Benson and Krausse 1984) (Section 2.3 – Equations 2.1 to 2.5). Respiration rates were normalised against total fresh mass weight for each shrimp. Statistical significance was identified at $p < 0.05$ as determined by one-way ANOVA and post-hoc Tukey-HSD.

4.4.5 Behavioural analysis

Behavioural analysis was conducted in identical experimental HP exposures in order to corroborate any observed changes in gene transcription and metabolism, and provide further insight into the effects of HP at the whole organism level. Ten shrimp were placed into the IPOCAMP system inside a PVC viewing cage mounted inside the HP chamber

(Ravaux et al. 2003; Shillito et al. 2006)(Sections 2.2.2 & 2.4). The IPOCAMP system was sealed and run at atmospheric pressure (0.1 MPa) for 1-hour prior to behavioural analysis to allow the shrimp time to acclimate and recover from any handling stress. Behaviour was measured by video recording through a viewing port in the IPOCAMP system lid (Shillito et al. 2006) using an endoscopic camera (Section 2.4). Behaviour was recorded; for 2-hours prior to experimental exposure (baseline measurements); during the 1-minute HP ramp, 2-hour HP exposure, and 1-minute decompression; and then for 2-hour periods at 1-hour post-exposure, 6-hours post-exposure, and 24-hours post-exposure. Behaviour was measured and characterised for 30-seconds at 5-minute intervals over each recording period, and throughout the HP ramp and decompression stages. Behaviour was characterised into distinct categories, as originally described by Oliphant et al. (2011), and adapted as described in Section 2.4. Shrimp behaviour was noted as "motionless", "active movement", or "tail flicking" (Section 2.4), and the position of the shrimp in the chamber was quantified according to Section 2.4.

4.5 Results

4.5.1 Gene expression analysis

The relative expression of 5 genes showed significant increases in comparison to an unstressed baseline measurement in head and abdomen sections of *Palaemonetes varians* at varying time points after exposure to a 2-hour, 10 MPa HP treatment, as shown by Figure 4.1. The relative expression of the *narg* gene showed significant increases against baseline measurements from immediately post-exposure (0-hours) up to 3-hours after exposure in the head section and up to 6-hours after exposure in the abdomen (Figure 4.1). The maximal level of expression in the head was reached 3-hours after exposure (RFC = 7.4), whereas in the abdomen section maximal expression of the *narg* gene was observed after 1-hour (RFC = 12.2). In the head section there was no significant difference between samples taken 6-, 8- or 24-hours post-exposure in comparison to the unstressed baseline measurements. Similarly, no significant difference was seen after 8-hours post-exposure in the abdomen.

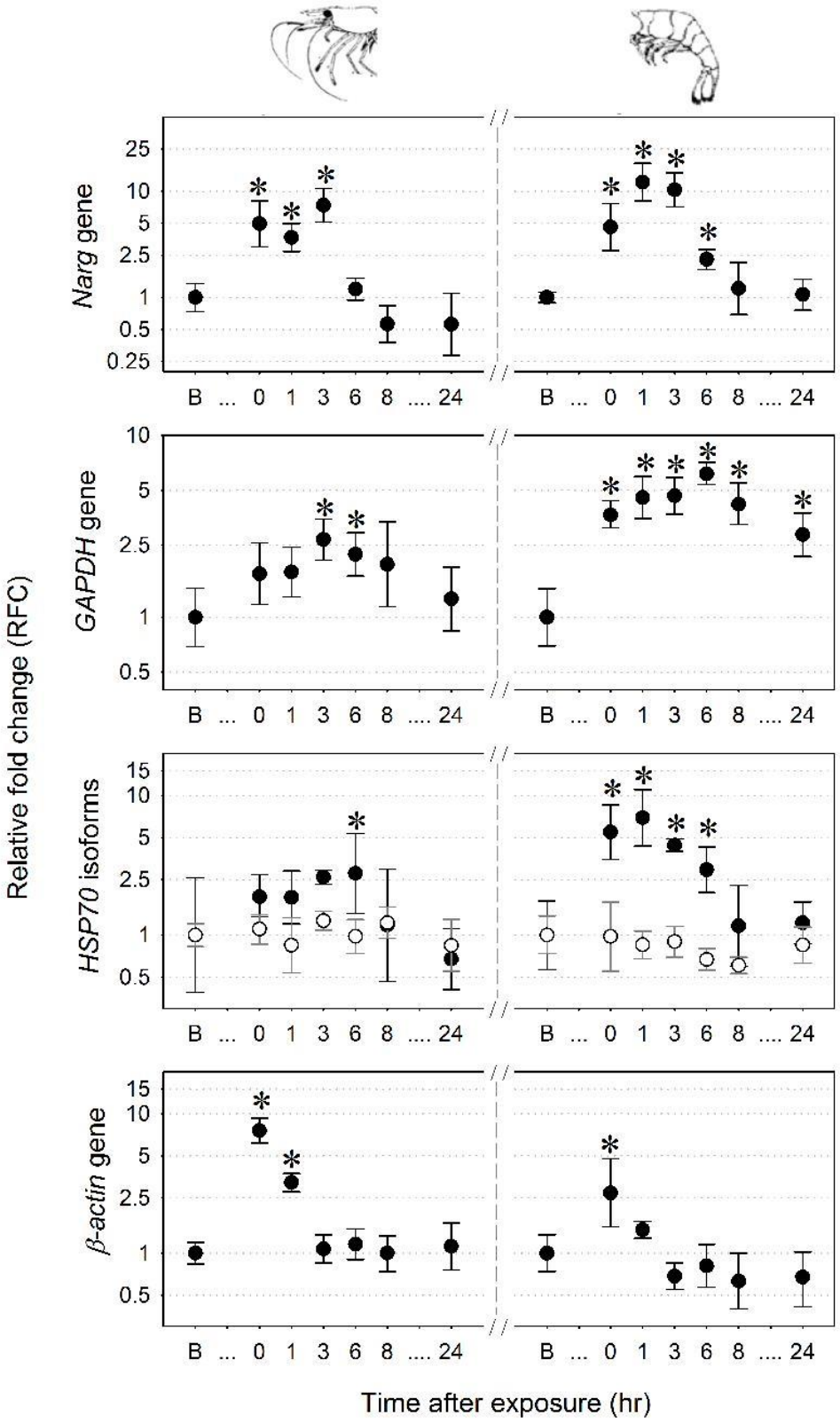
The *β-actin* gene showed significant, and maximal, expression immediately after experimental exposure in both the head (RFC = 7.6) and the abdomen (RFC = 2.7) (Figure 4.1). The head also showed significant expression of the *β-actin* gene an hour after

exposure, whereas the associated abdominal sample did not (Figure 1). There was no significant difference in relative expression of the *β-actin* gene 3-hours after exposure in the head, and 1-hour after exposure in the abdomen.

The *gapdh* gene showed significant increases in relative expression at all measured time points from 0-hours to 24-hours after exposure in the abdomen with maximal expression at 6 hours post-exposure (RFC = 6.1) (Figure 4.1). The head showed significant increases in relative expression of the *gapdh* gene at 3-, 6- and 8-hours post-exposure (Figure 4.1). However, no significant changes in relative expression were observed at 0-, 1- or 24-hours after exposure in comparison to the unstressed baseline measurements.

Two isoforms of HSP70 were tested. The *hsp70 f1* gene (form 1) showed significant increases in the relative expression only at 6-hours post-exposure in the head (Figure 4.1). In the abdomen, significant increases in the relative expression of the *hsp70 f1* gene were observed between 0- and 6-hours post exposure, with maximal expression seen at 1-hour post exposure (RFC = 6.9) (Figure 4.1). The second isoform, the *hsp70 f2* gene, showed no significant differences in relative expression at any time point in either the head or abdomen in comparison to baseline measures.

All individual head and abdomen samples were analysed using a nested general linear model (GLM) ("Time" nested within "Anatomical section" as independent variables) to provide a comparison of relative expression between head and abdomen sections at each time point (Table 4.1). Following this, the data were pooled to compare overall differences in expression between the head and abdomen, and significance was tested using an appropriate Bonferroni-corrected p-value. The *narg* gene showed no overall significant difference in relative expression between the head and abdomen, however there was significantly higher expression in the abdomen at 1-hour after exposure (Table 4.1). The *β-actin* gene showed significantly higher relative expression in the abdomen in comparison to the head immediately after exposure, and 1-hour post-exposure (Table 4.1). Likewise, the *gapdh* gene also showed significantly higher relative expression in the abdomen in comparison to the head at all experimental time points (Table 4.1). The *hsp70 f1* gene showed significantly higher expression in the abdomen than in the head up to 1-hour post-exposure, no significant difference in expression was seen between the anatomical sections at 3- and 6-hours post-exposure (Table 4.1). There was no significant difference in the relative expression of the *hsp70 f2* gene between the head and abdomen (Table 4.1).



Legend overleaf

Figure 4.1 Changes in relative fold change (RFC) in head (left column) and abdomen (right column) sections of; the *narg* gene, the *gapdh* gene, the *hsp70 f1* (black dots) and *hsp70 f2* genes (white dots), and the *β-actin* gene in; at; 0-hours, 1-hour, 3-hours, 6-hours, 8-hours, and 24-hours in *Palaemonetes varians* after a 2-hour, 10 MPa HP shock. Statistical comparisons were made against a baseline measurement (B) of each gene under non-stressful conditions, and graphs were scaled against the baseline measurements. Statistical significance is displayed as; * = $p<0.05$ determined by a GLM and a post-hoc Tukey-HSD test. Relative fold change and 95% confidence intervals are calculated from 5 biological replicates

4.5.2 Respiration rate analysis

The acute 2-hour HP exposure significantly ($p<0.05$) affected the respiration rate, or oxygen consumption (MO_2, μmol O_2 mg^{-1} h^{-1}) of the shrimp (Figure 4.2). In comparison to unstressed baseline measurements oxygen consumption was significantly higher during the exposure period, and also up to 3-hours into the recovery period (Figure 4.2). After 8-hours of recovery, oxygen consumption had returned to the baseline measurement, and after 26-hours of recovery oxygen consumption was significantly lower than all other measurement points including the unstressed baseline measurement (Figure 4.2).

4.5.3 Behavioural analysis

Behavioural data were not analysed statistically due to the complex nature of the observations, and the inherent problems of autocorrelation and pseudo-replication within the time-series observational dataset. It was considered to be more principled to simply present the data without statistical inference rather than pool observations and rely on pseudo-replication to gain statistical significance. Only clear behavioural patterns are noted and discussed.

Video analysis of vertical position of shrimp within the chamber (Figure 4.3) shows that the baseline measurement and recovery time periods have similar patterning. These distributions appear consistent throughout the 2-hour analysis in each case. For the baseline measurement, and the 1-hour, 6-hour and 24-hour recovery measurements, the majority of shrimp occupied the bottom half of the chamber. Conversely, during the 2-

hour HP exposure, the majority of shrimp occupied the top half of the chamber (Figure 4.3). Again, this observation is consistent throughout the 2-hour exposure period.

Video analysis of behavioural types throughout the experiment show similar patterns between the unstressed baseline measurement (Figure 4.4a), and 6- and 24-hour recovery time periods (Figure 4.4f and 4.4g). In these cases, the majority of shrimp remained motionless. During the HP ramp (Figure 4.4b) the shrimp showed no obvious changes in behavioural type until around 45-seconds into the ramp at a HP of approximately 7.5 MPa. Beyond 7.5 MPa, an increase in active movement and tail flicking behaviour was observed. Tail flicking was observed regularly for the first hour of the HP exposure (Figure 4.4c), and the majority of shrimp showed active movement throughout the HP exposure (Figure 4.4c). During the 1-minute decompression step, no clear change in behavioural type was observed with the majority of shrimp showing similar active movement behaviour at the start of the decompression (10 MPa), and at the end of the decompression (0.1 MPa) (Figure 4.4d).

Table 4.1 A nested comparison (Time nested within Anatomical section) between the relative fold change of each gene between head and abdomen sections of *Palaemonetes varians* at each time point. Comparisons were only made at time points that were significantly different from the control baseline treatment (/ = no comparison made). Statistical significance was determined by GLM and post-doc Tukey HSD test: * = $p<0.025$, ** = $p<0.01$

	narg	*gapdh*	*hsp70 f1*	*hsp70 f2*	*β-actin*
0-hours	No SD	Abdomen*	Abdomen**	/	Abdomen*
1-hour	Abdomen**	Abdomen**	Abdomen*	/	Abdomen*
3-hours	No SD	Abdomen*	No SD	/	/
6-hours	No SD	Abdomen**	No SD	/	/
8-hours	/	Abdomen*	/	/	/
24-hours	/	Abdomen*	/	/	/
Pooled	**No SD**	**Abdomen***	**Abdomen***	**No SD**	**Abdomen***

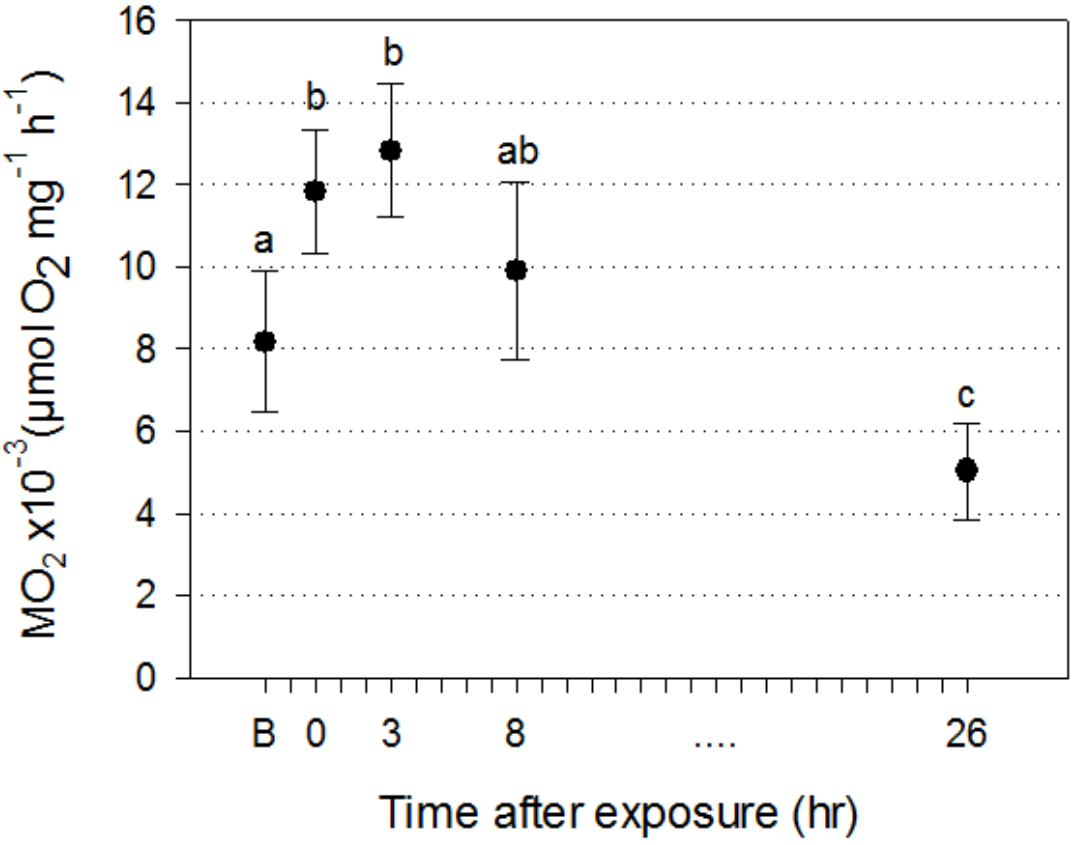


Figure 4.2 Changes in oxygen consumption of *Palaemonetes varians* (MO_2 = x10^{-3} µmol O_2 mg^{-1} h^{-1}) during the 2-hour acute 10 MPa HP exposure (E), and at 3-hours, 8-hours, and 26-hours into recovery. Exposure and recovery data are presented alongside a 2-hour unstressed baseline measurement of oxygen consumption (B). Statistical comparisons were made across all time periods, and statistically significant differences ($p<0.05$ one-way ANOVA and post-hoc Tukey-HSD, calculated using R statistical software) are denoted by a different letter: a, b or c. Error bars represent standard deviations. Oxygen consumption is calculated as the mean and standard deviation of 5 biological replicates against the mean of 3 control measurements

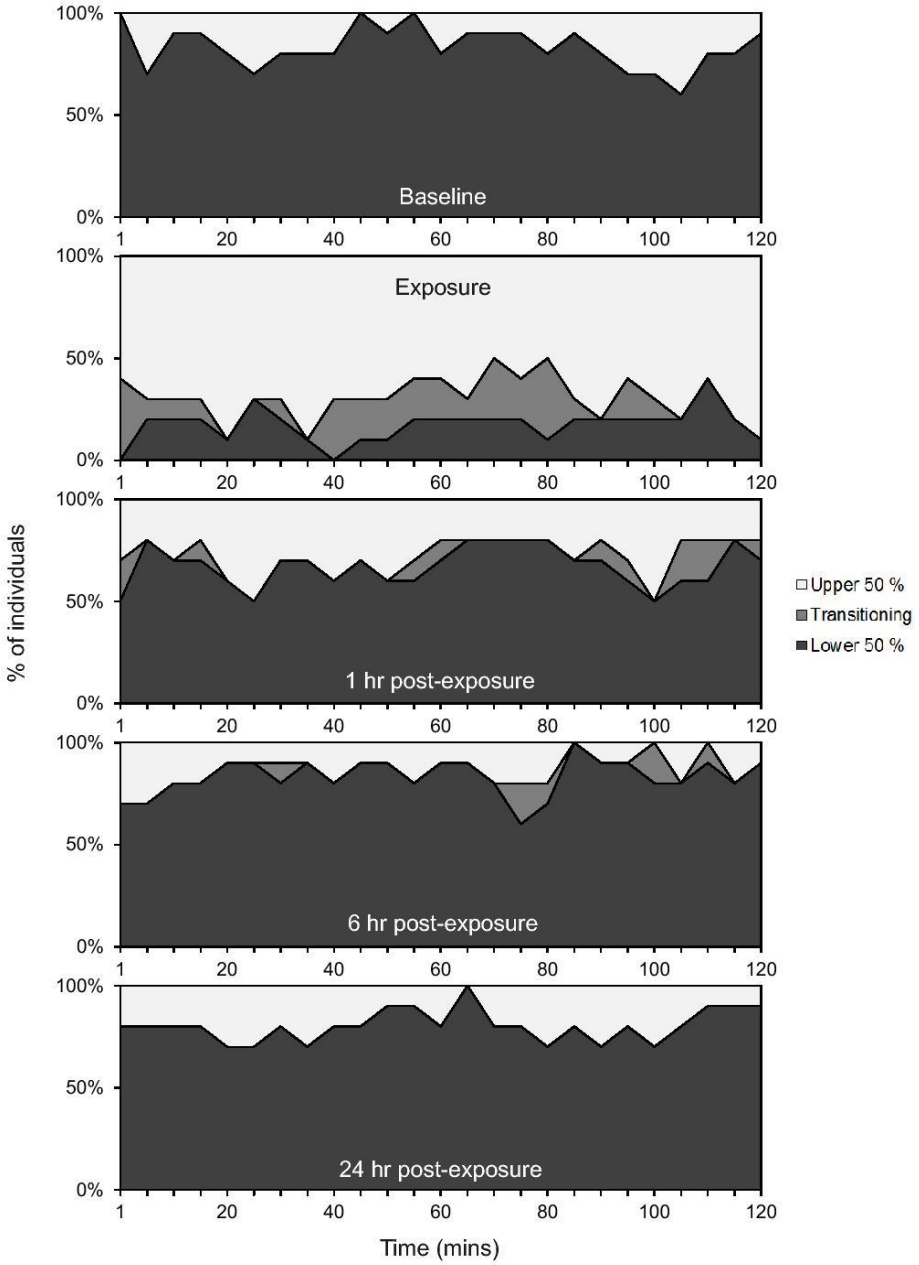
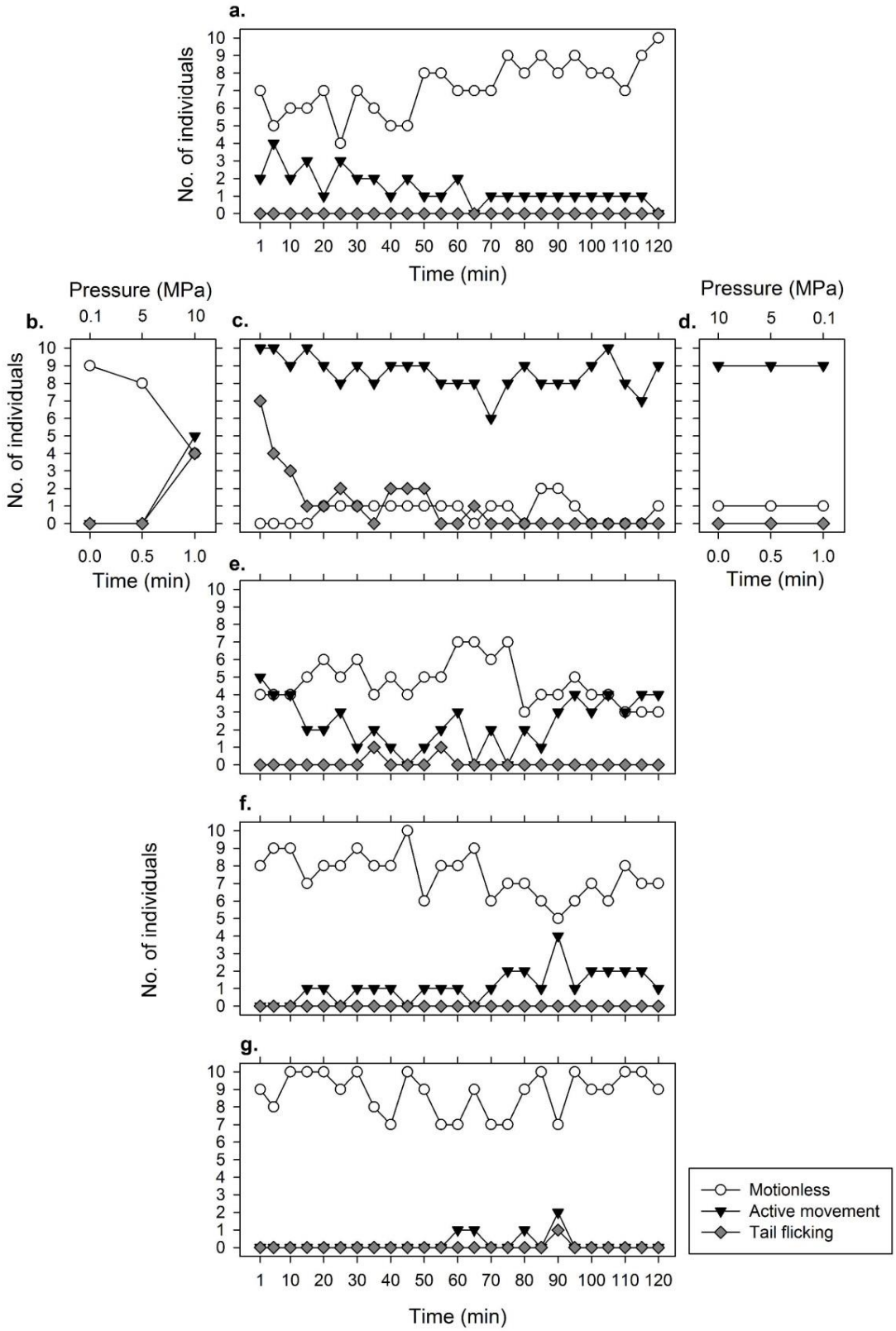


Figure 4.3 Stacked area plots representing changes in the observed vertical distribution of shrimp (*Palaemonetes varians*) in the high pressure (IPOCAMP™) system viewing chamber across time in; the 2-hour 10 MPa exposure; during the recovery period, and including a 2-hour unstressed (0.1 MPa) baseline observation. Vertical distributions are presented as % proportion of individuals, and calculated from 10 biological replicates. The dark grey area represents the proportion of individuals occupying only the lower 50% of the chamber. The light grey area represents the proportion of individuals moving between the lower and upper halves of the chamber, and the white area represents shrimp occupying only the upper 50% of the chamber



Legend overleaf

Figure 4.4 Changes in the observed behavioural category of the shrimp (*Palaemonetes varians*) at; **a.** a two hour unstressed baseline measurement; **b.** the 1-minute HP ramp from atmospheric pressure (0.1 MPa) to 10 MPa (rate = 10 MPa min^{-1}); **c.** the 2-hour 10 MPa HP exposure; **d.** the 1-minute decompression from 10 MPa to atmospheric pressure (0.1 MPa); **e.** 1-hour post-exposure; **f.** 6-hours post-exposure; and **g.** 24-hours post-exposure. Open circles represent "motionless", black triangles represent "active movement", and grey diamonds represent "tail flicking". Specific definitions of each behavioural category can be found in the Section 2.4

4.6 Discussion

In order to understand the potential for shallow-water ectotherms to undergo climate-driven bathymetric range shifts, a holistic understanding of the wide-ranging and multilevel effects of increased HP is required first. In accordance, this study presents an analysis of the effects of a 2-hour acute hydrostatic exposure in a shallow-water shrimp at multiple levels of biological organisation over the exposure period, and during the recovery phase. Although it is accepted that a 2-hour rapid exposure to HPs equivalent of 1000 m is by no means ecologically relevant in itself, the results of this study provide a solid basis for which longer-term, more ecologically relevant exposures can be conducted.

In this study, significant increases in the transcription of four genes; the *narg* gene, the *gapdh* gene, the *hsp70 f1* gene, and the *β-actin* gene were recorded in both head and abdomen of *Palaemonetes varians* at various time points from 0- to 24-hours following exposure to an acute two hour HP treatment. A second hsp70 isoform, the *hsp70 f2* gene, which has previously been characterised as non-inducible following a heat stress (Cottin et al. 2010), was not transcriptionally regulated at any of the time points. Further, respiration rates of the shrimp showed similar patterns to that revealed by the expression of the genes described above. This was supported by behavioural observations showing active movement during the acute HP exposure, which were coupled with a higher proportion of shrimp occupying the upper half of the viewing chamber. These data, from distinct levels of organisation, give a novel insight into the relative contribution of a variety of distinct stress responses that collectively contribute to what we term the overall stress response (OSR) upon exposure to a stressor, in this case an acute exposure to elevated HP.

Up-regulation of the *narg* gene with increased HP has been suggested as a proxy for the onset of physiological HP intolerances associated with high pressure neurological syndrome (HPNS) (Morris et al. 2015) (Chapter 3). The *narg* gene codes for an NMDAR-regulated protein. NMDARs have been shown to become over-active in response to elevated HP in non-adapted organisms (Rostain et al. 1986; Millan et al. 1989). NMDAR over-activity is associated with behavioural pathologies that likely prevent long-term survival under elevated-HP scenarios (Morris et al. 2015). Acclimation to elevated HP conditions may require a shift in NMDAR functionality in order to negate the identified behavioural pathologies associated with non-normal NMDAR functioning (Morris et al. 2015), one such homeostatic shift might be the transcription of novel NMDARs to counteract over-activity. NMDARs are well known to change density depending on functionality by a process of synaptic plasticity (Liu et al. 2004), yet no evidence has so far been presented showing novel NMDAR transcription in response to elevated HP. No differences in levels of relative expression of the *narg* gene were seen between head and abdomen sections. This is unsurprising as expression of this gene is associated with neural tissue, which is distributed throughout the organism: reports of the nervous system structure in caridean shrimp identified major ganglia and neural networks in both head and abdomen portions of the organism (McLaughlin, 1983). We hypothesise that *narg* gene expression represents a component of the cellular homeostatic response (CHR). This represents a shift in homeostatic-point in nervous tissue (receptor over-activity) that may be counteracted by a cellular homeostatic-type response.

70 kDa heat shock proteins (HSP70s) are commonly used markers of cellular stress. An increase in expression of genes coding for HSP70 isoforms may indicate an increase in intracellular macromolecular damage (Feder and Hofmann 1999), although care must be taken with such inferences in isolation (Morris et al. 2013; Chapter 1). Results of this study confirm that the *hsp70 f1* gene is responsive to acute HP stress. The relative fold change of the *hsp70 f1* gene shown in this study is lower, however, than observed in previous research in response to a large temperature shock (Cottin et al. 2010; Ravaux et al. 2012). The *hsp70 f2* gene showed no changes in expression at any time points, thus in response to HP stress alone; the gene remains constitutively expressed. The *hsp70 f1* gene showed significantly higher expression in the abdomen than in the head. The abdomen was also shown to exhibit a higher degree of expression in post-exposure samples in comparison to unstressed baseline measurements. Muscle-rich abdomen tissue may therefore be exhibiting a

particularly high degree of intracellular macromolecular damage. The expression of HSP70 isoforms has been well documented as being a vital component of the CSR (Feder and Hofmann 1999; Sorensen and Loeschcke 2007; Benarroch 2011). HSP70 expression occurs not only as a response to increasing macromolecular damage during stress exposures but predominantly post-exposure as a recovery mechanism during which macromolecular damage might still be prevalent within the cell (DiDomenico et al. 1982; Tomanek and Somero 2000). Although difficult to quantify, the heat shock response and CSR come with clear costs associated with the up-regulation of genes and production of proteins (Sorensen et al. 2003). These costs will have knock-on effects on energy budgets and distribution, and therefore metabolism. The data presented here suggest that these costs occur during an acute exposure to HP, but also, and to a greater magnitude, during recovery from exposure.

The *gapdh* gene codes for an enzyme involved in glycolysis. Up-regulation of the *gapdh* gene may signify an increase in cellular metabolism (Chapter 3). Significantly higher relative expression of the *gapdh* gene was seen in the abdomen section in comparison to the head section. Expression of metabolic-related genes is likely to be higher in tissues that have higher metabolic requirements (Barber et al. 2005). In this case, the difference in expression between head and abdomen sections is probably due to a higher density of metabolically-demanding muscle tissue in the abdomen region rather than the head. This metabolic proxy may represent an accumulation of the metabolic demands of all other components of the OSR; for example, behavioural responses, the CSR, and the CHR, each having their own metabolic costs. As expected, up-regulation of the *gapdh* gene occurs over time points covering the up-regulation of genes related to the other aspects of the OSR.

The *β-actin* gene encodes an isoform of the actin protein family. β-actin is a non-muscular cytoskeletal isoform, and is commonly used as a reference in qPCR studies. However, in a number of pilot studies to determine suitable reference genes, the *β-actin* gene was shown to be consistently up-regulated under increased HP scenarios in *P. varians* (Morris et al. 2015; Chapter 3). In this study, the *β-actin* gene showed the most transient response of all the genes tested, with up-regulation highest during the exposure. Although there is no clear evidence why such transcriptional regulation is observed, in humans, a mutation leading to loss of function in the gene coding for β-actin was found to be linked to the onset of dystonia (Procaccio et al. 2006), a movement disorder characterized by involuntary muscle contractions, similar in symptom to that observed in marine invertebrates under elevated HP (Oliphant et al. 2011). The actins of deep-sea adapted fish have been shown to differ

from shallow-water adapted fish, with the deep-sea adapted actins showing greater resistance to polymer dissociation under elevated HP (Swezey and Somero 1982). It has been hypothesised that increasing HP causes a loss of structure and function in non-HP adapted actins such as those found in shallow-water restricted marine invertebrates (Somero 1992). Loss of functional conformation in actin proteins may trigger further transcription of actin genes, leading to our observed transcriptional increases. HP increases have wide effects on genes at the transcriptional level many of which are still not fully understood, but are still important to consider, as they undoubtedly have metabolic costs associated with them, and this may have far reaching implications on the ability of shallow-water organisms to tolerate and acclimate to deeper waters.

Respiration rate measurements show an increase in respiration during HP exposure and correlate well with the gene expression data, suggesting that the acute HP increase is stressful not only at cellular and molecular levels, but also extends across whole organism physiology. Respiration rate further increases during the recovery period up to 3-hours after the exposure. Again, this matches well with the gene expression data showing maximal expression of CSR and metabolism related genes during the recovery period rather than the acute exposure period. The rise in oxygen consumption following elevated HP exposure suggests that an oxygen debt is being accrued over the exposure period that is then being recompensed during the recovery phase. Interestingly, 8-hours into the recovery period the oxygen consumption of the shrimp had returned to a similar level to the unstressed baseline measurement, yet at 26-hours after exposure the mean oxygen consumption of the shrimp was significantly lower than any other measurement, including the unstressed baseline. This drop in metabolism may represent another aspect of the recovery from an acute HP exposure, namely metabolic depression as an energy conservation mechanism (Guppy and Withers 1999). The transcriptional and respiratory responses of the shrimp during exposure are likely to have significant energy costs associated with them. Although the data presented here suggest that the oxygen debt accrued during the exposure was repaid within 8-hours of recovery, the energy debt may not have been, and thus the drop in metabolic rate at 26-hours may represent an energy conservation mechanism, or simply a response to dwindling energy reserves.

Further to the observations of transcriptional and respiratory responses to the acute 2-hour exposure, behavioural observations show a clear increase in active movement during the

acute exposure, throughout the 2-hour period. Active movement remained high immediately following the exposure and slowly decreased over a 3-hour period, correlating well with the observed oxygen consumption rates and gene expression over the experiment. Tail flicking, a commonly observed escape response in shrimp (Arnott et al. 1998), showed a pronounced increase during the final stages of the HP ramp, and over the first hour of the HP exposure. This may be a reaction to the HP increase itself rather than the high HP, or it is a response to HPs above a threshold (observed at around 7.5 MPa). The reduction in tail flicking behaviour may then be explained by the energy cost associated with it, this type of explosive reflexive contraction is likely to have a high energy cost in comparison to normal swimming behaviour (Arnott et al. 1998). Novel observations of vertical distribution of the shrimp in the viewing chamber across the experiment show a clear pattern: the majority of shrimp occupy the lower half of the chamber during unstressed baseline measurements, similarly for all recovery time periods, but occupy the upper half of the chamber during exposure. This pattern of vertical distribution in response to the HP exposure may represent a form of behavioural homeostasis (Johnson et al. 1992), in which the shrimp are actively seeking lower HPs (shallower waters) to counteract the negative physiological effects of the increased HP exposure. Behavioural homeostasis has been shown in marine ectotherms in response to hypoxia (Gorr et al. 2010) where, in response to hypoxia, organisms actively seek cooler water where oxygen saturation is higher by the nature of the temperature effect. Behavioural homeostasis is a whole organism response to a stressor such as HP that, if successful will have effects on other aspects of the stress response, for instance, negating the need for a pronounced CSR.

These results show that although each quantified response is distinct, they are all inexorably linked. Upon exposure to acute elevated HP, the first response observed was an increase in overt behaviours, such as active movement and tail-flicking escape responses. These behaviours are energetically costly and are followed by an increase in oxygen consumption and the transcription of metabolism-related genes. At the same time, elevated HP denatured intracellular macromolecules, promoting the induction of the CSR: another energetically costly mechanism that has implications on oxygen consumption and gene transcription. Transient responses to elevated HP abate once atmospheric pressure was restored; however, recovery mechanisms such as the CSR continue, requiring further energy. Putative initiation of CHR was also observed during acute exposure and continued into the recovery period. The continued energetic demands of these responses may

eventually lead to the induction of energy conservation mechanisms such as metabolic depression. Overall, the effects of a 2-hour acute elevated HP exposure can be observed up to at least 26-hours into recovery. This observed response will appear different depending on the time point observed, and the component of the OSR observed. Thus, the results highlight the importance of considering a number of aspects of the OSR, as well a number of time points during, and following, exposure in order to properly assess the effects of a given stressor or stress scenario.

4.7 Conceptualisation of the overall stress response (OSR)

Figure 4.5 shows a conceptual model describing the hierarchy and kinetics of a variety of stress response mechanisms. The model is based on responses observed to an acute elevated HP exposure in a shallow-water adapted marine invertebrate. However, as previously described, although stressors and stress scenarios differ dramatically, observed organismal responses tend to be highly conserved, and fall into distinct categories. The highly conserved nature of organismal responses to stress is undoubtedly due to the importance of stress in the processes of ecology and evolution (Bjilsma and Loeschcke 1997; Feder 1999), and because of this it is proposed that, although this model (Figure 4.5) is based on a specific stress scenario, the general responses, kinetics, and hierarchical patterns are likely to be applicable to other environmental stressors. Abiotic and/or biotic stressors elicit a variety of responses in stressed organisms. The type and severity of response is likely to depend on the type of stressor or stress scenario, as well as the duration and speed of onset. Response mechanisms are inextricably linked, but can be divided into functional components. In this example, the overall stress response (OSR) of an organism is split into seven distinct components;

Figure 4.5a Behavioural responses; the first response to a stressor may be a behavioural response. For instance, in response to small increases (1 to 50 mbar) in HP, larvae of a brachyuran crab *Rhithropanopeus harrisii* were shown to change their swimming patterns and increase swimming speed to invoke an active ascent (Forward and Wellins 1989). Similarly, the caridean shrimp (*Palaemonetes varians*) showed increases in active upward swimming activity, as well as the onset of a abdomen flicking propulsion mechanism, in response to large increases in HP (Oliphant et al. 2011; Chapter 4). Both examples are characteristic of a first behavioural response to the onset of a stressor. If the stressor is related to proximity,

then movement away from the area may alleviate the stressor before other mechanisms are initiated in order to counteract the detrimental effects. This type of behaviour is termed behavioural homeostasis (Johnson et al. 1992), and is characterised by a specific behavioural pattern that actively attempts to alleviate the negative physiological effects of the encountered stressor. Increases in motor activity characterised by behavioural responses to a stressor require an increase in metabolism and are thus linked to the metabolic response to stress (Figure 4.5d).

Figure 4.5b Cellular stress response (CSR); the CSR is undoubtedly the most widely studied of stress responses, because of the universal nature of its components (Kültz 2005). The heat shock response (HSR) is a classic example of this. The HSR is a highly conserved mechanism that responds to increases in the abundance of intracellular macromolecular damage (Sorensen et al. 2003) as a result of a stressor or stress scenario by inducing the transcription of heat shock proteins (HSPs). The CSR responds rapidly to the general cellular effects of stress, i.e. macromolecular damage or increase in reactive oxygen species. Initiation of transcription can occur within minutes, with protein synthesis following thereafter. Post-translational modifications of existing proteins can be even more rapid (for review se de Nadal et al. 2011).

The CSR has two components, response, and recovery. During stressor exposure, damaged macromolecules will accumulate in the cell triggering the transcription of HSPs (Craig and Gross 1991). This may be highly costly in terms of energy but will continue until either the stressor subsides, acclimatory mechanisms restore homeostasis (Figure 4.5c), or the stressor reaches critical/lethal levels and other mechanisms are induced (Figures 4.5e, 4.5f, and 4.5g).

After an acute stressor exposure, the CSR moves into a recovery phase. It is likely that damaged macromolecules remain and components of the CSR, such as HSPs, will continue to be transcribed until the pre-stressed cellular environment is restored. There is likely to be a significant cost to invoking components of the CSR, which will result in an increase in cellular energy demand and thus increased metabolism (Figure 4.5d).

Figure 4.5c Cellular homeostatic response (CHR); The CHR represents a stressor-specific mechanism of stress response (Kültz 2005). The CHR is triggered by stressor-specific sensors that act to restore pre-stress homeostasis with regards to stressor-induced perturbations. The CHR is a permanent response until environmental conditions change

once more. The CHR is likely to be a slower process, as it requires specific changes in gene expression and cellular composition. The CHR may also represent a more cost-effective response than the CSR. Its aim is to restore cellular homeostasis, albeit at a new homeostatic point in line with the stressor, at this new homeostasis the CSR will no longer be required (Tomanek 2011). For example, the onset of osmotic stress will trigger the CSR, at the same time specific CHR mechanisms such as transcription of osmo-responsive genes will act to re-establish intracellular ion homeostasis by altering intracellular levels of organic osmolytes (Woo et al. 2002). Once a new cellular homeostasis is reached there is no longer a need for the CSR, and the CHR will continue to regulate osmolyte concentrations until environmental conditions change once more. The CHR acts to maintain homeostasis in non-optimal scenarios and, as a result, will likely involve an increase in cellular metabolism (Figure 4.5d), although this will vary according to the stressor and specific response mechanism.

Figure 4.5d Increased metabolism; each of the above mechanism (Figure 4.5a, 4.5b, and 4.5c) is likely to result in an increase in cellular metabolism as their processes require energy. The above mechanisms are distinct but can act together or separately over the same timescales. Quantifying the response of each individual mechanism will give an idea of the degree to which one or each of the mechanisms is working. However, quantifying changes in cellular metabolism will give an overall idea of cost of the stressor or stress scenario and concomitantly the degree of effect on the fitness of the organism. Under stressful scenarios, energy may be diverted away from processes such as growth and reproduction to allow for greater expenditure towards a stress response (Bjilsma and Loeschcke 1997). Although this will have obvious detrimental effects on the long-term fitness of the organism, it will act to buffer the metabolic demand of the stress response in the short term, and may be a preferable strategy in response to acute stress exposures.

Figure 4.5e Metabolic depression; Metabolic depression has been observed in most major animal phyla in response to environmental stress. It is characterised by a number of observable changes at a molecular level: a decrease in cellular pH, the presence of latent mRNA as a result of a down-regulation in protein synthesis; the maintenance of ion pumping despite protein synthesis down-regulation; and changes in protein phosphorylation state (for review see: Guppy and Withers 1999). Metabolic depression is a time-limited state, the longer it remains the more detrimental it is to the organism's overall

fitness, however at critical stress levels it is likely to be a more beneficial mechanism than further depleting energy reserves by invoking a CSR or CHR (Sokolova 2013). If critical stress exposure continues it will eventually lead to organism death, however if pre-stress environmental conditions return, then the organism may be able to recover as a result of the energy reserved during metabolic depression.

Although this thesis as a whole focusses on the sub-lethal effects of stress on organismal physiology, the critical and lethal effects of stress are still of obvious importance and are noted below;

Figure 4.5f Cell cycle control shutdown; cell cycle control shutdown is the next step on from metabolic depression and involves the complete arrest of growth and reproduction. It is thought to work in combination with the CSR at critical levels of stress, diverting as much energy as possible towards processes of macromolecular stabilisation and repair. Again, this mechanism will have significant detrimental effects on the fitness of the organism if it survives, and is a time-limited state (for review see Kültz 2005).

Figure 4.5g Cellular apoptosis; cellular apoptosis is regarded as a final resort in the face of critical stress levels and involves the programmed death of cells. Apoptosis is not only a stress response, but also an important mechanism during multicellular organismal development (Kuriyama and Fukuda 2002) as well as a mechanism to limit disease progression. Despite being widely studied, there is still little consensus on the role of apoptosis, particularly in response to environmental stressors. Programmed cell death of particularly sensitive cells/tissues may improve the chances of whole organism survival, or it may just be a process leading ultimately to cell death (for review on apoptosis see Franco et al. 2009).

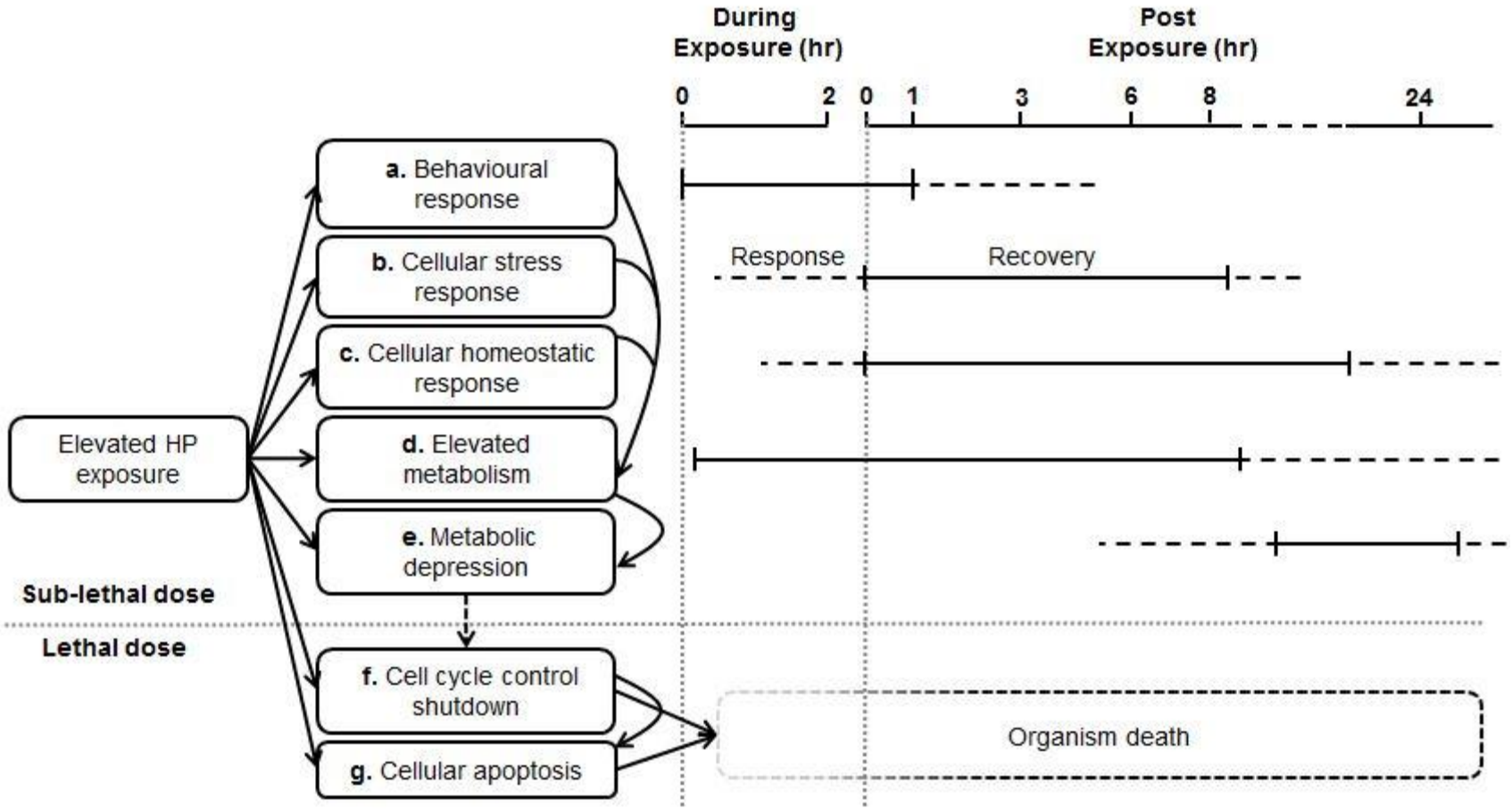


Figure 4.5 A conceptual model of the overall stress response (OSR) to an elevated hydrostatic pressure (HP) exposure. Showing seven distinct response mechanisms (**a.** to **g.**), and their interconnectivity. The timings of each response are taken from data presented earlier in this chapter and represented a solid lines. The dashed lines denote that the timings of each response is likely to be specific to the type, magnitude, and duration of stressor experienced.

4.8 Conclusions

Elevated HP acts upon shallow-water adapted organisms and produces responses from the transient universal cellular stress response (CSR), and the less transient cellular homeostatic response (CHR) at the cellular level, through to respiratory and behavioural responses at the whole organism level. Each of these responses is distinct yet interlinked, and as such I have termed them collectively as the overall stress response (OSR). Biomarkers of stress are used exhaustively in studies of organism physiology but in most cases likely only capture one aspect of the stress response (the CSR): HSP70s are a prime example of this, representing one of the most widely studied groups of genes. Results presented here show that the CSR is just one aspect of a large multilevel response to stress, and as such, the CSR (or any other component of the OSR) studied in isolation cannot provide a complete picture of the impact of a stressor. Quantifying more than a single aspect of the stress response with regards to specific stressors or stress scenarios will thus give a much greater insight into the impacts of single or multiple stressors on an organism

Bridging statement

Chapter 3 elucidated and quantified the expression of a number of novel gene biomarkers in response to HP and temperature stress. The changes in gene expression observed were indicative of HP intolerance in several important cellular mechanisms. Yet, justification of this was difficult as no higher order physiological measurements were taken leaving the link between transcriptional regulation and, for instance, respiratory response ambiguous. Further, synergistic effects between the two stressors (HP and temperature) were observed. However, without a full and multi-level analysis of the effects of HP alone, the relative contribution of each stressor to the overall stress level remained unknown. Chapter 4 has quantified the multi-level effects of an acute elevated HP exposure, in isolation, on adult *Palaemonetes varians*. Changes in gene expression correlated with higher-order changes in respiratory rate as well changes in overt behavioural characteristics. The concept of the overall stress response (OSR) was introduced highlighting the importance of considering more than a single aspect of the potential responses to stress, and also the importance of understanding the kinetics and hierarchy of such responses. Gene expression of the biomarkers introduced in Chapter 3 showed tissue-specific transcriptional regulation in the results of Chapter 4, highlighting that this is another important consideration for experimental design. The results of Chapter 4 provide a novel insight into the wide-ranging effects of elevated HP exposure, in isolation, on a shallow-water invertebrate. These results will help to better understand the relative contributions of HP and temperature to the overall observed stress level when in combination, and the apparent and putatively important synergistic effects between these two variables. The major constraint of Chapters 3 & 4 is the use of acute high HP exposure, and rapid pressurisation and decompression steps. The question remains as to whether the observed changes in physiology and gene expression are in response to the elevated HP exposure or are an artefact of the rapid pressurisation or decompression. Further, an acute 2-hour exposure to high HP is not of direct ecological relevance. To address this major constraint, the experiments of Chapter 5 were designed to test whether the responses observed in Chapters 3 & 4 would be observed once more in much longer, more ecologically-relevant, exposure periods, with slower, acclimation-based, pressurisation steps.

Chapter 5

Pressure stress: more than just an acute response

5. TOWARDS A MORE ECOLOGICALLY-RELEVANT PERSPECTIVE ON THE PHYSIOLOGICAL EFFECTS OF COMBINED PRESSURE & TEMPERATURE ON DEPTH-DISTRIBUTION LIMITS

[7-day exposure experiment under review for publication as; **Morris JP**, Cottin D, Oliphant A, Brown A, Ravaux J, Shillito B, Thatje S, Hauton C. (*under review*) The physiological viability of climate-driven bathymetric range shifts in a shallow-water ectotherm. Open Science]

5.1 Abstract

The physiological consequences for species facing global change can be succinctly summarised: move, acclimatise, adapt, or die. Movement, or range shifting, is of great potential importance as it requires no inherent change in phenotype or genotype. In light of current ocean-surface warming, many studies have focussed on the capacity of marine ectotherms to shift their ranges latitudinally. Bathymetric range shifts offer an important viable alternative, and may be the sole option for species already at high latitudes or those within enclosed seas; yet ecologically-relevant experimental data are scant. Hydrostatic pressure (HP) and temperature have wide ranging effects on physiology, importantly acting in synergy thermodynamically, and therefore represent key environmental constraints to bathymetric migration. This chapter presents data on transcriptional regulation in a shallow-water marine crustacean (*Palaemonetes varians*) at atmospheric and high HP following sustained HP (7-day) exposures at three temperatures across the organisms' thermal scope. Data are presented alongside previous behavioural studies conducted over same exposures. Further, two 28-day exposures were carried at a single temperature (10°C) close to the species optimum. The 28-day treatments represent the longest sustained experimental HP exposure of its kind in shallow-water species, and provide an important novel insight into the acclimation potential of the species. Changes in gene expression indicative of cellular macromolecular damage, elevated metabolism, and a lack of acclimation

were observed after prolonged exposure to high HP. These results validate previous studies and suggest that the combined effects of HP and temperature are not merely seen under acute shock exposures but also under prolonged acclimation-based exposures, highlighting their importance for our understanding on the potential for shallow-water marine ectotherms to shift their ranges bathymetrically in light of contemporary, rapid, ocean surface warming.

5.2 Pre-text

The 7-day exposure treatments across the temperatures described below were conducted prior to the start of this PhD thesis work. These exposures were initially conducted to test behavioural and heat shock responses, the results of which were published (Cottin et al. 2012). Shrimp abdomen samples that were not used in the initial study were stored with the aim of being utilised once additional molecular markers had been characterised allowing further investigations of the effects of such exposures (the work of this thesis). A single 28-day, 10 MPa exposure was also conducted prior to the start of this PhD; however, no studies were conducted on these samples, which were stored for future use. The results described in this chapter arise from nucleic acid extractions from previously unused samples, and involve different molecular protocols and qPCR assays to previously published work. A single 28-day, 0.1 MPa exposure was also conducted as part of the work of this chapter acting as a necessary control treatment to the previously conducted single 28-day, 10 MPa exposure.

5.3 Specific chapter hypotheses

$H_{5.1}$ – A similar pattern of gene expression will be observed in the same genes quantified over similar temperature and HP treatments between 7-day exposures (this chapter) and acute 2-hour exposures (Chapter 3).

$H_{5.2}$ – Transcriptional regulation of the genes studied will differ between 7-day and 28-day exposures at 10°C.

$H_{5.3}$ – Newly characterised metabolic markers will show differential expression across differing exposures and correlate with metabolic the state of the organism.

5.4 Introduction

Global change events are profoundly altering biology (Parmesan and Yohe 2003; Chen et al. 2011; Somero 2012; Beaugrand et al. 2014). Organisms occupying an environment where directional change is occurring must move, acclimatise, or adapt in order to prevent extinction (Somero 2012; Kelly and Hoffmann 2013). Significant progress has been made in documenting the role of latitudinal range shifts in response to environmental warming both in terrestrial (Chen et al. 2011) and marine habitats (Beaugrand et al. 2002; Edwards and Richardson 2004; Perry et al. 2005). In terrestrial systems there has also been significant research focussed on altitudinal range shifts, predominantly in plants, but also evidenced in mammals, insects, and other groups (Hill et al. 2002; Mortiz et al. 2008; Raxworthy et al. 2008; Feeley et al. 2011). In the majority of range shifting examples in the literature, temperature is implicated as the major driving force, although precipitation and drought are also regularly cited in terrestrial studies (Crimmins et al. 2011). Globally, surface temperature is on the rise, and this trend is projected to continue until at least the end of the 21st century (see Section 1.5). This directional change in temperature with time is generally observed in species distribution patterns as a poleward range shift, or an altitudinal shift towards higher elevations. Species are shifting their ranges in order to stay within their thermal scope as the planet's isothermal contours are shifting poleward, and to higher altitudes. Poleward and altitudinal trends appear widespread (Parmesan and Yohe 2003; Poloczanska et al. 2013) although not ubiquitous. Environmental drivers other than temperature can produce opposite directional effects, precipitation changes have been shown to cause a lowering of altitudinal ranges in plant species, for instance (Crimmins et al. 2011).

Our oceans account for almost 71% of the Earth's surface. Nearly 80% of the volume of the marine biosphere lies below 1000 m (Somero 1992). In our generally stratified oceans, temperature tends to decrease with depth, although there are many exceptions to this rule. Ocean surface waters are warming, deeper-waters are warming also, but not to the same extent (IPCC 2013; Rhein et al. 2013). Because of this, one might expect that bathymetric range shifts would feature heavily, as marine latitudinal range shifts do, in general range shift discussions. This is not the case, however, and poses the question of whether bathymetric range shifts are not a regular occurrence, or are simply not subject to the same intensive research as other range shift patterns. Ecological or physiological limitations may

act to reduce the viability of bathymetric migrations, and where latitudinal range shifts are not possible: acclimatisation or adaptation must occur instead in order to prevent extinction. Alternatively, bathymetric range shifts may simply be understudied, which could be due, in part, to the difficulty in obtaining down-water column measurements in comparison to across latitude measurement, or the technical difficulties of replicating deeper-water scenarios in a laboratory environment.

There is evidence in the scientific literature that some marine species are shifting their ranges bathymetrically. A 25-year study conducted in the North Sea on demersal fish assemblages observed a general bathymetric shift of 3.6 m per decade over the study period (Dulvy et al. 2008). Similarly, Atlantic surf clams (*Spisula solidissima solidissima*) from the North East coast of North America showed a significant deepening trend on a decadal timescale (Weinberg 2005). Further, a large scale analysis of coastal survey data around North America from 1968 to 2011 found that, for 360 marine taxa, there was a general latitudinal and depth shift with increasing temperature (Pinksy et al. 2013). As such, it seems that at least under certain circumstances ecological and physiological factors allow for bathymetric range shifts to occur. Bathymetric range shifts share the same potential physiological limiting factors, with the addition of HP. Therefore, experimental studies aimed at determining the potential for bathymetric range shifts in marine organisms must consider the effects of HP alongside other stressors.

HP is a thermodynamic variable, and as such affects all biological processes. It is of particular significance in the marine realm as pressure gradients are much steeper in water than in air. Consequently, the upper and lower depth distributions of marine organisms are delineated in part by HP (Brown and Thatje 2014). Despite its significance, HP is rarely considered as a stressor in the marine environment in comparison to other factors such as temperature. Undoubtedly, research involving HP is diminutive in part due to technological limitations. Yet, studies looking at the effects of acute elevated HP have shown that temperature changes can mediate the effects of HP (Chapter 3; Morris et al. 2015).

This chapter explores whether the apparent effects of HP and temperature, observed in acute experimental exposures (Chapter 3; Morris et al. 2015), are an artefact of rapid changes in these variables, or represent a truly ecologically-relevant response, with implications on our understanding of depth distributions and range shifts of marine ectotherms. This chapter has studied the transcriptional regulation of genes linked with the onset of a variety of pressure intolerances in the shallow-water shrimp *Palaemonetes varians* (Chapter 3; Morris et al. 2015), following a sustained, 7-day, hyperbaric exposure at three

temperatures across the species' thermal tolerance envelope, and also following a novel 28-day sustained pressure exposure at a single temperature.

5.5 Materials & methods

Adult *Palaemonetes varians* shrimp (4-5 cm total length) collected from Lymington salt marshes (Hampshire, UK) (Section 2.1.1) were acclimated from the temperature at which they were collected to 5, 10 or 27°C ± 0.5°C at a rate of 2°C per day. Shrimp were maintained for a further 3 days at acclimation temperature before pressure experiments.

5.5.1 7-day exposures

The IPOCAMP system (Shilitto et al. 2014; Section 2.2.2) was used to conduct 7-day HP and temperature exposures. The system was filled with aerated filtered seawater (salinity: ~32), and acclimated to 5, 10, or 27°C ±0.1°C. Shrimp were transferred into the hyperbaric chamber, and the system was set running at atmospheric pressure for one hour before the start of each exposure allowing time for recovery from handling stress. HP was then increased stepwise, at 1 MPa every 5-minutes to 10 MPa. Shrimp were held under these conditions for 7-days. 0.1 MPa control treatments were run over the same time period at each temperature. After exposure, the system was depressurised over one minute, and shrimp were snap frozen for RNA extraction (Section 2.5.1.1).

5.5.2 28-day exposures

The IPOCAMP system (Shilitto et al. 2014; Section 2.2.2) was, once more, used to conduct 28-day HP exposures. The hyperbaric chamber was filled with aerated filtered seawater (salinity: ~32), and acclimated to 10°C ±0.1°C. Shrimp were transferred to the hyperbaric chamber, and the system was run at atmospheric pressure for 1-hour prior the start of the exposures allowing time for recovery from handling stress. HP was then increased stepwise, at 1 MPa every 5-minutes to 10 MPa. Shrimp were held under these conditions for 28-days. 0.1 MPa control treatments were run over the same time period. ~30% water changes were made in the IPOCAMP system, via the reservoir tank (Section 2.2.2) every 2-days. The "at-pressure" filter was changed once, after 14-days to prevent unwanted further increases in HP due to poor filtration. The shrimp were monitored daily at 10am via the

endoscopic camera system (Section 2.2.2), and any mortality was noted. After exposure, the system was depressurised over 1-minute, and shrimp were snap frozen for RNA extraction (Section 2.5.1.1).

5.5.3 Characterisation of additional molecular markers

The results of Chapters 3 and 4 indicated that changes in metabolism may be key to understanding the effects of combined HP and temperature exposures on shallow-water marine invertebrates. For this reason, it was deemed beneficial to attempt to characterise two well-studied molecular markers of metabolism (citrate synthase and lactate dehydrogenase), and quantify their expression in addition to previously characterised molecular markers. Neither gene sequences nor expression sequence tags (ESTs) were available for the *cs* and *ldh* genes in *Palaemonetes varians*, so a technique of gene hunting by degenerate PCR (degenPCR) was employed to characterise ESTs in this species (Section 2.6.1). Translated protein sequences from a variety of eukaryotic taxa were obtained for the *cs* and *ldh* genes from the online GenBank database. The citrate synthase protein sequences used were (GenBank protein accession numbers): AB121881.1; AA127381.1; AAC25560.1; AA114139.1; AA31017.1. For lactate dehydrogenase protein sequences used were: AEC12822.1; AEK84522.1; ACY66479.1; XP_001900208.1; AAA67063.1; AAV80238.1; ABY60854.1. These sequences were aligned using the freely available Clustal Omega multiple alignment tool from the EMBL website (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al. 2011). A degenerate consensus sequence was then derived from each multiple alignment (Figure 5.1). Relatively conserved regions containing amino acids with fewer rather than greater nucleotide redundancies were selected as potential degenerate primer design regions.

Degenerate primers were designed on regions of the consensus sequences rich in the following amino acids: M,W,C,D,E,F,H,K,N,Q,Y (1 or 2 fold sites only); and preferably containing minimal L,S,R amino acids (6 fold sites) (see Table 5.1). Forward and reverse degenerate primer were designed to be 5-9 amino acids (15-27 base pairs) in length and produce an amplicon of between 200 and 1000 base pairs in length. Degeneracy at the 3' end was not favoured.

Once degenerate primer pairs had been designed (Table 5.2) they were used in degenPCR reactions made up of the following: 5 µl of 5x PCR buffer solution; 1 µl of stock dNTP mixture (10 mM); 1-4 µl $MgCl_2$ (1 mM); 1-3 µl FWD degenerate primer (25 nM); 1-3 µl

REV degenerate primer (25 nM); 1 µl *Palaemonetes varians* cDNA (~350 ng/µl); 0.125 µl TAQ polymerase; and nuclease-free H_2O up to 25 µl. The degenerate PCR mixture was run under conditions shown in Figure 5.2.

Table 5.1 A list of amino acids, their symbol, degenerate nucleotide code, and reverse compliment nucleotide sequence. Degenerate nucleotide symbols are as follows: R = A+G; M = A+C; S = C+G; W = A+T; H = A+T+C; B = G+T+C; N = A+T+C+G; Y = C+T; K = G+T; D = G+A+T; and V = G+A+C

Amino acid	**Symbol**	**Nucleotide sequence (with degeneracy)**	**Complement (for reverse primers)**
Methionine	M	ATG	TAC
Tryptophan	W	TGG	ACC
Cysteine	C	TGY	ACR
Aspartic acid	D	GAY	CTR
Glutamic acid	E	GAR	CTY
Phenylalanine	F	TTY	AAR
Histidine	H	CAY	GTR
Lysine	K	AAR	TTY
Asparagine	N	AAY	TTR
Glutamine	Q	CAR	GTY
Tyrosine	Y	TAY	ATR
Inosine	I	ATH	TAD
Alanine	A	GCN	CGN
Glycine	G	GGN	CCN
Proline	P	CCN	GGN
Threonine	T	CAN	TGN
Valine	V	GTN	CAN
Leucine	L	YTN	RAN
Arginine	R	MGN	KCN
Series	S	WSN	WSN

PCR products were separated by gel electrophoresis (Section 2.6.2.3), and distinct single bands of expected length were extracted according to Section 2.6.3. The extracted fragments were then cloned into an *E. coli* vector and grown on agar plates, as according to Section 2.6.4. Plasmids were extracted from selected colonies following positive identification by colony PCR (Sections 2.6.4 and 2.6.5). Extracted plasmids were then sent

for sequencing by conventional dideoxy-termination sequencing (Source Bioscience, UK), and returned sequences were cleaned and analysed using Geneious Pro™ R6 software (Section 2.6.6).

Figure 5.1 A Multiple-alignment of Lactate dehydrogenase (*ldh* gene) sequences (GenBank accession numbers): AEC12822.1; AEK84522.1; XP_001900208.1; AAA67063.1; AAV80238.1. Potential forward (F1 and F2) and reverse primers (R1) are highlighted. Alignment made using Clustal Omega (Sievers et al. 2011)

Table 5.2 Degenerate primer sequences used in *Palaemonetes varians* to isolate and characterise the *cs* and *ldh* genes. Optimised degenPCR conditions shown alongside sequences

Gene	Primer	Sequence	Conditions
Citrate synthase (*Cs* gene)	dCS F1	T GAY CAY GAR GGH GGH AAY GT	2 µl $MgCl_2$; 2 µl primers (25nM); 57.5°C annealing temp; 1 min elongation step
	dCS R1	TG DGC RTC YAC RTT RGG CCA	
Lactate dehydrogenase (*Ldh* gene)	dLDH F1	GTN GGN GGN CAR GTN GGN AT	2 µl $MgCl_2$; 2 µl primers (25nM); 54°C annealing temp; 1 min elongation step
	dLDH R1	SW RTC NCC RTG YTC NCC DAT	

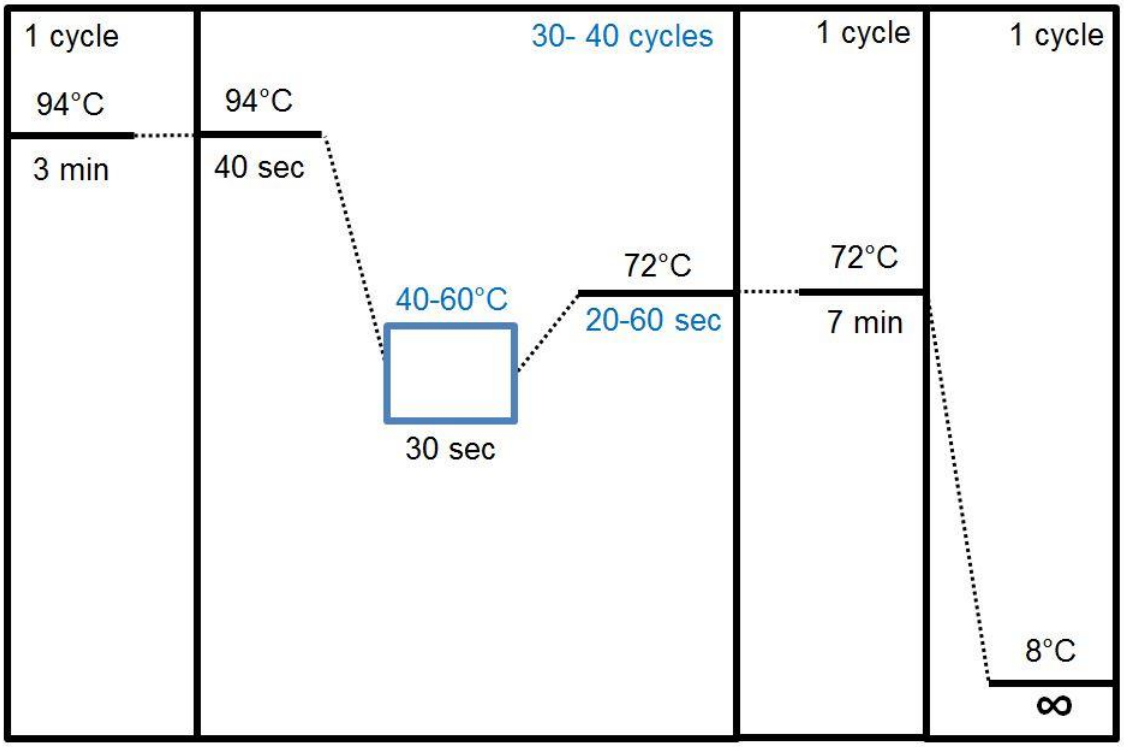


Figure 5.2 Standard degenerate PCR reaction profile, with step temperatures and timings, and the number of cycles per segment. Steps highlighted in blue are those that require assay-specific optimisation in order to yield single, distinct, reaction products

5.5.4 Nucleic acid preparation & qPCR

RNA extraction, DNase-treatment, and reverse-transcription were conducted and all necessary quality control measures were met, according to Morris et al. (2015) and Section 2.5. qPCR primers were designed and optimised in accordance with the MIQE guidelines (Bustin et al. 2009; Bustin et al. 2010). Primer sequences, concentrations, linear dynamic ranges, reaction efficiencies, are listed in Sections 2.8, A1.4, and Table 2.3. Assay specificity was confirmed by melt curve analysis (Section 2.8.4). Normalised relative quantities (NRQs) were calculated using qBase+ software (Section 2.8.7). NRQs were then scaled giving a value of relative fold change (RFC) (Section 2.8.7). The RFC of each gene was determined relative to the atmospheric control exposures at each temperature. Statistical significance was identified at $p<0.05$, determined by GLM and *post-hoc* Tukey-HSD test using R statistical software and the "multcomp" package (Horthorn et al. 2008).

5.6 Results

5.6.1 New molecular marker characterisation

A, 380-bp, mRNA fragment was isolated from gene-hunting by degenPCR and deposited in the EMBL-EBI database: Accession number LN713461. The fragment was putatively identified as coding for the Citrate synthase protein (*cs* gene), and further validation confirmed its identity as a protein coding sequence (Section 2.7.1.3).

A, 503-bp, mRNA fragment was isolated from gene-hunting by degenPCR and deposited in the EMBL-EBI database: Accession number LN713462. The fragment was putatively identified as coding for the Lactate dehydrogenase protein (*ldh* gene), and further validation confirmed its identity as a protein coding sequence (Section 2.7.1.4).

5.6.2 Experimental results

5.6.2.1 7-day exposures

The relative fold changes of 5 genes showed significant differences under elevated HP (10 MPa) across 3 temperatures (5, 10 and 27°C; figure 5.3a) after 7-days of exposure. To observe the effects of elevated HP at different temperatures, the RFC of each gene was determined relative to the atmospheric control exposures at each temperature. Further, the

atmospheric control treatment was scaled to a RFC of 1 in each case (Figure 5.3a). Consequently, Figure 1a presents the effect of elevated HP, and how that HP effect is influenced by temperature. These graphs are scaled to remove temperature effects at 0.1 MPa (i.e. effects of the 3 temperatures alone at atmospheric HP). For transparency, temperature-only effects at 0.1 MPa are shown as well (Figure 5.3b).

The *narg* gene, the *cs* gene, the *ldh* gene, the *gapdh* gene, and the *hsp70 f1* gene showed significant relative fold (RF) increase under elevated HP at 5°C and 10°C. The *ldh* gene showed a significant RF decrease under elevated HP at 5°C. The *hsp70 f2* gene showed no significant RFC under elevated HP across the 3 temperatures (Figure 5.3a). Further, the *narg* gene showed no significant RFC across temperatures at 0.1 MPa. The *cs* gene, and the *hsp70 f1* gene, showed significantly higher RFC under atmospheric HP at 27°C in comparison to 5°C and 10°C. A significant RF increase of the *gapdh* gene was seen at 10 and 27°C in comparison to 5°C. Significant RF decreases of the *ldh* gene, and the *hsp70 f2* gene were observed at 27°C in comparison to 5°C and 10°C (Figure 5.3b).

5.6.2.2 28-day exposures

The relative expression of 5 genes showed significant differences under elevated HP (10 MPa) in comparison to 0.1 MPa after 28-days of exposure at 10°C (Figure 5.4; white dots). The *narg* gene and the *ldh* gene showed significant RF increases after 28-days of exposure at 10 MPa in comparison to 0.1 MPa. The *gapdh* gene, *hsp70 f1*gene, and *hsp70 f2* gene showed significant RF decreases after 28-days of exposure at 10 MPa in comparison to 0.1 MPa. The *cs* gene showed no RFC after 28 days of exposure at 10 MPa in comparison to 0.1 MPa.

In comparison to equivalent 7-day exposures (Figure 5.4 black dots vs. white dots), the *narg* gene showed a similar pattern of expression being upregulated at both 7-day and 28-day time points under elevated HP. The degree of upregulation was higher after 28-days than after 7-days for the *narg* gene. The *cs* gene showed change in expression pattern between the 7-day and 28-day time points: significant upregulation was observed at elevated HP after 7-days, but no change in expression was observed at elevated HP after 28-days. The *ldh* gene showed a change in expression pattern between the 7-day and 28-day time points: no change in expression was observed after 7-days of elevated HP exposure, however a significant RF increase was observed under elevated HP after 28-days. The *gapdh* and *hsp70*

f1 genes both showed changes in their expression patterns between the 7-day and 28-day time points: significant upregulation was observed in both genes after 7-days of elevated HP exposure. After 28-days of elevated exposure, both the *gapdh* gene and the *hsp70 f1* gene showed significant decreases in RF at elevated HP. Finally, the *hsp70 f2* gene showed no change in RF after 7-days of exposure at elevated HP, but a significant down-regulation after 28-days of exposure at elevated HP.

The survival of the shrimp was monitored over the 28-day exposures at both 0.1 MPa and 10 MPa for 10 shrimp in each exposure (Figure 5.5). In both treatments, survival remained at 100% for the first 8-days. After 11-days survival had dropped to 70% in the elevated HP exposure (10 MPa), and remained at that level for the duration of the exposure period. 100% survival was observed at atmospheric pressure until day 18 of the exposure, and after 28-days of exposure the survival rate was 80% (Figure 5.5). A Kaplan-meier survival estimator (Goel et al. 2010) was used to calculate survival over time in regards to the proportion of remaining individuals. Further, a cox proportional hazards model using R statistical software was used to determine any differences between the survival estimates at 0.1 MPa and 10 MPa treatments over the 28-day period. However, as expected, the sample size (n=10) for only one repeat at each exposure was insufficient to provide any meaningful statistical inference. As survival measurements were not the primary reason for conducting the experiments, it was not deemed appropriate to conduct 4-months of additional IPOCAMP exposures in order to statistically estimate survival differences. The proportional survival plot is displayed (Figure 5.5) but will not be discussed in a statistical manner.

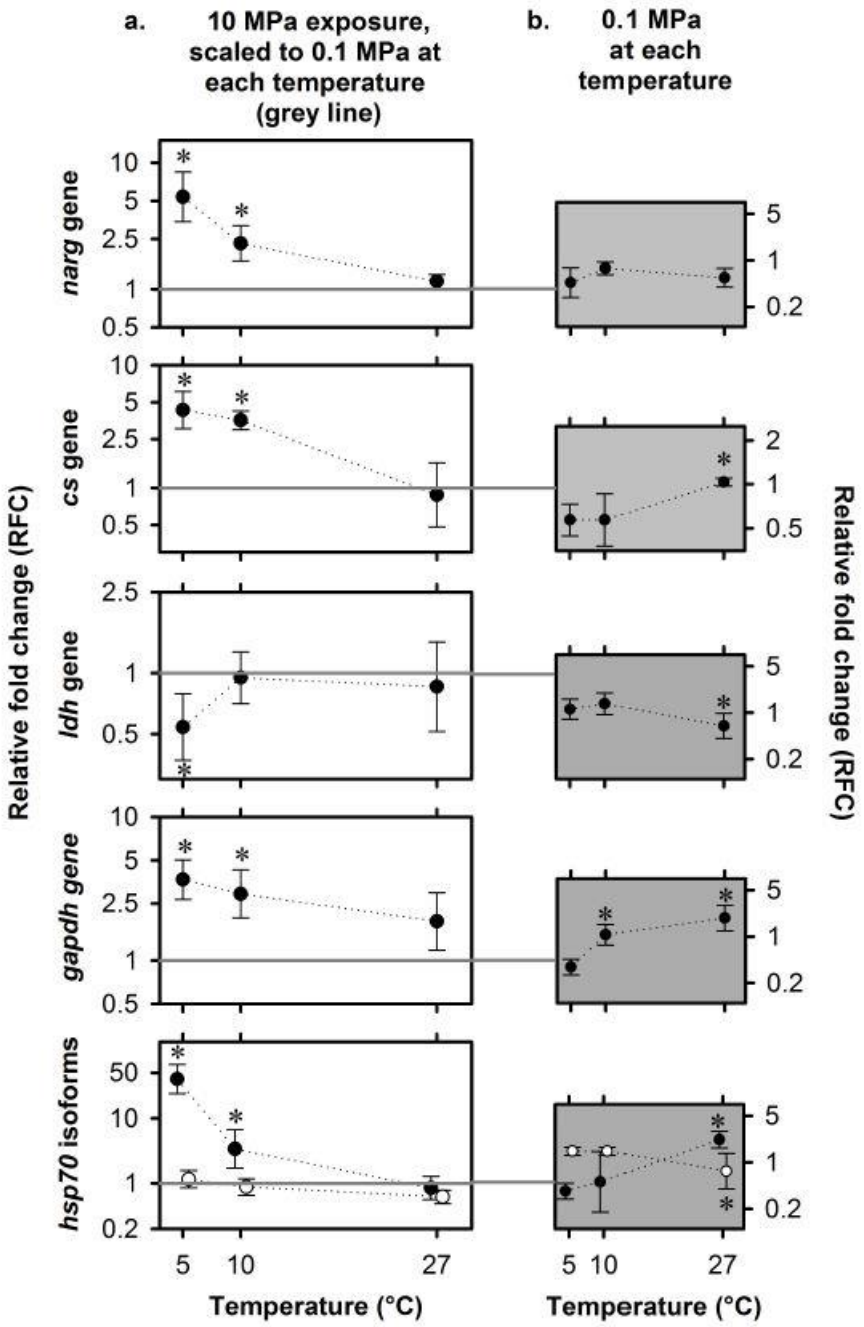


Figure 5.3 Relative fold change (RFC) of 6 genes after 7-day exposures at 0.1 MPa and 10 MPa across three temperatures: 5, 10 and 27°C. **a.** RFCs at 10 MPa compared to 0.1 MPa at 5, 10 and 27°C. RFCs are scaled; 0.1 MPa is represented by grey line in each graph. **b.** RFCs at 0.1 MPa across 5, 10 and 27°C (represented by straight grey line in **a.**). HSP70 F1 isoform is represented by black dots, whilst the HSP70 F2 isoform is represented by white dots. RFCs and 95% confidence intervals calculated from 5 biological replicates. Significance displayed as; *= $p<0.05$ determined by a GLM and a *post-hoc* Tukey-HSD test

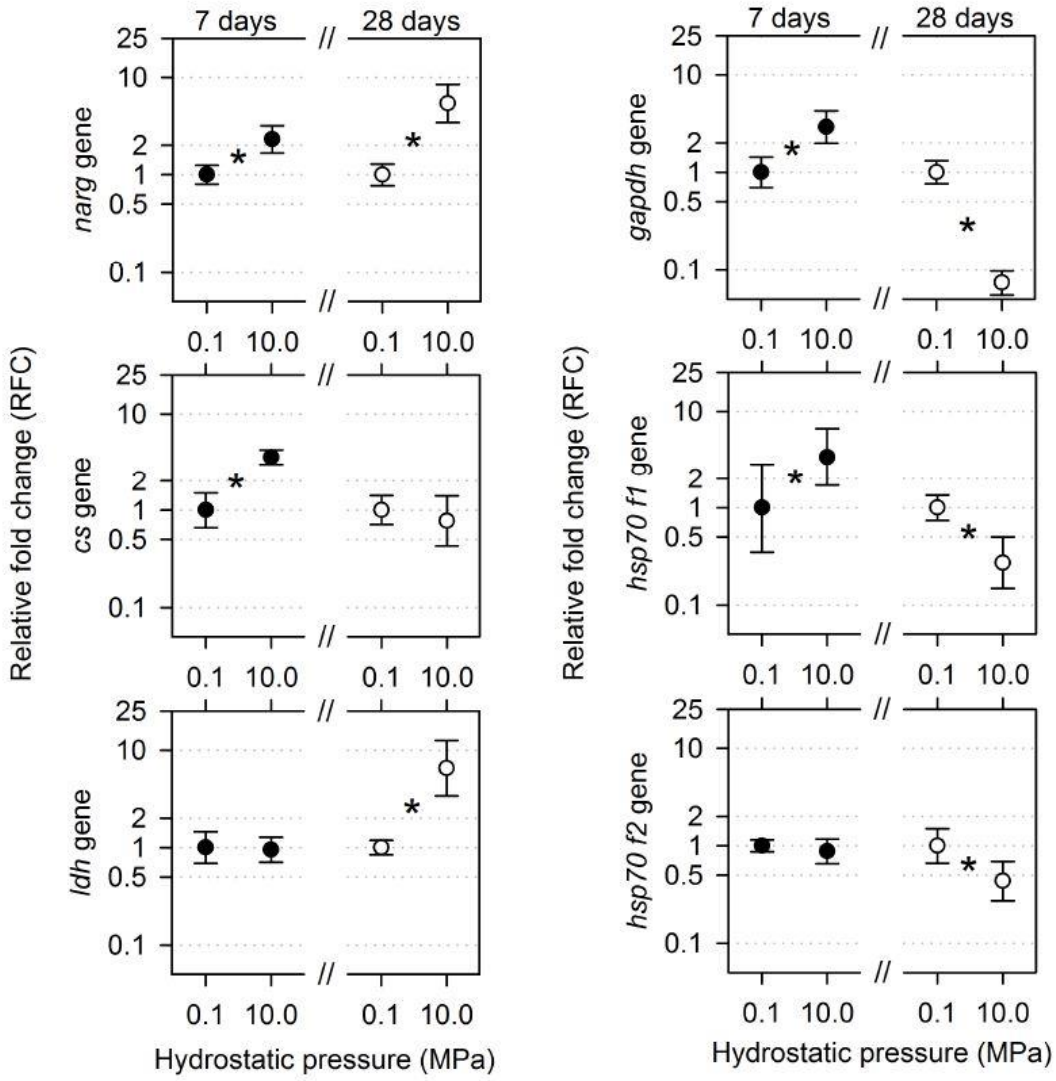


Figure 5.4 Relative fold change (RFC) of 6 genes after 7-days exposures at 0.1 MPa and 10 MPa at 10°C (black dots), and after 28-days at 0.1 MPa and 10 MPa at 10°C (white dots). RFCs and 95% confidence intervals calculated from 5 biological replicates. Significance displayed as; *= $p<0.05$ determined by a GLM and a *post-hoc* Tukey-HSD test

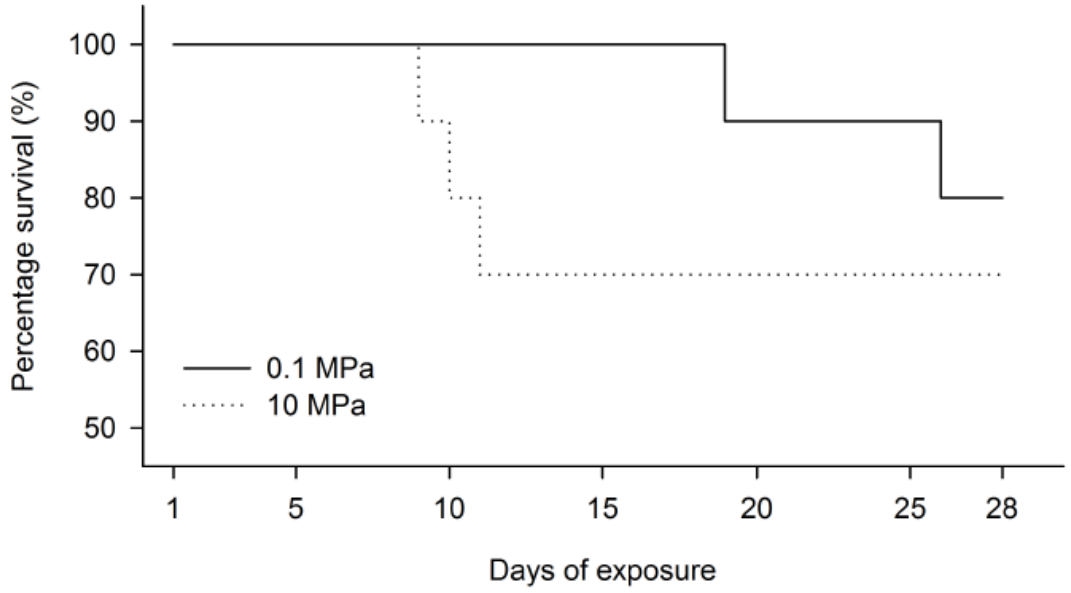


Figure 5.5 The percentage of shrimp surviving as a function of the days of exposure at 0.1 MPa (solid line) and 10 MPa (dotted line)

5.7 Discussion

5.7.1 7-day HP & temperature exposures

For a strictly shallow-water eurytopic invertebrate, large variation in temperature or HP, in isolation, has effects on the transcriptional regulation of genes involved in distinct physiological mechanisms. Importantly, these effects are considerably more pronounced when temperature and HP act in combination (Morris et al. 2015; Chapter 3). Tested over a 7-day exposure, the data in this chapter show, for the first time, that the antagonistic effects of HP and temperature in response to acute experiments (Morris et al. 2015; Chapter 3) are not merely artefacts of acute exposures. Therefore, these results have important implications on our understanding of physiological limits to the depth distributions of marine organisms, and their ability to shift distribution ranges. The thermodynamics of volume change reactions indicate that decreasing temperature and increasing HP both favour reactions in which volumes decrease, and vice versa (i.e. the thermal-pressure coefficient; Flory 1965). Volume change reactions are central to all biological processes,

and the efficiency of such reactions has implications across an organism's physiology (Stearn et al. 2011). If temperature and HP are considered a single entity: as they co-vary throughout the marine biosphere, then all aquatic organisms exist within a specific thermodynamic envelope that is determined by their physiology. Outside this physiological scope, survival would either be time-limited or not possible. Although ecological interactions are of clear importance in setting distribution limits, the physiological scope of an organism is likely to be of fundamental importance also (Somero 2012; Brown and Thatje 2014). When considering bathymetric migrations, the effects of the combination of temperature and HP must be considered.

Table 5.3 Each gene studied; their associated protein, and their significance as a marker

Gene (Accession no.)	Protein	Relevance	Ref
narg (FR667656)	NMDAR - regulated protein	Marker of HP intolerance, pre-cursor to the onset of symptoms characterised by high pressure neurological syndrome (HPNS).	Morris et al. (2015)
cs (LN713461)	Citrate synthase	Enzyme involved in the TCA cycle. Rate limiting marker of aerobic capacity / aerobic metabolism.	Goldenthal et al. (1998)
ldh (LN713462)	Lactate dehydrogenase	Enzyme that catalyses the conversion of pyruvate and lactate. Marker of anaerobic metabolism.	Crawford and Powers (1989)
gapdh (GQ120565)	Glyceraldehyde-3-phosphate dehydrogenase	Enzyme involved in glycolysis. Marker of aerobic metabolism.	Morris et al. (2015)
hsp70 isoforms form 1 (FJ875280) form 2 (FJ875279)	70kDa Heat shock protein isoforms	Molecular chaperones. Markers of the cellular stress response (CSR) and intracellular macromolecular damage.	Cottin et al. (2010)

Aerobic metabolism, as inferred by the *cs* and *gapdh* genes (Table 5.3), increases with higher temperature at atmospheric pressure (Figure 1b). Such trends are expected and well understood (Gillooly et al. 2001). However, under high HP the trend is reversed with increasing aerobic metabolic rate at lower temperature (*cs* gene, *gapdh* gene). Under a combination of elevated HP and low temperature, increases in aerobic metabolism are a sign of increased stress: increases in metabolism are a key aspect of the cellular stress response (CSR) (Kültz 2005). This is further corroborated by the induction of the *hsp70 f1* gene under high HP and low temperature (Table 5.3). The *ldh* gene, an anaerobic metabolic marker (Table 5.3), is down-regulated where maximal expression of aerobic markers is observed, which may be a consequence of high cellular-level aerobic activity. The *narg* gene

(Table 5.3) shows no change in regulation across temperatures at atmospheric HP, but the same trend under high HP conditions as the aerobic metabolism and CSR markers, inferring that the negative physiological effects of HP are greatest at low temperatures. Overall, the regulation of markers of aerobic metabolism, anaerobic metabolism, the CSR, and pressure-specific intolerances indicate that the physiological effects of high HP or low temperature are exacerbated in combination with one-another, in line with thermodynamic theory. Equally, the effect of high HP is ameliorated at higher temperature. Our results corroborate previously published behavioural analysis over the same experimental exposures (Figure A2.1). Cottin et al. (2012) demonstrated that at low temperature there is a reduction in locomotory activity at high HP (a sign of metabolic stress), compared to atmospheric HP. These data, considered alongside our observations of elevated aerobic metabolism at 5°C, indicate that high HP at lower temperatures produces the greatest detrimental physiological effects for temperate marine ectotherms. Cottin et al. (2012) also analysed the relative expression of the two HSP70 isoforms, and despite using different biological samples from different treatments, as well different qPCR assays and analysis techniques, the results presented here are comparable in terms of expression pattern when normalised in the same way, inferring the repeatability of these molecular observations. Current data show that elevated HP induces the CSR, increases aerobic metabolism, and induces pressure-specific physiological intolerances in shallow-water ectotherms. These effects can be ameliorated by higher temperatures within the organisms' thermal scope. Likewise, the effects of increasing HP are exacerbated at lower temperatures. For temperate ectotherms, bathymetric migration down a warm isothermal water column is a physiologically viable alternative to latitudinal migration. A cold isothermal water column would require inherent pressure tolerance or acclimatisation/adaptation counteracting the negative effects of HP alone, at least for temperate ectotherms. Shallow-water cold-adapted ectotherms may have inherently higher HP tolerance due to low temperature adaptation; this should be investigated further. Isothermal water columns currently exist at high latitudes and in some areas of enclosed seas and thus the potential for bathymetric migrations may be greatest there; coincidentally these are the same regions where latitudinal migrations are not possible. The more widespread stratified oceans, characterised by decreasing temperature with depth, will represent the greatest physiological challenge for shallow-water organisms attempting down-slope migration. Our results demonstrate that temperature and HP are particularly significant environmental factors in combination and,

as they co-vary throughout the ocean, it is important to consider them concurrently rather than in isolation.

5.7.2 28-day exposures

It is important to note that the shrimp were maintained at elevated and atmospheric pressures for the 28-day period without access to food. Currently, no appropriate feeding mechanisms are ready to be used as part of our IPOCAMP systems. Although the lack of feeding potential is clearly a confounding factor in the discussion presented below, it was deemed that these time-consuming exposures were, nonetheless, a useful comparison. Measures were taken to minimise the potential effects of lack of feeding on shrimp survival and gene expression. For instance, the gene expression results are only discussed where elevated HP relative fold values are normalised against the potential effects of starvation because such effects would affect shrimp in both exposures equally. This does not, however, control for the fact that starvation stress and HP stress combined could add up to more than the sum of each stressor in isolation (synergistic stressor effects). As such, the following discussion is tempered by such limitations. The 28-day exposures do, however, provide a first-of-a-kind insight into the effects of sustained HP exposures beyond 10-days in length, which is a significant step forwards despite the technological limitations of feeding.

The 7-day exposures have provided a novel insight into the physiological effects of sustained thermodynamics with regards to combined HP and temperature exposures. These results correlated well with expression patterns seen over acute 2-hour shock exposures. However, the fact that they correlate with acute exposures suggest that processes of acclimation are not occurring over a 7-day period. As acclimation is not an instantaneous process, likely involving macromolecular modification at a biochemical level, it would not be expected that the effects of any acclimation could be observed after 2-hour acute exposures. Thus, as the patterns of expression observed over 7-day exposures show the same pattern as the 2-hour exposures it can be suggested that acclimatory processes are not influencing the observed expression following 7-day exposures. For this reason, a 28-day exposure (and control) was conducted at a single temperature to determine whether the pattern of gene expression would change between 7-days and 28-days of elevated exposure, relative to atmospheric control exposures. Any change in expression pattern observed may be indicative of an acclimatory process. Likewise, no change in gene expression pattern

between 7-days and 28-days would indicate no acclimation, but also that the overall stress level experienced by the shrimp over the exposure period was not reaching critical levels, and was thus not a highly time-limited state. Further, a change in gene expression pattern may also be indicative of critical stress thresholds being reach, which would suggest that the overall stress level experienced made the experimental conditions a time-limited scenario for the shrimp.

The cellular homeostatic response (CHR) to stress introduced in Chapter 4 may describe the process of acclimation to specific stressors (Kültz 2005). The CHR is a response to stressor-specific perturbations, and acts to restore pre-stress homeostasis with regards to stressor-induced alterations to function. The CHR is an enduring response until environmental conditions change once more (Kültz 2005). The CHR is likely to be a slower process, as it requires specific changes in gene expression and/or biochemical alteration. In general terms, a stressor may act to shift a homeostatic point of a cellular mechanism or component away from optimal. A cellular homeostatic response will restore homeostasis to the optimal point despite the optimal point having shifted due to the stressor. An example of such a mechanism was observed by Woo et al. (2002). Osmotic stress has been shown to elicit a CSR, but, in conjunction, specific osmo-responsive genes are transcriptionally regulated, and once abundant will act to re-establish intracellular ion homeostasis in light of the osmotic stress. These osmo-responsive genes alter intracellular levels of organic osmolytes thus making a once stressful scenario non-stressful to the cell (Woo et al. 2002). This a form of CHR as it acts to maintain the cells optimal homeostatic point despite that point being shifted by a stressor.

In comparison, the 7-day exposure to elevated HP at 10°C in *P. varians*, *narg* gene expression shows the same pattern after 28-days of exposure. The *narg* gene has been suggested as an indirect marker of pressure intolerances associated with hyper-excitability of N-methyl-D-aspartate receptors (NMDARs). Over-activity of NMDARs has been implicated in, or suggested as a precursor to, the onset of behavioural pathologies under elevated HP scenarios characterised by spasm, convulsions, and nervous system malfunctioning, collectively termed high pressure neurological syndrome (HPNS) (Dingledine et al. 1999; Darbin et al. 2000; Morris et al. 2015; Chapter 3). The transcriptional regulation of the *narg* gene has been suggested to be a result of NMDAR over-activity (Chapter 3). One might expect evidence of acclimation to elevated HP to be shown by a reduction in the up-regulation of the *narg* gene as NMDAR over-activity

subsides and a new homeostatic point is reached. Alongside this, one might also expect general stress marker and metabolic marker up-regulation to reduce as, if acclimation has occurred, the need for a general CSR would no longer remain.

Results from the 28-day exposure in *P. varians* show that, in comparison to 7-day exposures, the *narg* gene remains transcriptionally up-regulated. This suggests that HP is still causing disturbances associated with nervous system perturbations, and thus acclimation, at least with regards to this aspect of HP tolerance, is not being observed. Further, a change in the transcriptional regulation of both aerobic metabolic markers (the *cs* gene and the *gapdh* gene) and general stress markers (the two *hsp70* isoforms) suggests that critical stress levels are being reached after 28-days of exposure. Critical stress levels are characterised by cessation of general responses to stress such as the CSR (Kültz 2005), and also a suppression of metabolic activity as an energy saving mechanism (metabolic depression) (Sokolova 2013). When compared to the 7-day exposures the gene expression patterns of *P. varians* after 28-days of exposure are indicative of both a cessation of the CSR, and the onset of metabolic depression. As further evidence of the potential onset of metabolic depression, transcriptional up-regulation of an anaerobic metabolic marker, the *ldh* gene suggests, unlike any of the 7-day exposures, that anaerobic metabolism is playing a role in energy provision within the shrimp. The use of anaerobic metabolism for energy provision is an unsustainable state (Sokolova 2013), and thus any organism relying on anaerobic metabolism is time-limited in terms of survival, and can be thought of as experiencing, or approaching, critical stress levels. Again, this gene expression pattern is indicative of a lack of acclimation to elevated HP in *P. varians* after 28-days of exposure.

5.8 Conclusions

In conclusion, sustained HP and temperature exposures over a 7-day period demonstrate that synergistic thermodynamics of these two variables play an important role in the overall stress levels experienced by shallow-water species such as *Palaemonetes varians*. Effects at the transcript level show the same pattern over 7-day exposures in comparison to results obtained previously over 2-hour exposures (Chapters 3 and 4). Results presented in this chapter suggest that the synergistic/antagonistic effects of HP and temperature are of ecological relevance in terms of bathymetric ranges and migrations. Shallow-water, temperate, marine ectotherms may be able to extend or shift their bathymetric distributions due to climate-forcing, in terms of physiology, down a temperate isothermal water column.

Decreasing water temperature with depth, or cold isothermy, presents a challenge to cellular physiology in temperate species, and may limit their potential for bathymetric range shifts. Further, following novel 28-day sustained HP exposures, no evidence of acclimation potential to HP was observed in *P. varians* at 10°C. Rather, observed changes in transcription with time suggest that the shrimp are reaching critical stress levels where general stress responses are supressed, metabolic depression is initiated, and energy is being obtained via anaerobic rather than aerobic pathways. The 28-day exposures represent the current technological boundary for keeping species such as *P. varians* under elevated HP in the IPOCAMP system due to feeding limitations.

Bridging statement

Chapters 3 and 4 elucidated and quantified a number of distinct and generalised responses to changes in HP and temperature in a shallow-water adapted marine ectotherm, *Palaemonetes varians*. Exposures were conducted over acute 2-hour periods with pressurisation steps, which although useful in identifying stressor responses, were not directly ecologically relevant. Chapter 5 has taken the work conducted in Chapters 3 and 4 and placed it in a more naturally relevant context by quantifying the expression of molecular markers over a set of sustained HP and temperature exposure. The exposures conducted in Chapter 5 extend our understanding of the responses to sustained HP exposures to the technological limits of currently existing hyperbaric systems.

Palaemonetes varians has now been subjected to a variety of experimental exposures and, in my opinion, represents the best eukaryotic model organism for HP physiology studies, with responses to both HP and temperature, along with other stressors, now well characterised in the species. However, the molecular markers characterised, stress responses quantified and ecological context discussed are of potentially diminished relevance to a wider scientific discussion unless such responses are comparable in other species and other scenarios. For this reason, Chapter 6 aims to elucidate and quantify molecular markers of the same response to HP stress now well characterised in *P. varians* in another marine ectotherm species. If our molecular markers can provide insight into the physiological limits to depth distributions and potential for bathymetric range shifts in shallow-water adapted marine ectotherms, then they may also be able to provide an insight into similar questions in non-shallow-water species. Chapter 6 aims to characterise and quantify the expression of molecular markers alongside other, higher-order, physiological measurements in a more eury-baric species, the Anomuran crab *Lithodes maja*. This will help to understand whether the responses shown in *P. varians* can be extrapolated as general concepts in hyperbaric physiology.

Chapter 6

Testing the generality of HP tolerance hypotheses: Deep-water species comparison

6. HYDROSTATIC PRESSURE TOLERANCE ACROSS ONTOGENY IN A MID-DEPTH KING CRAB *LITHODES MAJA*: DOES PRESSURE DELINEATE CURRENT & PAST DEPTH DISTRIBUTION LIMITS?

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6.1 Abstract

Most deep-sea fauna are thought to have originated from shallow-water origins at some point over biological history. Of the many potential ecological and physiological factors that may restrict the ability of marine organisms to shift their bathymetric ranges and potentially colonise deeper waters, hydrostatic pressure (HP) may be a key driver, and is certainly the most ubiquitous. HP may have a role in setting physiological depth distribution limits thus contributing to well documented bottlenecks and being, in part, responsible for driving the processes of evolution and speciation. This chapter assesses shifts in the tolerance of HP through early ontogenetic stages, and in mature adults, of the northern stone crab *Lithodes maja*. In its natural environment the species currently occupies a depth range in the North Atlantic of between 4 and 790 m. Two larval stages (Zoea I and Megalopa), the juvenile stage (Crab I), and mature adults were exposed to HPs of up to 30 MPa (≈3000 m water depth). The relative fold change of genes coding for an NMDAR-regulated receptor protein, 2 heat shock 70 kDa isoforms, and citrate synthase were quantified alongside measurements of respiratory rate in response to elevated HP. Results suggest a pronounced decrease in the acute elevated HP tolerance limit with age across ontogenetic stages, suggesting that age and organismal complexity may determine stress tolerance in relation to HP. Data are discussed in the context of the well characterised evolutionary history of the species.

6.2 Pre-text

Part of the work described in this chapter formed the research project of an MRes student, Catriona Munro (Munro 2014). The project was co-led by me, Alastair Brown, Sven Thatje, and Chris Hauton. Adult crabs were collected from Sweden and maintained in the laboratory by Alastair Brown. Subsequent larvae were reared predominantly by Catriona Munro, but also by Alastair Brown and myself. Experimental exposures were conducted by me and Catriona Munro. Gene-hunting of the *narg* gene and the 3 reference genes was conducted by both me and Catriona Munro. Initial nucleic acid extraction, preparation, and gene expression quantification was conducted by Catriona Munro under my supervision. Following the research project, I conducted further gene-hunting experiments (the *cs* gene, the *ldh* gene, and the *hsp70a* and *hsp70b* genes) and quantified the expression of additional molecular markers. All gene expression analysis was conducted by me.

6.3 Specific chapter hypotheses

$H_{6.1}$ - Transcriptional perturbations indicative of hydrostatic pressure (HP) intolerance are observed at HPs beyond the natural depth distribution limit of *Lithodes maja*.

$H_{6.2}$ - The apparent HP tolerance window of *Lithodes maja* narrows through ontogeny in line with analogous theories of thermal tolerance.

$H_{6.3}$ – Transcriptional regulation of gene markers associated with HP intolerance is observed in mature adult *Lithodes maja* after both short-term and long-term exposure periods.

6.4 Introduction

Although some suggest that early life may have originated around deep-water hydrothermal vent systems (Miller and Bada 1988), it is generally accepted that all complex deep-sea fauna originated from shallow-water origins (Crimes 1974). Climate-driven mass extinction events and subsequent re-colonisations are thought to have shaped both past and present deep-sea faunal assemblages (Jacobs and Lindberg 1998; Rogers 2000; Aquino-Souza et al. 2008). As such, the current deep-sea faunal assemblage comprises both young and ancient lineages (Raupach et al. 2009; McClain and Hardy 2010). One prevalent explanation of the shallow-water origin of deep-sea fauna suggests a mechanism of near-shore speciation and

innovation followed by migration offshore into unoccupied or under-represented niches, and potentially less competitive ecosystems (Jablonski et al. 1983; Lindner et al. 2008). There is evidence of repeated down-water column migrations of shallow-water taxa throughout the Phanerozoic (Horne 1999; Rogers 2000; McClain and Hardy 2010).

Clear zonation patterns can be seen along bathymetric gradients in the marine environment, particularly in benthic fauna where high species turnover occurs at the upper continental slope boundary (≈1000 m), and also at the upper abyssal boundary (2000-3000 m) (Carney 2005). Unimodal patterns of biodiversity have been observed at bathyal depths suggesting such depths to be a major site of speciation in the deep sea (Etter et al. 2005), large-scale environmental heterogeneity may play an important role in this, as proposed by the depth-differentiation hypothesis (Etter et al. 2011). HP and temperature may be two key factors, amongst many, that set species distribution limits and contribute to speciation (Brown and Thatje 2014; 2015). Studies testing hyperbaric tolerance in early life stages of shallow-water adapted invertebrates consistently find evidence for HP tolerance thresholds approaching bathyal depths (Brown and Thatje 2014). The synergistic thermodynamic effects of HP and temperature, as described by the thermal-pressure coefficient (Flory 1965) have been shown to play an important role in the HP tolerance limits of shallow-water adapted marine ectotherms (Morris et al. 2015; Chapters 3 and 5). Because of the similarity in their effects, theories of the response to changes in temperature have been extended to HP. However, significantly more is known about the effects of temperature than HP. Aerobic thermal tolerance limits have been shown to correlate with ontogeny in fish. Tolerance windows increase from the embryonic stage through to the juvenile stage, as diffusion across cell surfaces is replaced by a circulatory/ventilation system (Cossins and MacDonald 1989). Such observations are suggested as a mechanistic explanation for shifting HP tolerances with ontogeny in benthic invertebrates (Hazel 1995; Somero 1995).

Anomuran crabs in the family Lithodidae are thought to have radiated and speciated globally in cold waters. Lithodid evolution can be traced back to the Cenozoic era and the speciation of shallow-water populations of anomuran crabs in the North East Pacific. Two subfamilies have persisted: the Haplogastrinae and the Lithodinae (Section 1.5.3). Within the Lithodinae subfamily, members of the *Lithodes, Paralomis*, and *Neolithodes* genera are thought to have colonised deep waters before extending their distribution into other ocean basins (Hall and Thatje 2009). Bottlenecks in Lithodinae distribution have been thought to occur due to thermal tolerance limits, and differences in life-history traits between genera

(Hall and Thatje 2009). *Lithodes maja* is among lithodid species currently found in the North Atlantic which are thought to have derived from the lineages of the earliest Lithodinae to leave the North Pacific (Snow 2010). *Lithodes maja* shows lecithotrophic development (Section 1.5.3) through 3 demersal zoeal stages (zoea I, II, and III) and a putatively demersal megalopa stage, before moulting as a benthic, feeding, juvenile crab I stage (Anger 1996; Anger 2001; Thatje 2004; Thatje et al. 2005; Brown 2015). Growth, maturation and maximum age has not been resolved for this species.

This chapter investigates the effects of elevated HP on the transcription of 4 newly characterised genes in the species, alongside respiration rate measurements, initially in 3 distinct early ontogenetic stages (zoea I, megalopa, crab I), and also transcriptional regulation in mature adults of the species. Genes were selected *a priori* based on pressure intolerance signals shown in previously in *Palaemonetes varians* (Chapters 3, 4 and 5). Traditional markers of the cellular stress response (CSR), coding for 2 heat shock 70 kDa proteins, were characterised. A putative marker of aerobic metabolism, coding for the citrate synthase enzyme, was isolated and characterised. Finally, a putative pressure intolerance marker (coding for an NMDAR-regulated protein) was resolved. The expression of these newly characterised markers were then quantified under elevated HP scenarios to test the hypothesis that HP tolerance decreases with age, and maybe complexity, as seen in thermal tolerance studies (Cossins and MacDonald 1989).

The *narg* gene, coding for an NMDAR-regulated protein, has been suggested as a putative pressure intolerance marker associated with NMDAR hyper-excitability and a precursor to the onset of behavioural pathologies associated with high pressure neurological syndrome (HPNS) (Morris et al. 2015; Chapter 3). This gene marker has been observed to be responsive to elevated pressure scenarios in a shallow-water adapted marine ectotherm (Chapters 3, 4, and 5) but has not been tested in a species with a wider depth distribution pattern, and thus a wider HP tolerance window.

Members of the heat shock protein family respond to the general effects of stress at a cellular level, namely macromolecular damage. The proteins of the HSP70 family are chaperones that help with the assembly and folding of nascent proteins, and the re-folding and repurposing of developed proteins (Feder 1999; Feder and Hofmann 1999). Genes encoding HSP70 proteins have been shown to be highly transcriptionally regulated in response to the general effects of stress and can be thought of as biomarkers of intracellular macromolecular damage (Morris et al. 2013; Section 1.3).

The Citrate synthase (CS) enzyme (coded by the *cs* gene) is an important regulatory protein in the TCA cycle. It is considered a "pace-maker" enzyme in the formation of ATP (Goldenthal et al., 1998). Specifically, the citrate synthase enzyme catalyses the production of citrate from acetyl CoA and oxaloacetate: a rate-limiting process. It is important to note that although citrate synthase activity is localized in mitochondria, the gene encoding the enzyme is found within the nuclear genome in eukaryotes. Synthesis occurs in the cytoplasm before transportation to the mitochondrial matrix (Goldenthal et al. 1998). CS activity can be used as a proxy for aerobic respiration at the mitochondrial level, and hence of an organism's metabolic capacity (Seibel and Childress 2000). CS activity has been previously linked with depth, metabolic rate, food availability, and seasonally induced changes in physiological status (Childress and Somero 1979; Drazen and Seibel 2007; Seibel and Drazen 2007; Cullen et al. 2003; Dahlhoff and Menge 1996).

The quantification of transcriptional regulation of genes coding for 2 HSP70 isoforms, CS, and the NMDAR-regulated protein, alongside respiratory rate analysis, allow for an examination of the molecular physiological constraints acting upon different life stages of *Lithodes maja*, and will provide a novel insight into the degree to which HP may constrain bathymetric distributions in a mid-depth benthic marine invertebrate at various ontogenetic stages. The HP exposures were all conducted at a water temperature of 6°C. This represents the temperature of the deepest recorded observation of *Lithodes maja* (790 m and 6.6°C) (OBIS 2015).

6.5 Materials & methods

6.5.1 Adult sampling & maintenance

Adult specimens of *Lithodes maja* were collected during September and October 2011 using baited traps in Gullmarsfjord, Sweden at depths of approximately 60 m (approximate location: 58° 21' 0" N, 11° 34' 48" E) (Section 2.1.2). Before relocation to the National Oceanography Centre Southampton (NOCS, UK) the animals were maintained in an open aquarium system (temperature ~10°C, natural light cycle, atmospheric pressure) at the Sven Lovén Centre for Marine Sciences – Kristineberg, Sweden, in seawater from a 32 m deep intake. The adults were transferred to Southampton in a temperature-controlled van at 6°C, and travel time was less than 24-hours. The animals were starved for several days prior to

transportation, and were transported individually in polystyrene boxes lined with wetted towels. Subsequently, animals were maintained in a recirculating aquarium (temperature 6°C, 24-hour darkness, atmospheric pressure) at the NOCS. The maintenance temperature was chosen to match the temperature in the field. Constant darkness was chosen to reflect the conditions observed at the depth ranges tested by the hyperbaric exposures.

6.5.2 Larval rearing

Larvae were obtained from two gravid females. Individuals used for gene expression studies were obtained from a single female that moulted during the night of the 3rd April 2012 and oviposited the following day (Section 2.1.2). Although the use of offspring from a single female may result in reduced genetic variation between individuals, the lengthy brooding period combined with extended hatching periods imposes a practical limit on the number of larvae from different parents that can be reared and maintained simultaneously. For further details of larval rearing see Section 2.1.2. The larval development of *L. maja* is lecithotrophic (Anger 1996) and therefore larval stages were maintained without food; the first juvenile crab stage was fed freshly hatched *Artemia salina* nauplii *ad libitum* every other day and the water was changed before feeding. The juvenile crabs (Crab I) were starved for 3 days prior to starting pressure treatments to avoid molecular contamination from gut contents.

6.5.3 Larval pressure exposures

Acute HP exposures were performed on individuals from the zoea I, megalopa, and crab I stages at 5 different pressures: 0.1, 10.0, 20.0, 25.0 and 30.0 MPa. Five individuals were used for most HP treatments, except where numbers were limiting and only four individuals were used - these are highlighted in the results. In separate, yet comparable, treatments, respiration rates (MO_2, μmol O_2 $mg^{-1}h^{-1}$) were measured to assess respiratory response to acute increases in HP. Respiration rates were assessed following an adaptation of established protocols (Benson and Krause 1984; see Section 2.3 for further details). Due to larval mortality, fewer crab I were available for these treatments, therefore fewer HP treatments were carried out: 0.1, 10.0, and 15.0 MPa. Three control vials without individuals were used for each HP treatment to control for microbial respiration within the seawater.

Individuals were placed in separate 2.8 ml cryovials filled with seawater pre-incubated to 6°C. The vials were filled until they overflowed, and were carefully capped underwater to avoid trapping air within the vial. The vials were placed within a steel pressure vessel (Section 2.2.1), which was filled with fresh water. Both the water and the pressure vessel were pre-incubated for 24 hours at 6ºC. The vessel was then pressurised to the selected HP within 10-seconds (Section 2.2.1). The vessel was kept at constant temperature (6°C) and HP for 4-hours. For transcriptional analysis, individuals were removed from the pressure vials, transferred to 1.5 ml centrifuge tubes, and flash frozen whole in liquid nitrogen, within 5-minutes of instantaneous depressurisation. Samples were stored at -80°C until further analysis.

6.5.4 Adult pressure exposures

Adult *Lithodes maja* crabs were subjected to elevated HP exposures of 4-, 24-, and 216-hours at 0.1 MPa (atmospheric control) and 7.5 MPa (elevated HP exposure). Further, 216-hour exposures were conducted at 10 MPa and 12.5 MPa, all using the IPOCAMP system (Section 2.2.2). The system was filled with filtered seawater (Salinity 32-34) and set running at atmospheric pressure overnight to stabilise at 6°C prior to the start of the exposure. Once stabilised, an individual crab was placed on a platform inside the hyperbaric chamber which was sealed and run for 24-hours at 0.1 MPa prior to the exposure to allow time for the crab to recover from any handling stress. The system was then pressurised to the desired HP (0.1 MPa, 7.5 MPa, 10 MPa, or 12.5 MPa) at a rate of 0.5 MPa every 10-minutes and then maintained for either: 4-, 24-, or 216-hours. Following the desired exposure period the system was depressurised over a period of 1-minute. The crab was removed and muscle tissue was sampled for transcriptional analysis. The 3rd right pereopod was severed at the merus, transferred to an RNase/ DNase-free 1.5 ml microcentrifuge tube and snap frozen in liquid nitrogen before being transferred to -80°C storage. Tissue samples were always preserved within 10-minutes of the HP exposure in order to minimise the potential effects of depressurisation or post-exposure recovery on transcriptional regulation.

6.5.5 RNA extraction & reverse transcription

For larval experiments, whole individuals were homogenised for total RNA extraction due to the small amount of tissue mass available. For adult experiments, muscle tissue from the 3rd right pereopod was homogenised for total RNA extraction. In all cases, tissue samples were transferred from -80 °C storage to liquid nitrogen and then to a 5 ml tube containing 2-3 ml TRI-reagent (based on available tissue mass), homogenised, and total RNA was extracted, as according to Section 2.5.1. Total RNA was assessed for purity and integrity using a Nanodrop spectrophotometer and Experion™, respectively (Section 2.5.1.1). Subsequently, total RNA was DNase treated (Section 2.5.2), and 0.68 µl of DNase-treated total RNA was reverse-transcribed in a 20 µl reaction using oligo $(dT)_{23}$ primers according to Section 2.5.3.

6.5.6 Primer design

Degenerate PCR-based gene hunting techniques were used to generate *Lithodes maja* specific gene sequences for 7 genes: the *narg* gene, *cs* gene, two *hsp70* isoforms, and four candidate reference genes, *rps26, tubulin a1*, *eef1a* and *rpl8*. Degenerate primer pairs were designed for each gene from alignments of sequences from crustacean and insect species available in sequence databases (GenBank, EMBL-EBI) using Clustal Omega (Sievers et al. 2011) (see Chapter 5, section 5.5.3 for further details). The putative target genes were amplified by PCR reactions using the degenerate primers and *L. maja* template cDNA. The resulting amplicons were gel extracted (Section 2.6.3) and cloned using the pGem-T™ easy vector kit (Section 2.6.4). Colonies with the appropriate insert were grown, plasmids were extracted (Section 2.6.5), and sequences were determined using conventional dideoxy-termination sequencing (Section 2.6.6). Primers for use in qPCR were designed from consensus *L. maja* gene sequences (Section 2.8; Table 2.4) using standard criteria and Primer Express software (Section 2.8.3). The *rps26* gene and *ldh* gene degenerate primers did not provide extractable PCR fragments in this species despite various optimisation strategies, and consequently were not included further in the study.

6.5.7 Quantitative PCR

To ensure primer specificity and efficiency, each qPCR primer pair was optimised in order to determine the optimum primer concentrations and reaction efficiencies, ensuring 90-110% efficiency across the predicted cDNA concentration range (Appendix A1.4) in accordance with Bustin et al. (2009; 2010) (Section 2.8.5). A melt curve analysis was also applied to ensure that all samples produced a single distinct product (Section 2.8.4).

All reactions were carried out on the Mx3005P qPCR system (Section 2.8.1.2). Each 25 µl reaction contained 12.5 µl Precision 2x qPCR Master mix containing SYBR green fluorescent dye, 1 µl (~34 ng) of template cDNA, and 0.5 µl ROX reference dye (Section 2.8.1.2). Two technical replicates were run for each sample. Standard qPCR conditions were applied as described in Section 2.8.6 (Figure 2.11).

Quantification cycle (Cq) values were analysed using qBase+ software (Section 2.8.7). The Cq values for each of the genes of interest (*narg*, *cs*, *hsp70a,* and *hsp70b* genes) were normalised against the best combination of reference genes (*eef1a* and *rpl8* genes) from 4 potential endogenous reference genes, as determined using the geNorm analysis (Section 2.8.8). These normalised relative quantities were scaled relative to the 0.1 MPa atmospheric control treatment for each gene and presented as relative fold change (RFC) values. Statistical analysis of all gene expression data was conducted in R (R core team, 2013).

Table 6.1 Degenerate primer sequences used in *Lithodes maja* to isolate and characterise: the *cs* gene; the *ldh* gene; the *hsp70* gene; the *narg* gene; the *rpl8* gene; the *rps26* gene; the *tub1a* gene; and the *eef1a* gene. Optimised PCR conditions shown alongside sequences

Gene	Primer	Sequence	Conditions
Citrate synthase (*Cs* gene)	dCS F1	T GAY CAY GAR GGH GGH AAY GT	2 µl $MgCl_2$; 2 µl primers (25nM); 57.5°C annealing temp; 1 min elongation step
	dCS R1	TG DGC RTC YAC RTT RGG CCA	
Lactate dehydrogenase (*Ldh* gene)	dLDH F1	GTN GGN GGN CAR GTN GGN AT	2 µl $MgCl_2$; 2 µl primers (25nM); 54°C annealing temp; 1 min elongation step; (Failed)
	dLDH R1	SW RTC NCC RTG YTC NCC DAT	
HSP70 *(hsp70* gene)	dHSP F1b	GTG GAR ATC ATC ATG CCA AYG AYC ARG G	2 µl $MgCl_2$; 2 µl primers (25nm); 54°C annealing temp; 1 min elongation step
	dHSP R1	CCN CGR TCY TTN GTN GCY TG	
NMDAR P1 (*narg* gene)	dNMDA F2	TGY TGG CAY GTN TAY GG	3 µl $MgCl_2$; 2 µl primers (25nM); 55°C annealing temp; 20 sec elongation step
	dNMDA R2	CCA CAT RCA YTG CAT YTC RTT	
RPL8 (*rpl8* gene)	dRPL8 F1	GTN CAT TTY MGN GAY CCN	2 µl $MgCl_2$; 2 µl primers (25nM); 55°C annealing temp; 30 sec elongation step
	dRPL8 R1	GG RTG YTC NAC NGG RTT	
RPS26 (*rps26* gene)	dRPS26 F1a	AAR CAY GGN MGN GGN CAY	3 µl $MgCl_2$; 2 µl primers (25nM); 56°C annealing temp; 20 sec elongation step; (Failed)
	dRPS26 R1	NCK RTT NCK NAC NAC YTT	
Tubulin 1α (*tub1a* gene)	dTub F1	GNA ARG ARG ACG CNG CNA	2 µl $MgCl_2$; 2 µl primers (25nM); 54°C annealing temp; 30 sec elongation step
	dTub R1	TYA TGG TNG AYA AYG ARG CNA	
EEF1α (*eef1a* gene)	dEEF1a F1	GGN GAR TTY GAR GCN GG	3 µl $MgCl_2$; 2 µl primers (25nM); 56.5°C annealing temp; 30 sec elongation step
	dEEF1a R1	CCN CAN GCR CAN GTY TG	

6.6 Results

6.6.1 HP effect on larval gene expression

The effect of HP on the relative transcription of all genes correlated with ontogenetic stage (Figure 6.1). In the zoea I stage, there were no significant relative fold changes (RFC) of the *narg* gene or the *hsp70b* gene, but both the *cs* gene and *hsp70a* gene showed a significant relative fold (RF) increase at 30.0 MPa. In the megalopa stage, significant RF differences were seen at 25.0 and 30.0 MPa in the *narg* gene, the *cs* gene, and the *hsp70a* gene; there was also a significant RF increase of the *hsp70b* gene at 30.0 MPa (Figure 6.1). Finally, in the crab I stage, significant RF increases were seen at 25.0 and 30.0 MPa in the *narg* gene, and the *hsp70b* gene. Significant RF increases were also seen at 20.0, 25.0 and 30.0 MPa in the *cs* gene, and the *hsp70a* gene (Figure 6.1).

6.6.2 HP effect on adult gene expression

In mature adult *Lithodes maja,* no significant RFCs were observed between 0.1 MPa atmospheric control exposures and 7.5 MPa elevated HP exposures in the *narg* gene, the *cs* gene, the *hsp70a* gene, or the *hsp70b* gene following 4-, 24- and 216-hour sustained pressure exposures at a constant 6°C (Figure 6.2).

The 216-hour exposures were also conducted at 10 MPa and 12.5 MPa (Figure 6.3). Significant RF increases were observed in the *narg* gene, the *hsp70a* gene, and the *hsp70b* gene at 12.5 MPa in comparison to the 0.1 MPa control exposure. Significant RF increases were observed in the *cs* gene at both 10 MPa and 12.5 MPa in comparison to the 0.1 MPa control exposure.

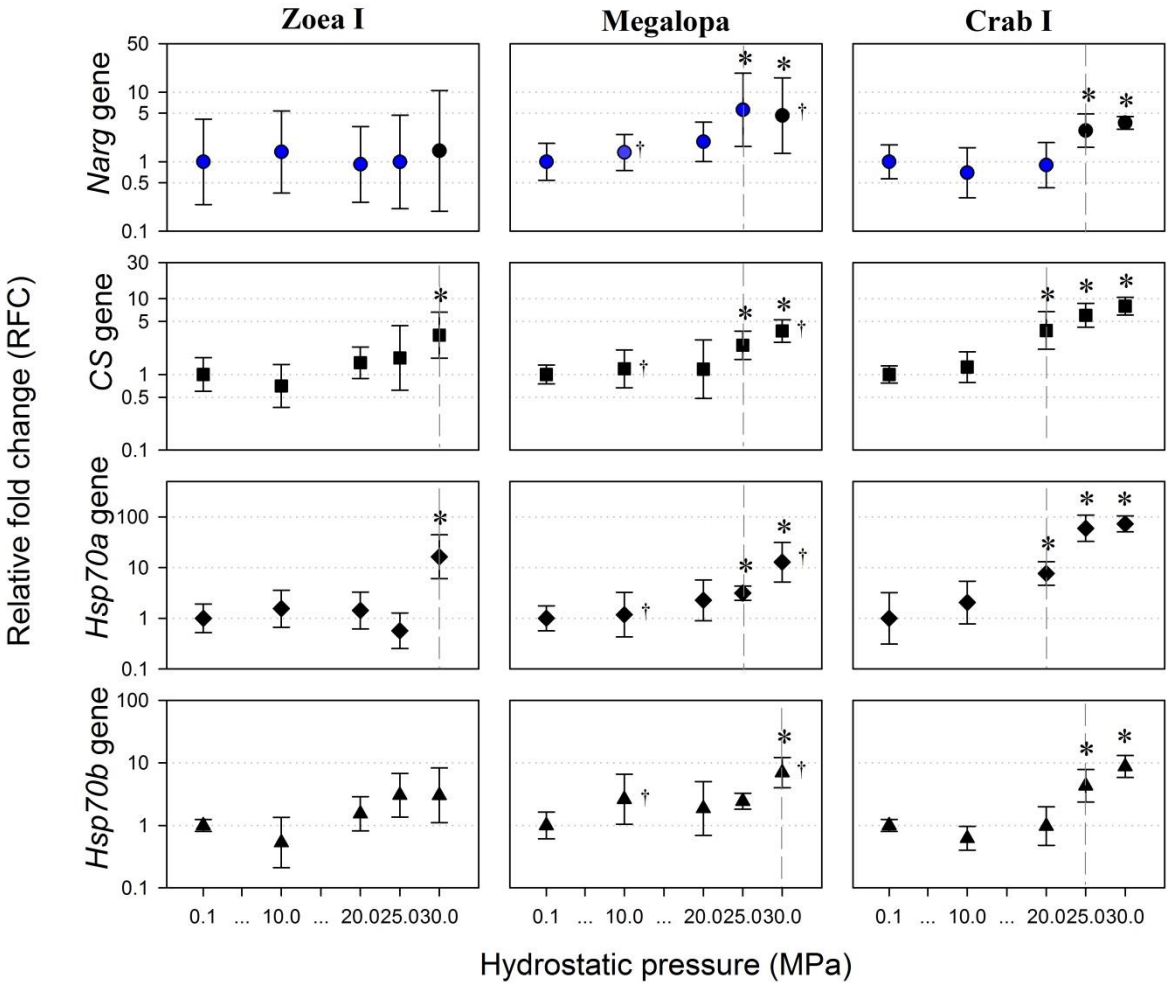

Figure 6.1 Relative fold changes (RFC) and 95% CIs in the northern stone crab *Lithodes maja* of 4 genes; the *narg* gene (circles); the *cs* gene (squares); the *hsp70a* gene (diamonds); and the *hsp70b* gene (triangles) across three early ontogenetic life stages at hydrostatic pressures ranging from 0.1 MPa (atmospheric control) to 30 MPa (equivalent of 3000 m water depth). Blue points represent previously published data points (Munro 2014). Black points represent the work of this Chapter. RFCs scaled to the 0.1 MPa control treatment. Statistical significance is displayed as: * = $p<0.05$, calculated from 5 biological replicates unless otherwise stated. † = 4 biological replicates. Vertical dashed lines visually emphasise the hydrostatic pressure at which significant differences are first observed

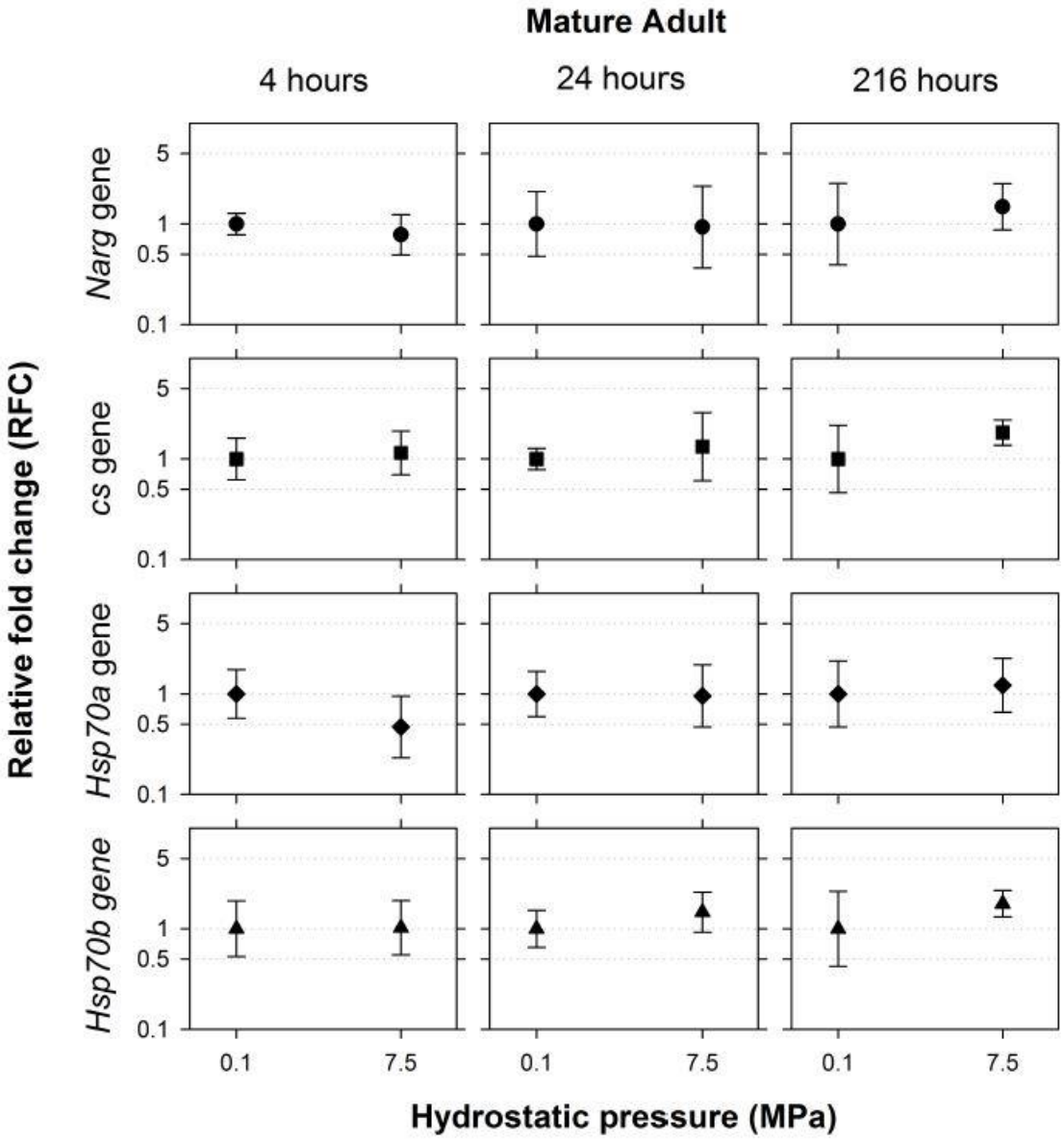

Figure 6.2 Relative fold changes (RFC) and 95% CIs of 4 genes; the *narg* gene (circles); the *cs* gene (squares); the *hsp70a* gene (diamonds); and the *hsp70b* gene (triangles) in mature adult *Lithodes maja* at 0.1 MPa (atmospheric control) and 7.5 MPa (around the natural distribution limit of species). Elevated HP exposures conducted over 4-, 24- and 216-hour periods at 6°C. RFCs scaled to the 0.1 MPa control treatment. Statistical significance is displayed as: * = p <0.05, calculated from 5 biological replicates

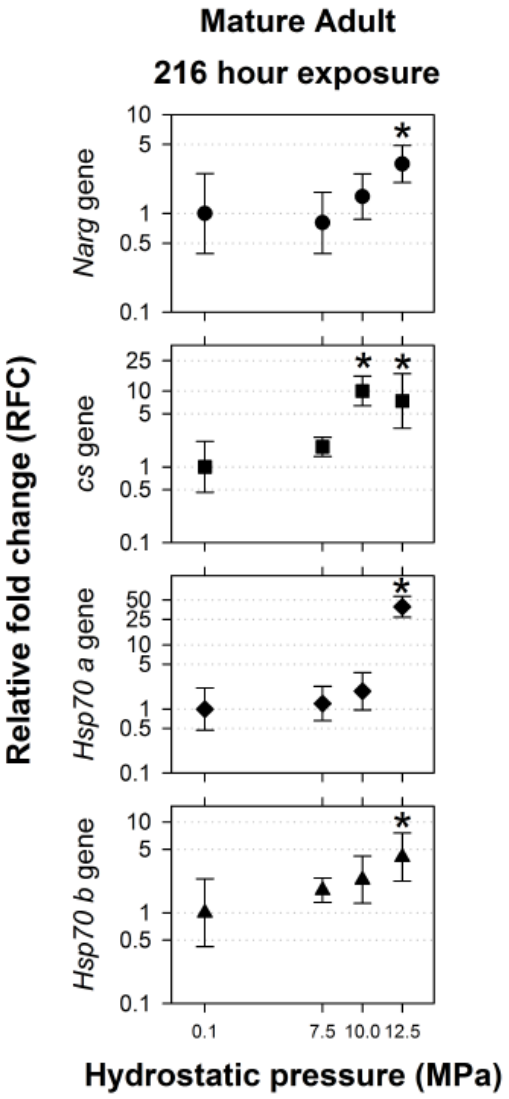

Figure 6.3 Relative fold changes (RFC) and 95% CIs of 4 genes; the *narg* gene (circles); the *cs* gene (squares); the *hsp70a* gene (diamonds); and the *hsp70b* gene (triangles) in mature adult *Lithodes maja* at 0.1 MPa (atmospheric control) and 7.5, 10 and 12.5 MPa following a 216-hour sustained exposure at 6°C. RFCs scaled to the 0.1 MPa control treatment. Note that the y-axis is scaled differently for each gene to aid visualisation. Statistical significance is displayed as: * = $p<0.05$, calculated from 5 biological replicates

6.7 Discussion

This chapter provides a novel quantification of patterns in transcriptional responses to hydrostatic pressure (HP) in a continental slope-depth species. Firstly, a decrease in the acute HP tolerance limit was demonstrated as ontogeny advances. Secondly, an up-regulation of genes associated with intolerance to HPs was observed narrowly beyond the natural depth distribution limit of the species in mature, reproductively active, adults following sustained acclimation-based exposures. This chapter represents a significant advance in describing the potential physiological mechanisms that control bathymetric distributions. Such knowledge could be used to assess the ability of species to respond to environmental change by shifting their bathymetric distributions. This may also be useful

for the understanding of the physiology of known bottlenecks in the radiation history of *Lithodes maja* and related species.

6.7.1 Early life response to elevated HP

In the acute ontogeny experiment (Figures 6.1 and A3.1), all three early ontogenetic stages of *Lithodes maja* were able to tolerate exposure to HPs beyond that of their adult depth distribution maximum (790 m); however, the HP at which respiratory and transcriptional effects were first observed decreased with advancing ontogeny between the zoea I, megalopa, and the crab I stage. These results are consistent with a number of studies that have examined the effects of HP on the embryonic and larval forms of several benthic invertebrates (see Brown and Thatje 2014 for review). General trends in ontogenetic HP tolerance shifts have previously been observed: initial increases in tolerance from early embryonic through to early larval forms are followed by decreases in tolerance in later life history stages (Mestre et al. 2013).

Acute elevated HP led to transcriptional regulation of genes associated with the cellular stress response (CSR), namely two 70kDa heat shock protein isoforms (the *hsp70a* and *hsp70b* genes). These results are consistent with previous studies demonstrating transcriptional regulation of genes coding for heat shock proteins in response to the combined effects of elevated HP and changing temperature (Cottin et al. 2012; Morris et al. 2015; Chapters 3 & 5). Elevated HP is thought to affect protein conformation and *de novo* protein folding (Somero 1992; Silva and Weber 1993). The up-regulation of two HSP70 isoforms provides evidence for an increase in macromolecular damage in early *L. maja* life stages in response to elevated HP beyond its natural distribution range.

Respiratory responses observed support the transcriptional inferences of an ontogenetic shift in the upper HP tolerance in early *L. maja* life stages. Elevated HP typically results in an increase in oxygen consumption, inferring a stress-induced increase in metabolism, followed by a subsequent decrease (metabolic depression) (Brown and Thatje 2011; Oliphant et al. 2011; Thatje and Robinson 2011; Smith and Thatje 2012). As with aerobic thermal windows, an acute HP increase away from an organisms optimal point is suggested to lead to increases in homeostatic effort, and a corresponding increase in metabolism (Frederich and Pörtner 2000; Brown and Thatje 2011). In the absence of adaptations to elevated HP, impaired membrane and enzyme functionality can lead to metabolic

depression and a mismatch between oxygen supply and demand, resulting in a shift from aerobic to anaerobic metabolism (Oliphant et al 2011; Weiss et al 2012; Chapters 4 & 5). Whilst tolerable in the short-term, the scenario represents a time-limited state, and prolonged survival is not possible unless conditions change, or acclimatory processes occur (Pörtner 2002). The change in mitochondrial activity in response to HP may be reflected in the expression of the *cs* gene, coding for the citrate synthase enzyme which is involved in a rate limiting phase of the TCA cycle (Goldenthal 1998).

Further evidence of cellular damage may be inferred by the up-regulation of the *narg* gene, putatively coding for an N-methyl-D-aspartate receptor (NMDAR)-regulated protein. Under elevated HP scenarios beyond natural distribution limits, neuronal cell death leads to an up-regulation of genes associated with NMDAR activity (Morris et al. 2015; Chapter 3). Significant up-regulation of the *narg* gene in response to elevated HP in the temperate shallow-water shrimp *Palaemonetes varians* is proposed as an indicator for the onset of pressure intolerances associated with neuronal tissue sensitivity (Morris et al. 2015; Chapters 3 and 4). This up-regulation is proposed to be a precursor to behavioural signs of pressure intolerance such as 'loss of equilibrium' (Oliphant et al. 2011; Morris et al. 2015). Results of this chapter support the hypothesis that HP affects critical biological processes associated with neuronal activity.

Because of similarities in the general effects of HP and temperature in terms of thermodynamics, namely the way both factors interact with volume change reactions (Simonato et al. 2006), theories of thermal tolerance have been extended to HP tolerance (Airriess and Childress 1994). One such theory is the change in stress tolerance with ontogeny. In fish, thermal tolerance limits widen through early ontogeny before narrowing with age in adulthood (Pörtner and Farrell 2008). Pörtner and Farrell (2008) suggest that the observed narrowing of thermal tolerance windows with age may be due to the increases in metabolic demand and functional complexity that accompanies age in many species. Complexity, however, is a very general concept that is particularly hard to underpin in a specific enough sense to prove experimentally that is the causative factor in changes in a parameter such as thermal tolerance. For this reason, experimental data proving or supporting such a concept is sparse. A narrowing of HP tolerance through ontogeny in *L. maja* may, however, be associated with a number of morphological and functional changes. Many crustacean larvae experience a shift in cardiac function through early development. The shrimp *Metapenaeus ensi*s and isopod *Ligia oceanica* show a transition in cardiac control (from myogenic to neurogenic) (Yamagishi and Hirose 1997; McMahon et al. 2002). Such

an example adds weight to the theoretical model of ontogenetic shifts in the HP tolerance of marine invertebrates (Hazel 1995). In *Lithodes santolla*, cardiac and gastric regions differentiate between the megalopa and juvenile stage (McLaughlin et al. 2001). This differentiation may coincide with a transition in cardiac function (Spicer 2001). In *L. maja*, there are significant differences in biomass, oxygen consumption, and chemical composition between the zoea, megalopa, and juvenile stages (Anger 1996), that may be indicative of a shift towards greater structural (as suggested by biomass) and functional (as suggested by oxygen consumption) complexity with age.

Of the 3 deep water lithodid genera, *Lithodes* has the shallowest distribution range (Snow 2010). The results of this chapter show that early life stages of *L. maja* were able to tolerate acute elevated HP exposures beyond the HPs experienced at the depth limits of their natural adult distribution, which is reported to be 790 m, or the equivalent of 7.9 MPa. Respiration rate measurements for the crab I stage in *L. maja* showed a significant drop from control measurements (a sign of intolerance) at 15 MPa, the first of the HP exposures conducted that fell outside the species natural range limits.

HP tolerance may affect ontogenetic migration patterns in lithodids. Although *Lithodes* is an understudied genus, there is evidence of ontogenetic migration in *Lithodes ferox* (Abelló and Macpherson 1991). In this species, juveniles and adults have almost completely distinct bathymetric distributions, with juveniles occupying deeper waters than adults of the species (Abelló and Macpherson 1991). In *Paralithodes camtschaticus*, seasonal migration into shallow waters is related to larval release, thought to be driven by their planktotrophic larval development (Jørgensen and Nilssen 2011). Further, *Lithodes santolla* shows the opposite pattern to *Lithodes ferox* with juveniles found in kelp holdfast habitats in surface waters (3-5 m), whilst adults are found over a range of depths but usually deeper than 5 m (Cárdenas et al. 2007).

The larval ontogeny tolerance experiment presented here (Figures 6.1 and A3.1) is an important first step towards fully understanding the role of HP tolerance in the evolution and ecology of this group of benthic marine taxa. This study provides an important precursor for longer-term exposure studies in adults (as described below), and provides an indication of the short-term role of HP limitation in early ontogenetic stages.

6.7.2 Mature adult response to elevated HP

Transcriptional regulation of the same set of markers used in the larval ontogeny experiment (Section 6.7.1) was quantified in mature adults of the same species. Over 4-hour acute exposures no sign of HP intolerance was observed at 7.5 MPa compared to atmospheric control treatments, as suggested by a lack of transcriptional regulation in any of the gene markers quantified. Water depths equivalent to 7.5 MPa (750 m) represent the approximate natural distribution limit of the species (790 m) (OBIS 2015). It is therefore to be expected that HPs of such magnitude are able to be tolerated by *L. maja*. Such a result provides further support that the gene markers quantified are useful and ecologically relevant markers of the onset of pressure intolerance in that they are not responsive to HPs within the species natural distribution window. Further, sustained HP exposures of 24- and 216-hours at 7.5 MPa result in the same lack of transcriptional regulation as seen in the 4-hour exposures. Again, this suggests that the results of acute exposures such as those in the larval ontogeny experiment which (Section 6.7.1), despite not being directly ecologically relevant, do corroborate well with more relevant sustained HP exposures and therefore can be used to provide insight into the processes of depth distribution delineation and range shifting.

In the larval ontogeny experiment (Section 6.7.1) transcriptional regulation indicative of HP intolerances were observed at HPs well beyond the natural distribution of the species. The disparity between the experimentally observed onset of HP intolerance and natural distribution limits reduced through ontogeny. If this reduction in ontogeny is a result of increasing organismal complexity, be it structural, functional, or both, then it would be expected that this disparity would further reduce in mature adults of the species. Indeed, following sustained HP exposures (216-hour) in mature adult *L. maja*, signs of HP intolerance indicative of cellular macromolecular damage, specific HPNS-associated nervous system sensitivities, and stress induced metabolic elevation were observed at 12.5 MPa. Over the same sustained exposures, Brown (2015) analysed cardiac activity and respiratory rate, the results of which further suggest that the HP tolerance window of *L. maja* adults is significantly narrower than earlier ontogenetic life stages. Further, evidence of elevated aerobic metabolism was observed at HPs equivalent of 1000 m both in transcriptional regulation analysis (the *cs* gene: Figure 6.3) and in respiratory rate analysis (Brown 2015). Cardiac activity which reduced step-wise with increasing HP across the sustained HP exposures suggests a mismatch between oxygen supply and demand that may

be indicative of an oxygen- and capacity-limited hyperbaric tolerance in *L. maja* (Brown 2015). The theory of thermal tolerance being oxygen- and capacity-limited (Pörtner 2010; Sokolova 2013) may extend to HP, as the negative effects of the two stressors share many similarities (Airriess and Childress 1994; Simonato 2006; Chapters 3 and 5). Certainly, results presented in this chapter suggest that hyperbaric intolerance is associated, in part, with unsustainable increases in aerobic metabolism as suggested by the increase in relative expression of the *cs* gene and corroborated by the results obtained by Brown (2015). It should be noted that larval gene expression assays were conducted on whole organism homogenisations whilst adult gene expression assays were conducted solely on leg muscle tissue. Whole organism homogenisations were a necessity due to low tissue mass of the larvae. Although gene expression signatures might be different between the organ-incorporated homogenisations and muscle tissue homogenisations, the work of Chapter 4 suggests that, in a shallow-water shrimp, the targeted gene expression pathways studied here were comparable in their magnitude and pattern of expression following hyperbaric exposures.

6.8 Conclusions

In summary, there is a shift in the short-term acute high-HP tolerance limit between the zoea I, megalopa, and crab I stages of *Lithodes maja* as evidenced from changes in gene expression and aerobic metabolism. These results provide an indication of the molecular response to HP in the early ontogeny of a continental slope-depth adapted species, and indicate that bathymetric distribution may become more constrained as ontogeny advances. Such constraints may be driven by increasing structural or functional complexity, as theorised to be the driver of thermal tolerance narrowing with age in some marine taxa. Further, comparative evidence suggests that HP tolerance limits in mature adult *L. maja* represent an additional narrowing of the HP tolerance window. Over a sustained HP exposure period in mature adults of the species, transcriptional regulation inferring HP intolerance was observed at the first tested elevated HP point beyond the naturally experienced HP limit of the species, and equivalent to within ~200 m of their natural depth limit. This result suggests that, despite numerous and undoubtedly important ecological factors, physiological tolerance, and particularly HP tolerance, may be a strong driver in setting the depth distribution limits in mid-depth marine ectotherms such as *L. maja*.

Chapter 7

Synthesis & Conclusions

7. HYPERBARIC PHYSIOLOGY & CLIMATE CHANGE

7.1 Hydrostatic pressure (HP) responses

The experiments highlighted in Chapters 3, 4, and 5 have demonstrated that elevated hydrostatic pressure (HP) elicits a variety of sub-lethal responses at the cellular and organismal level in a shallow-water-adapted caridean shrimp, *Palaemonetes varians*. These results provide a valuable insight into stress tolerance windows, physiological limits to distribution ranges, and the viability of shifting distribution ranges in light of changing environmental conditions. Previous research on the subject using the same study organism has focussed primarily on the heat shock response (HSR) (Cottin et al. 2010; Cottin et al. 2012) or behavioural responses to elevated HP (Oliphant et al. 2011; New et al. 2014). This thesis has drawn on these previous observations and elucidated a set of distinct yet connected responses to elevated HP in order to further our understanding of the multi-level effects of HP on shallow-water ectotherms. Figure 7.1 highlights four categories of response to elevated HP: behavioural responses, cellular stress responses (CSR), homeostatic responses, and metabolic responses. Each of these distinct categories has a subset of responses that vary; depending on the timing and magnitude of the HP exposure; the effects of other stressors in combination; the prior physiological state (fitness) of the organism, and the magnitude of response from each of the other categories. It is important to consider each of these categories and how they relate to one another in order to fully understand the overall effect of a stressor or stress scenario on an organism. It is this overall effect that is of relevance in extending our appreciation of how stressors perturb organisms in an experimental laboratory setting, and helps in understanding the contribution of organismal physiology to a species' ecology and evolution. Elevated HP in the shallow-water-adapted shrimp *P. varians* elicits behavioural responses in 2 distinct forms (Figure 7.1a). The escape response is characterised in this species as an increase in directional active movement and an increase in reflexive locomotory contractions termed tail-flicking (Chapter 4).

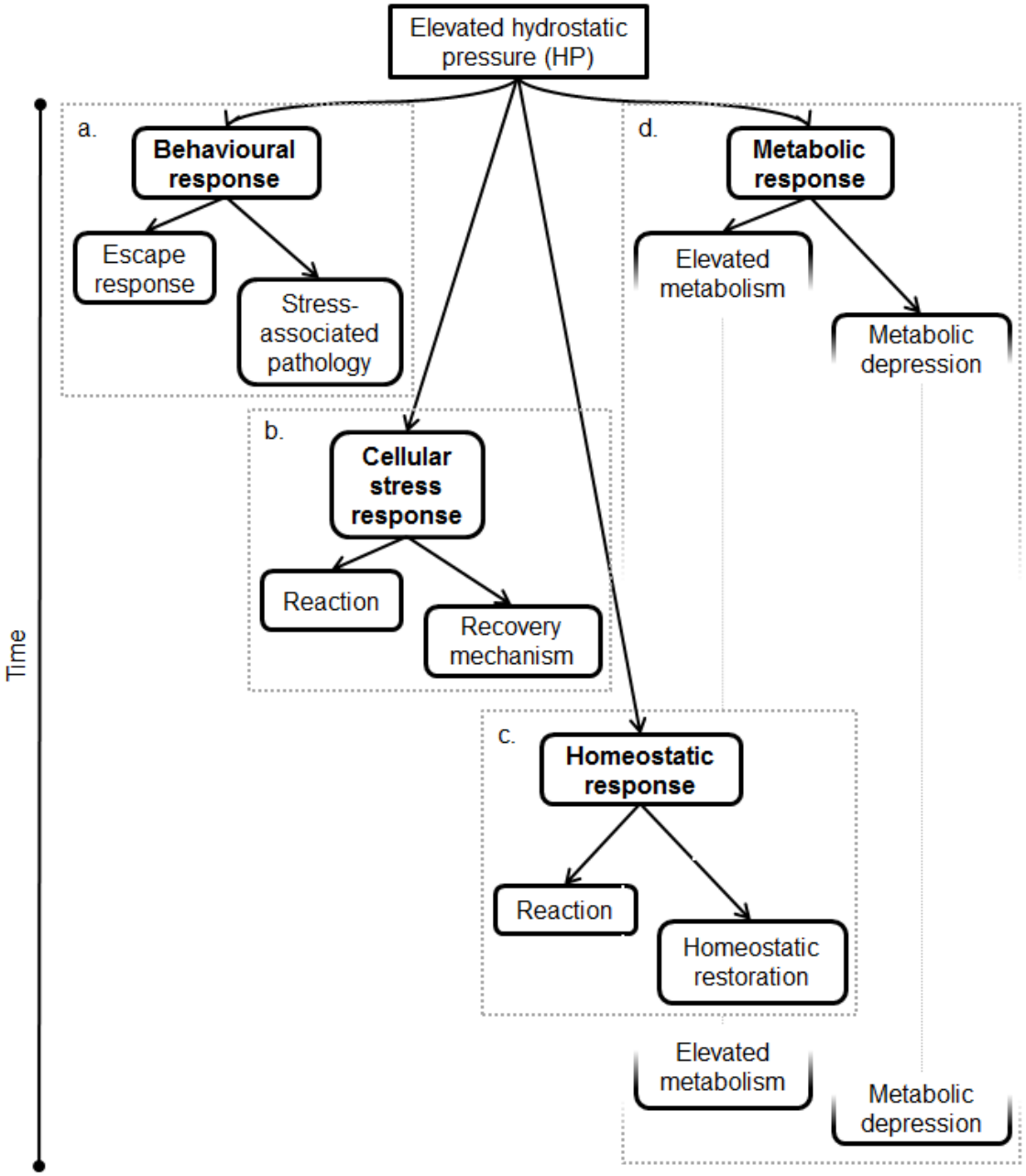

Figure 7.1 A conceptual model showing four categories of stress response that each contribute to the overall stress response of the organism: **a.** the behavioural response; **b.** the cellular stress response (CSR); **c.** the homeostatic response; and **d.** the metabolic response. Each response is shown relative to its hierarchical position over time with behavioural and metabolic responses being the first observed responses to elevated HP, followed by the CSR, and then the homeostatic response. Each response has a sub-set of responses that can be observed distinctly

These escape behaviours are likely to be the first response to elevated HP and occur instantaneously once the stressor reaches a threshold level and is sensed. An increase in active upward swimming as a direct response to elevated HP (Chapter 4) can be thought of as a behavioural homeostasis mechanism (Johnson et al. 1992) and has been observed previously in response to small increases in HP in shallow-water invertebrate larvae (Bayne 1963). This behaviour in the environment would act to reduce the negative physiological effects of elevated HP by directing movement towards lower HP environments (shallower-waters), by doing so, and if successful, this response will curtail the other categories of response that do not react so instantaneously (Figure 7.1a). The reflexive tail flicking behaviour again increases locomotion in order to move away from the stressor. However, this appears to be a more generalised stressor response as the movement does not appear to directional or to be specific to elevated HP exposures having been observed in response to a variety of biotic and abiotic stressors in shrimp and other crustaceans (Bellman and Krasne 1983; Arnott et al. 1998; Herberholz et al. 2000). The second sub-category of behavioural responses observed can be termed stress-associated pathologies (Oliphant et al. 2011; Cottin et al. 2012; Chapters 4, 5). In response to elevated HP in *P. varians* this is seen as 'loss of equilibrium (LOE)' (Oliphant et al. 2011), and spasming and convulsions. This is a negative physiological response, unlike escape responses which are positive, and is brought about by failure of the behavioural escape response to move away from the proximity of the stressor, in this case to shallower waters and lower HP. Behavioural responses are inherently connected to other response categories, as demonstrated in Figure 7.2. Escape responses in the shrimp increase active locomotion, an energy consuming mechanism (Arnott et al. 1998), and are therefore linked to an increase in metabolism (a metabolic response – Figures 7.1d and 7.2). Further, reflexive muscle contractions such as tail-flicking (escape response) or spasming (stress-associated behavioural pathology) can elicit the production of heat shock proteins (Paulsen et al. 2007) inducing the CSR (Figure 7.2).

The CSR, and in particular the induction of heat shock proteins (HSR) is an important response to elevated HP, and is amongst the most intensively researched general responses to stress in biology (Section 1.3). Even the fastest transcriptional responses take at least several minutes to alter the mRNA concentration of a cell, and thus induce the response (de Nadal et al. 2011), although stress-induced genes have been shown to be responsive within 10 minutes of stressor exposure (Boehm et al. 2003; Miller et al. 2011).

Nevertheless, the CSR is not an instantaneous response, and its induction is therefore dependent on the success or failure of positive behavioural responses. The CSR has two distinct sub-categories which can be determined as: reaction, and recovery (Figure 7.1b). The induction of HSP70 genes has been observed across numerous elevated HP exposures in *P. varians* (Cottin et al. 2010; Cottin et al. 2012; Morris et al. 2015; Chapters 3, 4, and 5). During exposure to elevated HP, the expression of HSP70 genes can be seen as a reaction to increased levels of intracellular macromolecular damage (Section 1.3). If the stressor subsides, or a behavioural escape response is successful after the CSR has been induced, then the expression of HSP70 genes will continue as part of a recovery phase as damaged macromolecules may still persist for some time once the stressor has abated (Feder and Hofmann 1999). Further, the cellular stress response, including the expression of HSP70 genes is energetically costly (Sorensen et al. 2003), and will elicit an increase in metabolism (metabolic response – Figures 7.1d and 7.2). Observations of induction of the CSR must therefore be considered with the knowledge that behavioural responses and metabolic responses are linked to it and may positively or negatively affect its magnitude (Figure 7.2), and likewise with behavioural and metabolic responses (Figure 7.1d). Transcriptional regulation of HSP70 genes was observed following elevated HP exposures (from 5 MPa upwards) from as little as 2-hours, up to 28-days in length (Chapters 3 and 5). This suggests that the CSR is an important reactionary response to HP. It was also observed up to 6-hours into recovery following a 2-hour HP exposure (Chapter 4) suggesting an important role in recovery mechanisms also. Homeostatic responses are an important but comparatively understudied group of stressor responses. Homeostatic responses elicit enduring changes in physiological function that act to restore homeostasis at a new point in light of disturbances from a stressor or stress scenario (Figure 7.1c). Restorative homeostatic mechanisms are likely to be the basis of an organism's acclimatory or acclimatisation response to changing environmental parameters (Kültz 2005). Homeostatic responses can be observed at the cellular (Woo et al. 2002) and transcript level (Kültz 2005). Again, this category can be split into two subsections: reaction, and homeostatic restoration. A reaction may be observed as an increase in the working of a certain mechanism under challenge from a stressor. In this thesis, expression of the *narg* gene was seen under a variety of elevated HP exposures in the shrimp *P. varians* (Morris et al. 2015; Chapters 3, 4, and 5).

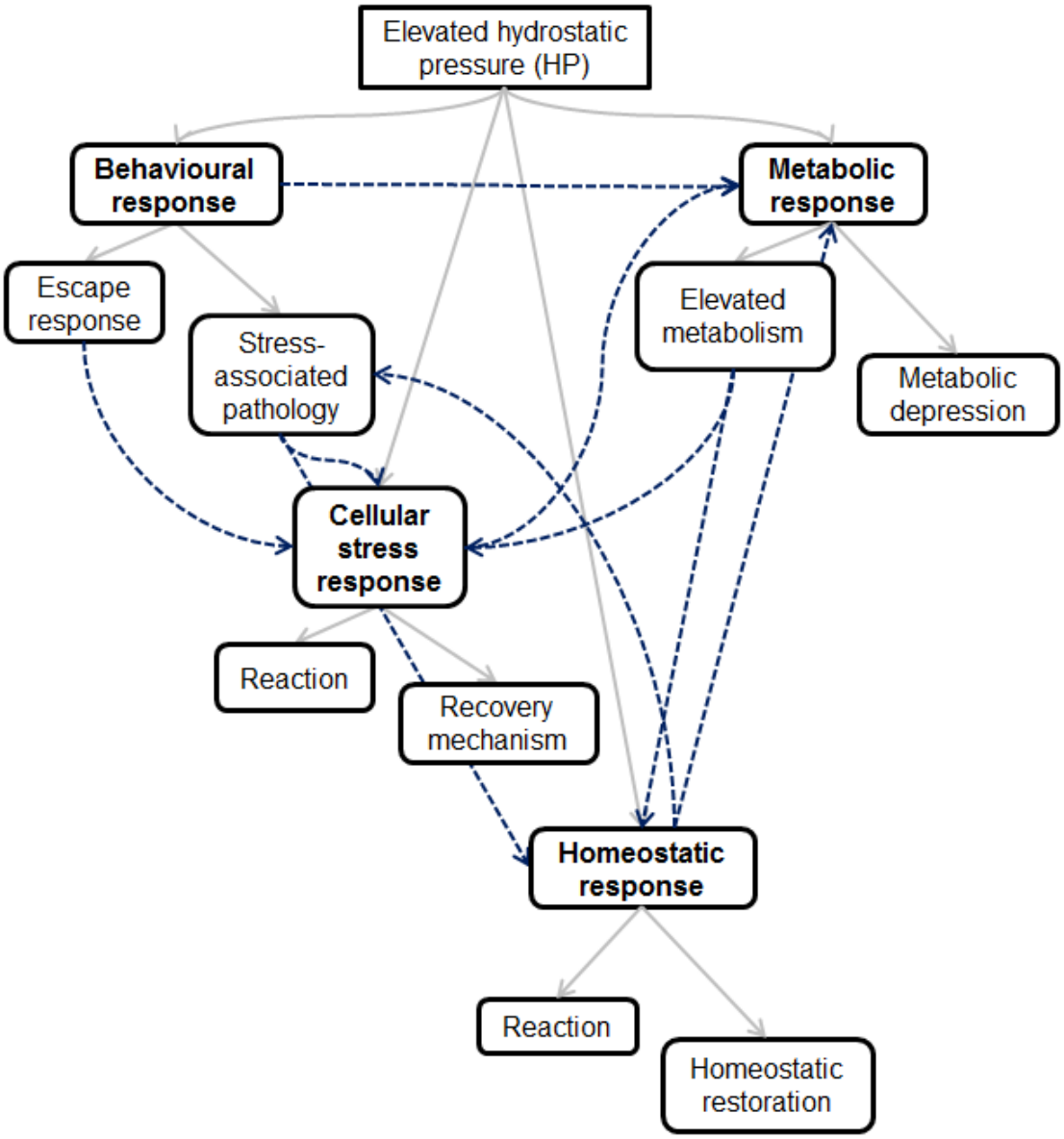

Figure 7.2 Based on the conceptual model of the 4 categories of stress response that each contributes to the overall stress response of the organism (Figure 7.1). The blue-dashed arrows denote the categories and sub-categories that can positively influence or induce other responses

Chapters 3 and 4 suggests that *narg* gene up-regulation, associated with HP-induced NMDAR over-activity, is a reactionary homeostatic response where the homeostatic point of NMDAR performance is shifted away from optimal by elevated HP. NMDAR over-activity may initiate the transcription of new receptors to compensate for the over-activity of existing receptors, and restore neuronal performance. Chapters 3 and 4 observed the transcriptional regulation of the *narg* gene under various HP scenarios following 2-hour

exposures. Chapter 5 quantified expression of the *narg* following 7-day and 28-day exposures and showed a continued up-regulation of the gene under elevated HP. As such, acclimation, in terms of inferred NMDAR activity in *P. varians*, was not observed at any time point up to 28-days of exposure as it would be expected that *narg* gene regulation would return to pre-stress levels if specific acclimation had occurred. The results of Chapter 5 suggest, under stressful elevated HP exposures, *P. varians* shows no sign of acclimatisation to specific neuronal pathologies associated with elevated HP and HPNS. In another study conducted using the same samples and exposures used in Chapter 5, no sign of acclimation, in terms of lipid membrane composition, was observed after 7- or 28-days of elevated HP exposure in *P. varians* (Oliphant *pers. comm.*). It may be that *P. varians* does not have the potential to acclimate to elevated HP. However, the species has shown acclimation to a variety of temperatures (Oliphant et al. 2011; Ravaux et al. 2012; New et al. 2014) and is a eurytopic species: able to tolerate large fluctuations in a number of ecological stressors (Oliphant 2014). It is generally thought that eurytopic species are more readily able to acclimate/acclimatise to changing conditions than stenotopic organisms (Pörtner and Farrell 2008; Somero 2010, 2012). As such, it may be that 28-day exposures, the current technological limit of experimental hyperbaric systems, are simply not long enough to observe any significant effects of acclimation to elevated HP (as described in Chapter 5).

Figure 7.2 shows that homeostatic responses influence, and are influenced by other categories of response. Therefore, a homeostatic response may not be observed if a behavioural escape response has been successful, or if the CSR has mitigated the negative physiological effects of the stressor over the time period of the exposure (Figure 7.2). Likewise, in enduring stressor exposures if acclimation is possible, the homeostatic restorative response may mitigate the specific negative effects of the stressor, thus alleviating the need for the CSR and stopping any stress-associated behavioural pathologies. Further, a homeostatic response will initially be an energetically costly mechanism, as functional change is involved, and will thus increase metabolism (metabolic response – Figures 7.1d and 7.2). However, if the homeostatic restorative mechanism was successful over time then the metabolic costs associated with this process, and also the CSR and behavioural pathologies, would be alleviated, thus reducing metabolic demand.

Metabolic responses make up the fourth category of stressor response (Figure 7.1d). Metabolic responses are, as described in the previous paragraphs, inexorably linked to the other three categories of response (Figure 7.2). This linkage is due to the simple fact that all physiological functions and alteration mechanisms require energy, and are, therefore,

affected by the energy state of the organism (Lodish 2000). The energy state of an organism is a complex concept that is dependent on life-stage, life-history, hereditary, and biotic and abiotic interactions to name but a few factors (Nisbet et al. 2000). The metabolic response to a stressor will include contributions from all other categories of response, and will in itself affect all other categories as well. The degree to which metabolic responses can react to a stressor will depend on the point at which an organism is placed along a stressor-performance curve, be it optimal, non-optimal, or critical (Pörtner and Farrell 2008; Sokolova 2013). This will be discussed in Section 7.2.

Chapter 4 quantified the metabolic responses of *P. varians* to elevated HP at the transcript level and at the whole organism respiratory level. As expected from the explanation above, the metabolic proxy gene showed up-regulation at all time-points where gene markers of other responses were observed. Over the same exposure and recovery period the respiratory response initially matched the metabolic gene marker, and then dropped to lower than the control at the 24-hour recovery point. This difference was thought to represent a mismatch between the energy allocated to stress recovery responses such as the CSR and CHR, and reductions through energy conservation mechanisms. Chapter 5 quantified the expression of additional metabolic markers including the rate-limiting *cs* gene, and the anaerobic metabolism marker coding for the lactate dehydrogenase enzyme. These two genes were isolated and characterised to probe the different sub-categories of metabolic response; elevated metabolism, and metabolic depression (Figure 7.1d). These genes also helped differentiate between aerobic and anaerobic metabolism. Chapter 5 showed, once more, that where the CSR, CHR, and behavioural responses were observed there was a concurrent response in aerobic metabolism; in this case following a 7-day exposure period. Interestingly, after 28-days there was a pronounced change in metabolic marker transcription that suggested a switch from elevated metabolism and aerobic metabolism, to metabolic depression and a reliance on anaerobic metabolism. This observation, alongside inferences of a lack of specific elevated HP acclimation, suggested that *P. varians* was experiencing critical stress levels and was time-limited in its survival under such conditions. Without an understanding of the other categories of response, the metabolic observations after the 28-day exposure may have been interpreted as a lack of stress, however, alongside observations of the other categories of response, the idea of critical stress levels being reached was much more plausible, and was further supported by observations of mortality over the latter stages of the exposure. This highlights the

importance of considering more than a single aspect of the overall stress response of an organism. Figure 7.3 highlights that the sub-categories metabolic response can differentially affect the other categories of response. Elevated metabolism can promote behavioural responses, the CSR, and homeostatic responses by allowing more energy to be made available and potentially diverted to these important processes (Figure 7.3 - blue arrows). On the contrary, metabolic depression, which is associated with stress-induced energy conservation mechanisms, can suppress the potential of the other responses, as less energy is allocated to them (Figure 7.3 – red arrows). This example has been observed at critical stress levels with the cessation of cell cycle control and the down-regulation CSR mechanisms (Guppy and Withers 1999; Kültz 2005; Sokolova 2013).

In conclusion, the responses observed under a variety of elevated HP scenarios in *P. varians* can be regarded as four categories within an overall stress response. Importantly, each of these responses is inexorably linked to other responses to the extent that studying a single aspect of the stress response, without consideration for others, may lead to erroneous inferences on the state or stress level of the organism. To my knowledge, *P. varians* represents the only marine invertebrate where a variety of responses to elevated HP have been observed across numerous differing exposures, and also in conjunction with other stressors such as temperature. The next section will discuss the concept of stressor-performance curves, and how they can be used to understand the consequences of synergistic stressor interactions on stress tolerance windows.

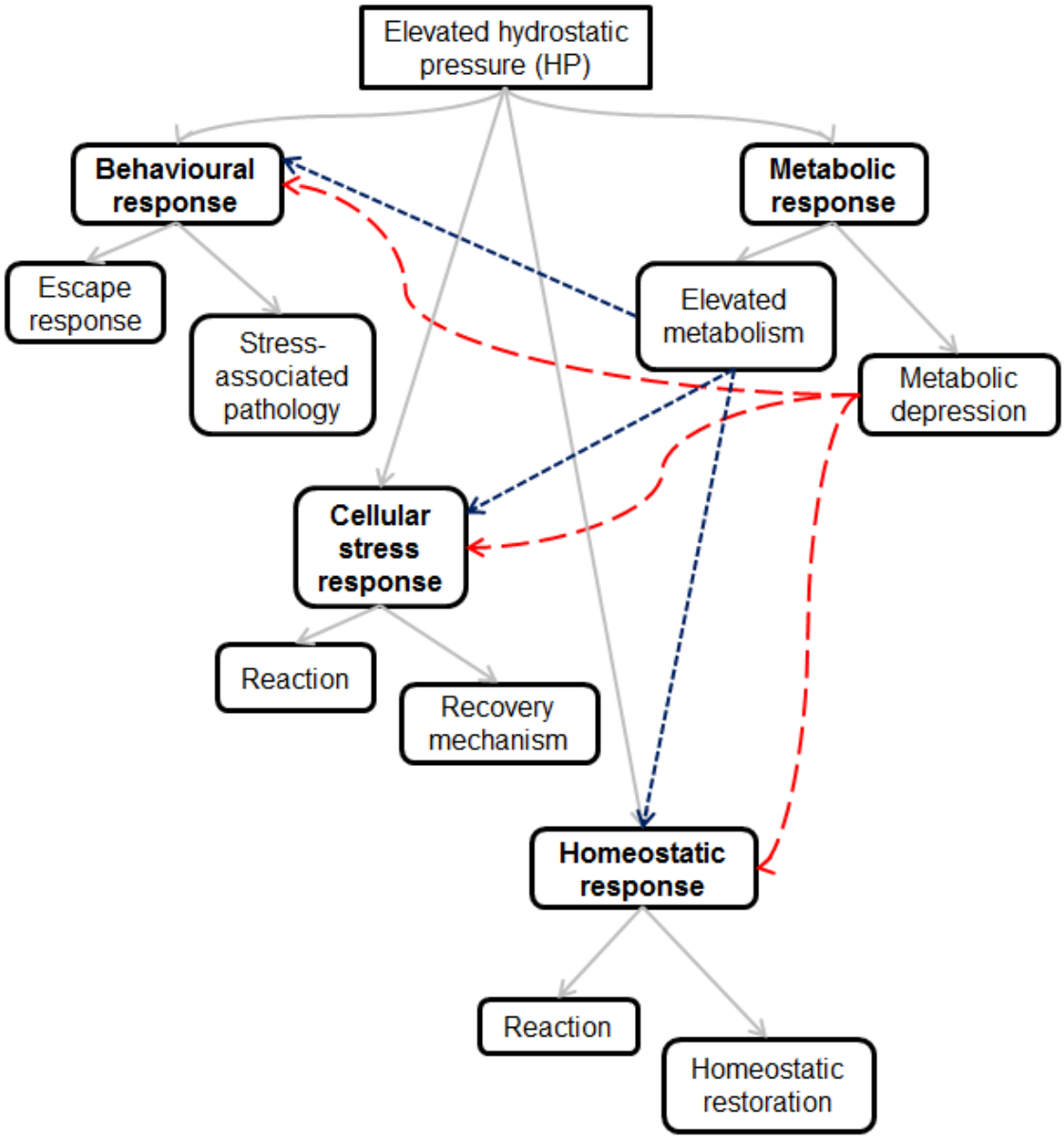

Figure 7.3 Based on the conceptual model of the 4 categories of stress response that each contributes to the overall stress response of the organism (Figure 7.1). The blue-dashed arrows denote the categories that are positively influenced or induced by increases in metabolism. The red-dashed arrows denote the categories that are negatively influenced or suppressed by a decrease in metabolism, or metabolic depression

7.2 HP & temperature tolerance windows & synergistic effects

The concept of thermal performance curves, or thermal-fitness curves, are now well established in ecophysiology, and can provide important inferences on how changes in temperature may affect species or populations (Pörtner 2001; Pörtner et al. 2007; Storch et al. 2009). Performance inferences can also be helpful in determining when and where species might show migration, acclimatisation, or adaptation in order to avoid death (Kelly and Hofmann 2013). Such a concept, because of its simplicity, can be extended to other environmental parameters, and has been done so successfully (Sokolova 2013). Such extensions will be referred to herein as stress- or stressor-performance curves.

The basis of the concept suggests that there are performance zones in relation to stressor intensity that determine how available energy can be partitioned and utilised by the organisms. The use of energy for various key functions can be used to derive a prediction of organismal performance or fitness under a variety of environmental conditions. This concept has been termed oxygen-limited and capacity-limited 'thermal' tolerance (OCLTT) (Pörtner 2010, 2012; Sokolova et al. 2012) and incorporates the concept of dynamic energy budget (DEB) models (Kooijman 2010). Under optimal conditions, aerobic metabolism supplies all energy needs, which sufficiently covers all vital energy-demanding functions such as basic survival, activity, reproduction/maturation, growth/development, and the provision of energy reserves (Sokolova et al. 2012; Sokolova 2013). The available energy from aerobic metabolism decreases away from an optimal performance point, and thus the allocation of energy to vital physiological functions changes in a hierarchical manner (Figure 7.4). At critical stress levels, only the most basic processes of survival are allocated energy, and energy is obtained from aerobic and anaerobic metabolism. The further an organism moves away from its optimal zone towards the critical zone the lower its performance/fitness becomes, and the more-time limited survival becomes (Sokolova et al. 2012) (Figure 7.4).

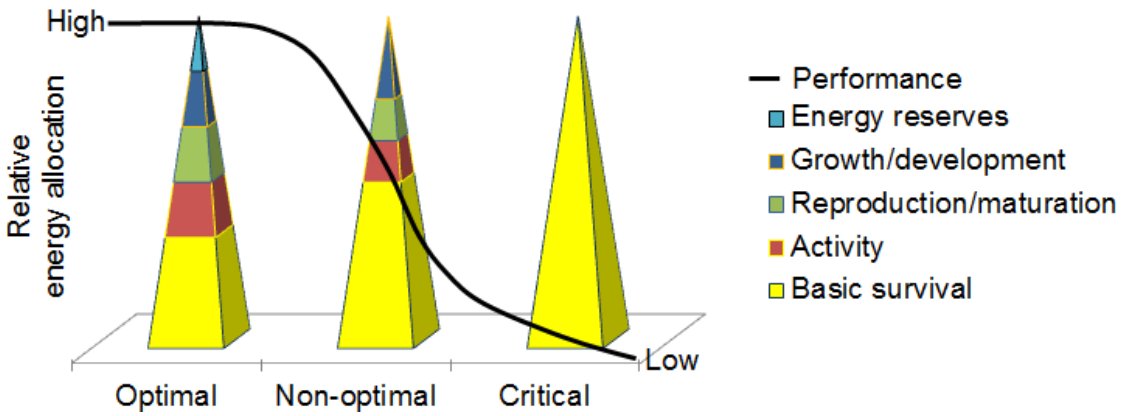

Figure 7.4 The concept of differential energy allocation under changing environmental zones. In the optimal zone of an environmental parameter such as temperature; theoretical organismal performance is high, and energy is distributed optimally between basic survival, activity, reproduction/maturation, growth/development, and energy reserves. In non-optimal conditions performance is reduced and energy is re-allocated in a way that reflects the hierarchy of physiological functions. Under critical conditions performance is low, and energy is allocated exclusively to basic survival. Adapted from the concept of oxygen- and capacity-limited thermal tolerance (OCLTT) (Pörtner 2010, 2012; Sokolova et al. 2012)

Considering this concept in relation to temperature and HP in *P. varians* may help extend our understanding of the data collected on HP and temperature tolerance limits in Chapters 3, 4, and 5.

The thermal limits of *P. varians* have been studied indirectly and directly on several occasions. Lofts (1956) reported environmental temperature fluctuations from 0°C - 33°C in a ditch in Cardiff Bay - UK, known to harbour an established *P. varians* population. Likewise, Oliphant (2014) measured temperature fluctuations of ~0°C to ~26°C in the ditch where *P. varians* was collected for the research in this thesis (see Section 2.1.1). Ravaux (2012) and Oliphant et al. (2011) experimentally established the upper thermal limit of *P. varians* to be around ~33°C. Further, Smith et al. (2013) showed the thermal preference of *P. varians* to be ~17°C when presented with a gradient of between 10°C and 25°C. Oliphant et al. (2011) found high mortality at -1°C and low mortality at 0°C, and unpublished experimental observations found 100% mortality at 35°C, and low mortality at

30°C (Oliphant *pers. comm.*). Maturation of gonad only occurs above 5°C (Hindley 2001), and the initiation of moulting is thought to occur only above 9°C (Jefferies 1964). In preliminary experiments conducted by myself prior to the experiments of Chapter 3, high larval mortality was observed at development temperatures of 10°C and 20°C, with very low mortality at 15°C (Morris *unpub. obs.*). From all the above, it can be suggested that, in shallow-waters, the thermal performance curve of *P. varians* ranges from ~0°C to ~33°C, with non-optimal temperatures being from ~5°C to ~10°C, and ~20°C to ~25°C, and optimal temperatures being around 12°C to 18°C, as displayed in Figure 7.5.

The results of Chapters 3 and 5 suggest that temperature is an important factor in determining the physiological effects of elevated HP in *P. varians*. Results from numerous studies (Cottin et al. 2010; Cottin et al. 2012; New et al. 2014; Morris et al. 2015; Chapters 3, 4, and 5) of elevated HP exposures using *P. varians* can be used to derive a HP-performance model for the species. At an optimal isothermal temperature of ~15°C, not accounting for adiabatic heating, experiments have shown no signs of HP intolerance at the behavioural, respiratory, cellular or transcriptional level below HPs of 5 MPa. From 5 MPa onwards signs of behavioural pathologies and stress-induced transcriptional regulation have been observed (Chapter 3). Also, from between 15 MPa and 20 MPa, critical behavioural pathologies and respiratory rate changes have been observed (Oliphant et al. 2011). Figure 7.6 shows the derived HP-performance curve of *P. varians* under optimal temperature.

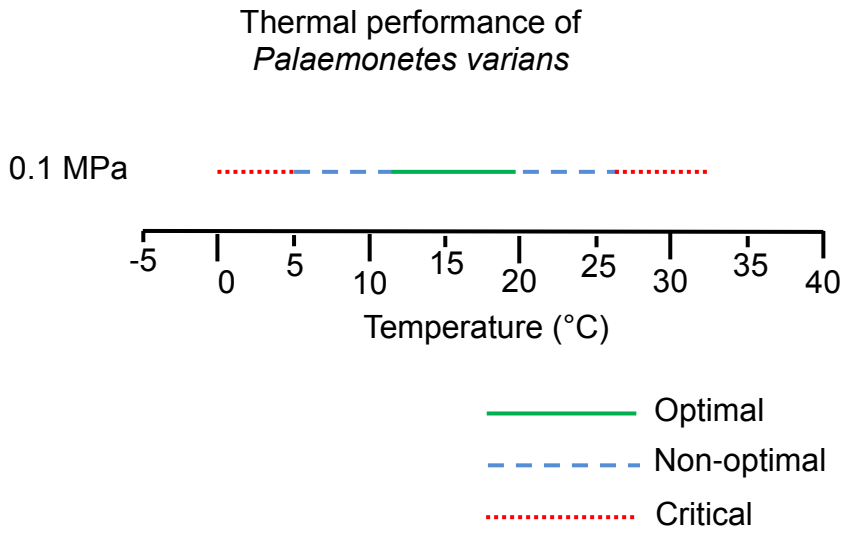

Figure 7.5 The predicted thermal performance model of *Palaemonetes varians* with optimal conditions for fitness/performance of between ~12°C to ~18°C, non-optimal conditions from ~5°C to ~10°C, and ~20°C to ~25°C, and critical thermal conditions from ~0°C to ~5°C, and ~26°C to ~33°C.

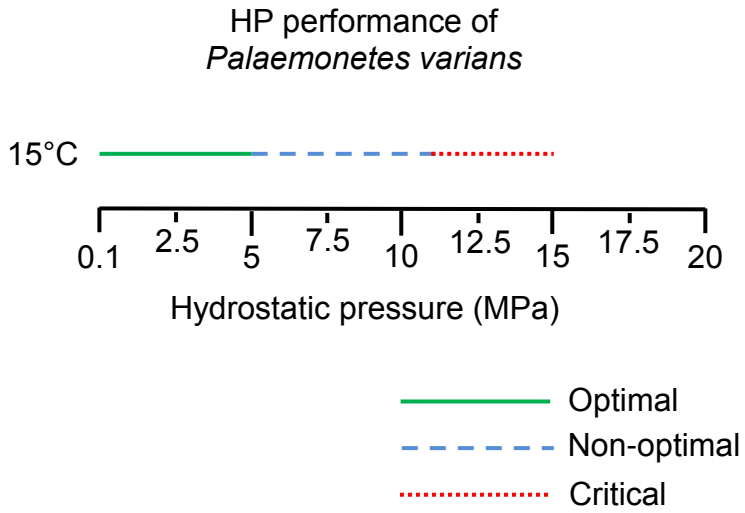

Figure 7.6 The predicted HP performance model of *Palaemonetes varians* at optimal temperature, showing the optimal HP to be between 0.1 MPa and 5 MPa, the non-optimal zone to be between 5 MPa and 11 MPa, and critical HPs of greater than 11 MPa.

Temperature and HP co-vary throughout the ocean and are known to share synergistic thermodynamic effects. The biological consequences of these effects have been shown to influence prokaryotic and eukaryotic physiology (Simonato et al. 2006; Cottin et al. 2012; Morris et al. 2015; Chapters 3 and 5). The results of Chapter 5, in particular, highlight the importance of temperature as a mediator for HP tolerance. From all the available data on *P. varians* temperature and HP experiments, Figure 7.7 shows the concept of combined temperature/HP tolerance windows and provides a logical next-step forwards from Figures 7.5 and 7.6.

Figure 7.7 highlights an idea touched upon in Chapters 3 and 5, and suggests that although temperature and HP, as potential stressors in the marine environment, are of undoubted relevance in isolation. When considering a change in temperature with depth, as is generally the case with bathymetric migrations, temperature and HP should be considered in combination as a single stress-scenario. It is likely to be erroneous to consider the effects of HP down a non-isothermal water column without considering how those effects are mediated by temperature. Likewise, an appreciation of the effects of HP on an organism without some knowledge of its thermal tolerance window and optimal zone will limit the potential understanding of its physiological ability to move across depths.

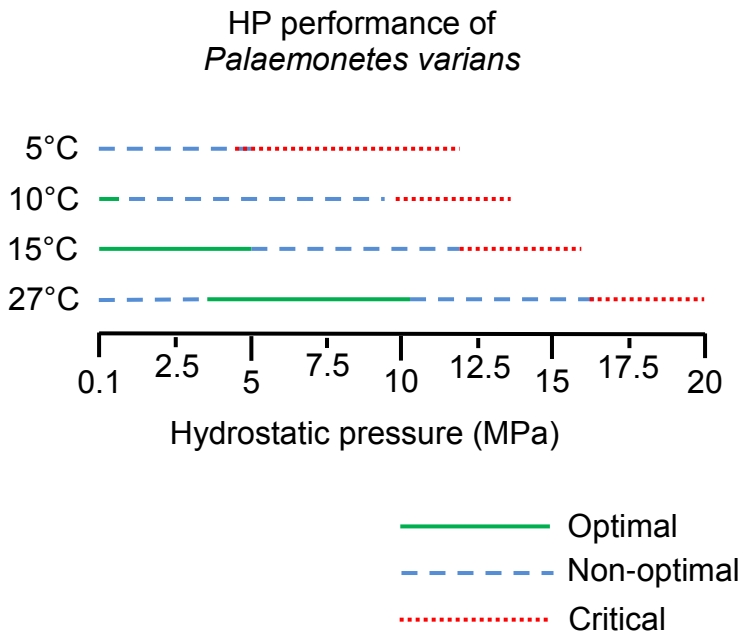

Figure 7.7 The predicted HP performance model of *Palaemonetes varians* at various temperatures within the organisms predicted thermal tolerance window. At the organism's optimal temperature (15°C), stress tolerance falls with the increasing HP. At 10°C, which is at the edge of the organism's thermal optimum at 0.1 MPa, stress tolerance enters the non-optimal zone at lower HPs. At 5°C, the organism is already in its non-optimal zone at 0.1 MPa (Figure 7.4). With the addition of increasing HP, critical stress levels are reached at lower HPs. At 27°C, the shrimp are in the non-optimal zone at 0.1 MPa also, however with increasing HP, and considering the thermodynamic synergies between the two stressors, *P. varians* may move into its optimal performance zone under elevated HPs as volume change reactions move in line with what would be expected at lower HPs and optimal temperature

For shallow-water adapted temperate marine ectotherms such as *P. varians*, climate-driven bathymetric range shifts are viable, from the standpoint of temperature and HP physiology, down warm isothermal water columns or water columns with increasing temperature with depth. These two types of temperature-depth profile allow *P. varians* to maintain optimal performance because of the thermodynamic synergies between temperature and HP. To the same extent, bathymetric range shifts down a cold isothermal water column, or a water column with decreasing temperature with depth, are not possible because of physiological restrictions imposed on the organism by its physiology. Temperature acclimation has been shown in *P. varians* (Ravaux et al. 2012), which acts to shift the thermal performance curve (Figure 7.5) to warmer or colder temperatures depending on the direction of acclimation. Considering this, temperature acclimation to colder waters may increase the elevated HP

tolerance potential of the organism. Further, cold-adapted marine ectotherms may have an inherently greater HP tolerance directly because of their lower range of temperature tolerance (Figure 7.7).

The origins of life in the deep sea are thought to have resulted from migration and colonisation of shallow-water organisms at various points in biological history (Jablonski et al. 1983). These have been associated with deep-sea anoxic mass-extinction events, and the subsequent increase in habitat and resource availability that follows (Rogers 2000). Periods of deep-sea species innovation have occurred on several occasions in combination with halo-thermal circulation (HTC) rather than the current thermo-haline circulation (THC) (Waelbroeck et al. 2002). HTC is characterised by warm waters at depth, and the late Mesozoic/early Cenozoic Eras may have seen warm-isothermal water columns at low latitudes. During the boundary of these two Eras temperate shallow-water ectotherms may have exploited warm-isothermal water columns as physiologically viable colonisation routes into the deep sea. In fact, this period is thought to have been a time of significant deep-sea species innovation (McClain and Hardy 2010), including the colonisation of ancestors of current hydrothermal vent species (65 to 147 Ma) such as *Rimicaris exoculata*, and *Mirocaris fortunata* (Little and Vrijenhoek 2003; Van Dover et al. 2002). Although not clearly resolved, these two caridean shrimp share some phylogenetic relation to palaemonid shrimp (Tokuda et al. 2006; Li et al. 2013), and may have, in part, exploited the synergistic thermodynamic effects of warm waters with depth to colonise deep-sea hydrothermal vent habitats from shallow-water origins at lower latitudes.

7.3 *Palaemonetes varians* as a model for HP & thermal physiology

A model organism can be classified as a species that has been studied extensively to better understand a particular biological area of research or biological phenomenon (Twyman 2002). There are two major stipulations for model organisms, it must be comprehensively studied in the particular field of interest, and it must be expected that the observations made on the model organism will provide insight into the mechanisms of other organisms (Fields and Johnson 2005). Further, model organisms should afford relative ease of study in comparison to other organisms of interest. A few model organisms have been used exhaustively in biology. The first of these was the bacterium *Escherichia coli* which was

initially used as a model for gene structure and regulation studies (Keseler et al. 2005). The eukaryotic yeast *Saccharomyces cerevisiae* has been widely used to study cell biology because of its rapid growth rate and easy maintenance. The fruit fly *Drosophila melanogaster* is comprehensively studied in physiology because of its short generation times and the ease of which congenital traits can be traced across generations (Powell 1997), and the roundworm *Caenorhabditis elegans* is widely studied in developmental biology and neurobiology (Leung et al. 2008). The above examples are the most widely studied of a more extensive group of organisms that are broadly considered as general biological model organisms. Their utility in biological research is unquestionable, yet in some more specialised fields little insight into specific mechanisms can be gained from such models. The marine realm is greatly under-represented in any of the model organism subsets, be it genomic models, genetic models, or experimental models (Twyman 2002). Further, marine invertebrates are under-represented within the marine model organism subset. It is impossible to compare the research effort focussed on *Drosophila melanogaster* to that of arguably the best studied marine model *Danio rerio* (Briggs 2002), yet *Danio rerio*, being a marine species, obviously provides a better insight into the marine habitat and marine processes than *D. melanogaster* does. Hyperbaric physiology is a relatively small research area and, as such, has no model organism advocate. The studies that have investigated hyperbaric physiology have done so through a range of organisms from; bacteria research related to food sterilisation (Simonato et al. 2006); to rat studies used to infer potential responses of humans to elevated HP exposures; and through to starfish in marine ecological studies (Benitez-Villalobos et al. 2006).

Over the course of the experiments documented in this thesis, and from previous research, it is now apparent that elevated hydrostatic pressure (HP) elicits a number of important physiological responses in the shallow-water marine ectotherm, *Palaemonetes varians*. Similar responses have been shown in numerous other marine ectotherms at HPs beyond those naturally experienced, including more eury-baric ectotherms, such as *Lithodes maja*.

P. varians arguably now represents the single best studied shallow-water ectotherm in terms of hyperbaric physiology: with a variety of physiological responses documented over numerous different temperature and HP exposures; from different acclimation points; and over a variety of exposure periods. Understanding the order and timeline of physiological responses in the context of the overall stress response (OSR) to elevated HP has provided a solid platform for future studies on the species under hyperbaric conditions but also in other species, using a *P. varians* as a model.

P. varians has also been used extensively in physiology studies associated with temperature (Cottin et al. 2010; Oliphant et al. 2011; Ravaux et al. 2012; Oliphant et al. 2013; Smith et al. 2013; New et al. 2014; Oliphant 2014), and the experiments in this thesis have been improved greatly because of this extensive understanding. *P. varians* has also been studied in relation to other ecologically relevant stressors including; salinity (Lofts 1959; Nugegoda and Rainbow 1989), oxygen concentration (Nielsen and Hagerman 1998), and metal toxicity (Rainbow et al. 2006; Gonzalez-Rey et al. 2007; Gonzalez-Rey et al. 2008). Such knowledge will be useful in extending future research in multi-stressor scenarios.

In order to be a model organism for an area of study, the responses observed must be comparable in other species and under other scenarios. Chapter 6 (Munro, Morris et al. 2015) explored the transcriptional response of gene markers derived from *P. varians* experiments, and demonstrated their usefulness in a non-shallow-water-adapted eury-baric species, *Lithodes maja*. The results from Chapter 6 (Munro, Morris et al. 2015), and also from Brown (2015), demonstrated that responses previously observed under elevated HP in *P. varians* are a useful predictor of the responses of the eury-baric *L. maja* under HPs greater than its natural depth limit. *P. varians* has also been compared, in terms of physiology, to deep-sea shrimp: *Chorocaris chacei*, *Mirocaris fortunata*, and *Rimicaris exoculata* (Gonzalez-Rey et al. 2008; Cottin et al. 2010; Oliphant et al. 2011; Cottin et al. 2012; Smith et al. 2013; New et al. 2014). Although it is not expected that *P. varians* itself is likely to undergo climate-driven bathymetric range shifts, being from a shallow-water salt marsh environment, it is considered that the responses observed in *P. varians* are comparable in shallow-water-adapted, eury-baric, and deep-sea species that may be forced into bathymetric migrations because of ocean warming (Brown and Thatje 2014; 2015). Although not fully resolved, *P. varians* has been suggested to show phylogenetic relations with deep-sea hydrothermal vent shrimp of the family Bresiliidae (Tokuda et al. 2006) and, more recently, shrimp in the genus *Periclimenes* (Li et al. 2011) which have deep-sea representatives. I advocate *P. varians* as an experimental model organism in hyperbaric and thermal physiology, and believe that continued research on the species will yield further insight into the processes of bathymetric migrations, depth distribution limitations, and the processes of colonisation and maintenance of life in the deep sea. A model organism in the areas of hyperbaric physiology and deep-sea biology is particularly important because of the complexity of sampling and maintaining true deep-sea organisms.

7.4 Future directions

This thesis has shown the utility of targeted gene expression studies in identifying how transcriptional physiology, supplemented by higher-order physiological measurements, can be used to determine limits to climate-driven range shifts (Chapters 3, 4 and 5). To the same extent, this approach has been shown to provide valuable insights into how environmental factors acting on organismal physiology play a role in determining observed distribution range limits (Chapter 6; Munro, Morris et al. 2015), and how the physiology partly controlling these distribution limits changes, for instance, with ontogeny (Chapter 6; Munro, Morris et al. 2015).

There are, however, a number of limitations to such an approach that can be addressed in future research in order to better understand what insights physiological studies can provide in our understanding of the ecology and evolution of marine species, as well as potential responses to contemporary climate change. These future directions are listed below:

- **Multi-generational hyperbaric exposures:** Chapter 5 presented data from the longest sustained hyperbaric exposure of its kind on a shallow-water adapted marine invertebrate. 28-days of sustained exposure to elevated hydrostatic pressure (HP) provided an insight into how the overall stress response of an organism changes over a prolonged period of stressor exposure, and also whether any processes of acclimation were occurring. Although, contemporary climate change is occurring at an unprecedented, and previously unseen, rate in the marine environment (IPCC 2013), and has been shown to be affecting marine ectotherm distributions within a single generation (Perry et al. 2005; Dulvy et al. 2008), 28-day exposures may not be sufficiently lengthy to identify acclimatory processes, and do not match the much slower rate of expected distributional shifts in the marine environment (Pinksy et al. 2013). Technological limitations, particularly the lack of a feeding mechanism for the IPOCAMP™, have prevented longer exposure being conducted. However, an *in situ* feeding mechanism is in development and will provide future research with potential for much longer-term exposures. Such studies would provide a more powerful assessment of the acclimation potential of shallow-water adapted species to hyperbaric conditions. Of particular interest, would be whether the processes of reproduction; oogenesis, spermatogenesis, spawning, fertilisation, and brooding are possible under elevated HP exposures. These reproductive and developmental mechanisms

would be vital in the processes of multi-generational bathymetric range shifts, and particularly the colonisation of the deep sea (Jablonski et al. 1983; Jacobs and Lindberg 1998; Raupach et al. 2009). Previous studies have shown that early developmental stages in shallow-water marine invertebrates are able to tolerate acute elevated HP exposures (Benitez-Villalobos et al. 2006; Aquino-Souza et al. 2008; Mestre et al. 2009), and this thesis has demonstrated temperature-dependent elevated HP tolerance in juvenile and adult *P. varians*, and across ontogeny and in adult *Lithodes maja*. However, such studies are limited by their "snap-shot" nature. Future research, once technological advancements allow, should focus on the reproductive potential of such species by multi-generational hyperbaric exposures.

- **Deep-sea restricted species comparison:** Not only shallow-water adapted species have HP tolerance windows (Chapters 3, 5), eury-baric species such as *Lithodes maja* also show signs of HP intolerance at HPs beyond their natural distribution limit (Chapter 6; Munro, Morris et al. 2015). Just as many shallow-water organisms are adapted to low-ambient HPs and show signs of intolerance at greater HPs, some deep-sea-adapted organisms are considered to be obligate barophiles: requiring high ambient HP to survive (Somero 1992). Although there is little research on the subject, Mickel and Childress (1982) observed cardiac, muscular, and nervous disturbances in the vent crab *Bythograea thermydron* upon decompression to atmospheric pressures. These observations are strikingly similar to the reported pathologies associated with high pressure neurological syndrome (HPNS) in shallow-water adapted organisms. Such observations suggest that adaptations to elevated HP are specialised to the point that, once adapted, low HP environments become inaccessible. Research concerning the potential for bathymetric range shifts, and acclimation or adaptation to deep-sea conditions may benefit from better understanding the cellular level physiology of deep-sea-adapted organisms. Studying the physiology of bathymetric range shifts from both a shallow-water and a deep-sea perspective may help to link the processes that must have allowed ancestors of many current deep-sea lineages to colonise the deep sea from shallow waters (Jablonski et al. 1983; Jacobs and Lindberg 1998; Raupach et al. 2009).

- **Multi-stressor exposures:** A large body of research, including this thesis, has shown that a variety of abiotic factors play an important role in setting the physiological

distribution limits of organisms and range shifts in light of climate change (Somero 2012). Temperature in particular has been shown to be an important driver of range shifts in both marine (Pinksy et al. 2013) and terrestrial habitats (Colwell et al. 2008), and this thesis advocates HP as an important physiological factor in depth distribution delineations. There are, however, many other potential physiological stressors that are likely to affect the potential for bathymetric range shifts in marine ectotherms.

Chapter 4 highlights the importance of understanding the variety of potential physiological responses to a single stressor before addressing multi-stressor scenarios. This thesis as a whole, but particularly Chapter 4, has quantified a number of distinct responses to elevated HP. Chapters 3 and 5 have quantified the combined effects of changes in temperature and HP, showing synergistic effects between the two. As a next step towards laboratory-based experiments that mimic the conditions expected with bathymetric range shifts, additional stressors added in a step-wise fashion will increasingly provide a more realistic perspective on the potential for this type of climate change response. With increasing depth, sunlight fades, but is visible through the euphotic zone (up to 200m) and the dysphotic zone (up to 1000m) depending on water turbidity (Levinton 2001). Beyond 1000m (the aphotic zone) no light penetrates (Levinton 2001). The deep sea is also characterised by oxygen minimum zones and areas of reduced oxygen content. These regions have been shown to coincide with species-rich areas, and hypoxia/anoxia is thought to promote allopatric speciation and sustained separation by stress-induced gene flow limitation (Jacobs and Lindberg 1998; Rogers 2000). I believe that light and water oxygen concentration may also be of importance in understanding the physiological viability of bathymetric range shifts. Future research on bathymetric range shifts may benefit from adding a physiological understanding of changes in light and oxygen concentration in combination with changing HP and temperature.

- **Genome-wide transcriptomic analysis:** This thesis demonstrated that small-scale targeted gene-expression analysis can provide an insight into multiple physiological mechanisms in non-model marine organisms including: the CSR by heat shock proteins and metabolic markers; the cellular homeostatic response (CHR) by stressor-specific markers; and metabolism by aerobic and anaerobic metabolic markers. However, it is now well understood that gene expression is controlled by more than just the regulation of the specific genes and promoters, and can be regulated epigenetically (Jaenisch and Bird 2003), by nuclear microRNAs (Salmanidis et al. 2014), or by long non-coding RNAs (Rinn and

Chang 2012) as just a few examples. Targeted gene expression analysis undoubtedly misses potentially important transcriptional signals, even within specific mechanisms. As an example, the heat shock response may involve many heat shock isoforms that respond differently during exposure to a stressor (Morris et al. 2013): 88 HSP70 isoforms have been identified in the oyster *Crassostrea gigas* for instance (Zhang et al. 2012). This thesis has quantified the expression of two differentially expressed HSP70 isoforms under a variety of stress scenarios which has provided a valuable insight into the CSR yet may have missed certain aspects through not quantifying all the potential heat shock isoforms. The same goes for understanding metabolism by a few key metabolic markers; although a recent review has suggested that this may be a particularly effective approach with metabolic enzymes (Marden 2013). This is a cost effective approach, commonly used in non-model physiology studies in both the terrestrial (Bahar et al. 2013) and marine realm (Pineda et al. 2012). Yet genome-wide transcriptome sequencing is becoming increasingly cost-effective and feasible in non-model organisms, and has been demonstrated to provide an insightful whole-genome perspective on the effects of climate change on non-genome-sequenced marine invertebrates (De Wit and Palumbi 2013). Future research should exploit such available technologies, in order to gain a full transcriptomic understanding of the true number of physiological mechanisms that are affected by elevated HP and temperature changes in shallow-water species such as *P. varians*.

- **Proteomic analysis:** There are ongoing disagreements in the literature concerning what mechanisms predominantly control specific protein levels within a cell. Kristensen et al. (2013) have suggested that protein synthesis rather than gene transcription is the predominant regulator of a cell's overall protein level. Further, some studies have found a weak correlation between gene expression regulation and protein accumulation (Maier et al. 2011). More recently, however, it has been suggested that transcription plays a comparatively more important role in determining cellular protein level as opposed to protein synthesis (Jovanovic et al. 2015; Li and Biggin 2015). It seems that either approach in isolation is unlikely to fully elucidate the true physiological response of an organism to an environmental stressor. Future studies may benefit from quantifying both gene expression and cellular protein levels in order to provide more powerful physiological results on the effects of elevated HP and temperature in marine ectotherms.

7.5 Final conclusions

As contemporary climate change continues to affect global biology, species will increasingly show responses at all levels of organisation. These can be classed into one of the following groups: migration, acclimation/acclimatisation, adaptation, or death/extinction. Physiology can help to predict which of these responses may be shown by species in different habitats by way of measuring tolerance limits and acclimatisation ability. Bathymetric migration is a viable response to ocean surface warming, in terms of physiology, for some shallow-water marine ectotherms. However, this is dependent on temperature and the thermal scope of the organism in question. Current stratified oceans, with temperature decreasing and hydrostatic pressure (HP) increasing with depth, represent a significant physiological challenge to bathymetric migrations for temperate marine ectotherms, which would require long-term acclimatisation, or adaptation to overcome. HP and temperature, in combination, undoubtedly play an important role in setting the depth distribution limits of shallow-water and eury-baric species, and also are important in controlling the potential for bathymetric range shifts in these species. Continued effort in elucidating the potential for bathymetric range shifts is vital for our understanding of the responses of marine ectotherms to contemporary climate change, and plays an important role in improving our understanding of the colonisation, evolution, and maintenance of life in the deep sea.

Appendices

A1. APPENDIX FOR CHAPTER 2

A1.1 Full list of available NCBI GenBank entries for *Lithodes maja* (11 entries) and *Palaemonetes varians*

A1.1.1 *Lithodes maja*

1. *Lithodes maja* voucher TE-004-01 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial. 658 bp linear DNA. FJ581746.1 GI:220030265

2. *Lithodes maja* voucher TE-004-02 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial. 658 bp linear DNA. FJ581745.1 GI:220030263

3. *Lithodes maja* voucher TE-004T153-01 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial. 658 bp linear DNA. FJ581744.1 GI:220030261

4. *Lithodes maja* voucher TE-004T22-01 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial. 658 bp linear DNA. FJ581743.1 GI:220030259

5. *Lithodes maja* voucher TE-004T88-03 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial. 658 bp linear DNA. FJ581742.1 GI:220030257

6. *Lithodes maja* voucher GSL31-24 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial. 658 bp linear DNA. FJ581741.1 GI:220030255

7. *Lithodes maja* voucher GSL31-25 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial. 658 bp linear DNA. FJ581740.1 GI:220030253

8. *Lithodes maja* 12S ribosomal RNA gene, partial sequence. 1,288 bp linear DNA. AF425309.1 GI:16798332

9. *Lithodes maja* cytochrome oxidase subunit II gene, partial cds; mitochondrial gene. 565 bp linear DNA. AF425369.1 GI:16566480

10. *Lithodes maja* NADH dehydrogenase subunit 1 gene, partial cds. 810 bp linear DNA. AF425330.1 GI:16566440

11. *Lithodes maja* 28S ribosomal RNA gene, partial sequence. 292 bp linear DNA. AF425350.1 GI:16417775

A1.1.2 *Palaemonetes varians*

1. *Palaemonetes varians* 70 kDa heat shock protein form 2 mRNA, partial cds. 1,840 bp linear mRNA. FJ875279.1 GI:238890529

2. *Palaemonetes varians* 70 kDa heat shock protein form 1 mRNA, partial cds. 1,687 bp linear mRNA. FJ875280.1 GI:238683638

3. *Palaemonetes varians* cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial. 652 bp linear DNA . KC763188.1 GI:514435196

4. *Palaemonetes varians* 18S ribosomal RNA gene, partial sequence. 1,714 bp linear DNA. KC763180.1 GI:514435180

5. *Palaemonetes varians* voucher ICMD-20111108-07 16S ribosomal RNA gene, partial sequence; mitochondrial. 538 bp linear DNA . JQ042301.1 GI:388565086

6. *Palaemonetes varians* voucher PAL-040 histone H3 (H3) gene, partial cds. 328 bp linear DNA. JN674374.1 GI:383285232

7. *Palaemonetes varians* voucher PAL-040 16S ribosomal RNA gene, partial sequence; mitochondrial. 400 bp linear DNA . JN674357.1 GI:383285201

8. *Palaemonetes varians* chitinase mRNA, partial cds. 216 bp linear mRNA. FJ685994.1 GI:238890527

9. *Palaemonetes varians* actin-related protein mRNA, partial cds. 384 bp linear mRNA. FJ769185.1 GI:238684066

10. *Palaemonetes varians* crustapain mRNA, partial cds. 394 bp linear mRNA. FJ769184.1 GI:238684064

11. *Palaemonetes varians* 15 kDa selenoprotein mRNA, partial cds. 353 bp linear mRNA. FJ654551.1 GI:238683730

12. *Palaemonetes varians* 60S ribosomal protein L23a mRNA, partial cds. 248 bp linear mRNA. FJ654550.1 GI:238683728

13. *Palaemonetes varians* 60S ribosomal protein RPL23 mRNA, partial cds. 207 bp linear mRNA. FJ654547.1 GI:238683722

14. *Palaemonetes varians* ribosomal protein S14 mRNA, partial cds. 248 bp linear mRNA. FJ654546.1 GI:238683720

15. *Palaemonetes varians* ribosomal protein L35A mRNA, partial cds. 242 bp linear mRNA. FJ654545.1 GI:238683718

16. *Palaemonetes varians* elongation factor 1 alpha mRNA, partial cds. 165 bp linear mRNA. FJ654544.1 GI:238683716

17. *Palaemonetes varians* clone 4462256 hemocyanin mRNA, partial cds. 479 bp linear mRNA. FJ654543.1 GI:238683714

18. *Palaemonetes varians* clone 4462213 hemocyanin mRNA, partial cds. 255 bp linear mRNA. FJ654542.1 GI:238683712

19. *Palaemonetes varians* clone CL12contig1 hemocyanin mRNA, partial cds. 687 bp linear mRNA. FJ654541.1 GI:238683710

20. *Palaemonetes varians* esterase D/formylglutathione hydrolase mRNA, partial cds. 277 bp linear mRNA. FJ654540.1 GI:238683708

21. *Palaemonetes varians* fatty acid binding protein mRNA, partial cds. 252 bp linear mRNA. FJ654539.1 GI:238683706

22. *Palaemonetes varians* NADH dehydrogenase mRNA, complete cds. 437 bp linear mRNA. FJ654538.1 GI:238683704

23. *Palaemonetes varians* NADH dehydrogenase flavoprotein 1 mRNA, partial cds. 456 bp linear mRNA. FJ654537.1 GI:238683702

24. *Palaemonetes varians* proteasome 26S non-ATPase subunit 2 mRNA, partial cds. 293 bp linear mRNA. FJ654536.1 GI:238683700

25. *Palaemonetes varians* cytochrome c oxidase subunit I mRNA, partial cds; mitochondrial. 723 bp linear mRNA. FJ654535.1 GI:238683698

26. *Palaemonetes varians* clone CL5contig1 cathepsin L mRNA, partial cds. 749 bp linear mRNA. FJ654534.1 GI:238683696

27. *Palaemonetes varians* clone CL4contig1 cathepsin L mRNA, partial cds. 743 bp linear mRNA. FJ654533.1 GI:238683694

28. *Palaemonetes varians* NADH dehydrogenase subunit 4 mRNA, partial cds; mitochondrial. 123 bp linear mRNA. FJ654531.1 GI:238683690

29. *Palaemonetes varians* putative SERF-like protein mRNA, complete cds. 404 bp linear mRNA. FJ654530.1 GI:238683688

30. *Palaemonetes varians* cuticle proprotein mRNA, partial cds. 443 bp linear mRNA. FJ654529.1 GI:238683686

31. *Palaemonetes varians* actin 1 mRNA, partial cds. 166 bp linear mRNA. FJ654528.1 GI:238683684

32. *Palaemonetes varians* skeletal muscle actin 7 mRNA, partial cds. 419 bp linear mRNA. FJ654527.1 GI:238683682

33. *Palaemonetes varians* clone 4461920 skeletal muscle actin 6 mRNA, partial cds. 223 bp linear mRNA. FJ654526.1 GI:238683680

34. *Palaemonetes varians* clone 4461887 skeletal muscle actin 6 mRNA, partial cds. 474 bp linear mRNA. FJ654525.1 GI:238683678

35. *Palaemonetes varians* paramyosin mRNA, partial cds. 206 bp linear mRNA. FJ654524.1 GI:238683676

36. *Palaemonetes varians* myosin 1 light chain mRNA, complete cds. 549 bp linear mRNA. FJ654523.1 GI:238683674

37. *Palaemonetes varians* slow muscle myosin S1 heavy chain mRNA, partial cds. 658 bp linear mRNA. FJ654522.1 GI:238683672

38. *Palaemonetes varians* slow-tonic S2 myosin heavy chain mRNA, partial cds. 427 bp linear mRNA. FJ654521.1 GI:238683670

39. *Palaemonetes varians* clone CL3contig1 sarcoplasmic calcium-binding protein 1 mRNA, partial cds. 270 bp linear mRNA. FJ654520.1 GI:238683668

40. *Palaemonetes varians* ribosomal protein P1 mRNA, complete cds. 469 bp linear mRNA. FJ654519.1 GI:238683666

41. *Palaemonetes varians* ribosomal protein S19 mRNA, partial cds. 210 bp linear mRNA. FJ654518.1 GI:238683664

42. *Palaemonetes varians* ribosomal protein L4 mRNA, partial cds. 458 bp linear mRNA. FJ654517.1 GI:238683662

43. *Palaemonetes varians* ribosomal protein S3 mRNA, partial cds. 567 bp linear mRNA. FJ654516.1 GI:238683660

44. *Palaemonetes varians* ribosomal protein L37 mRNA, partial cds. 229 bp linear mRNA. FJ654515.1 GI:238683658

45. *Palaemonetes varians* ribosomal protein S10 mRNA, partial cds. 258 bp linear mRNA. FJ654514.1 GI:238683656

46. *Palaemonetes varians* hypothetical ATP-dependent transporter mRNA, partial cds. 258 bp linear mRNA. FJ654513.1 GI:238683654

47. *Palaemonetes varians* NADH dehydrogenase subunit 1 mRNA, partial cds; mitochondrial. 424 bp linear mRNA. FJ654511.1 GI:238683650

48. *Palaemonetes varians* NADH dehydrogenase subunit 5 mRNA, partial cds; mitochondrial. 310 bp linear mRNA. FJ654512.1 GI:238683652

49. *Palaemonetes varians* ATP synthase subunit g mRNA, complete cds. 357 bp linear mRNA. FJ654510.1 GI:238683648

50. *Palaemonetes varians* hypothetical ATP synthase F0 subunit 8 mRNA, partial cds; mitochondrial. 239 bp linear mRNA. FJ654509.1 GI:238683646

51. *Palaemonetes varians* ATP synthase F0 subunit 6 mRNA, partial cds; mitochondrial. 279 bp linear mRNA. FJ654508.1 GI:238683644

52. *Palaemonetes varians* cytochrome c oxidase subunit III mRNA, partial cds; mitochondrial. 504 bp linear mRNA. FJ654507.1 GI:238683642

A1.2 CLUSTAL Omega alignments for all isolated genes in *Palaemonetes varians* and *Lithodes maja*

A1.2.1 *Palaemonetes varians*

Narg gene

```
CLUSTAL O(1.2.0) multiple sequence alignment


Palaemonetes varians    gi|301016605|emb|FR667656.1|          EIIEDQFDFHTYCMRKMTLRSYVELLRLEDVLRNNRFYWDAART----------------
Drosophila grimshawi    gi|195059508|ref|XP_001995651.1|      EIIEDQFDFHTYCMRKMTLRAYVGLLRLEDVLRRHPFYFKAAKCAIELYIHLYDKPLKSE
Drosophila mojavensis   gi|195398779|ref|XP_002057998.1|      EIIEDQFDFHTYCMRKMTLRAYVGLLRLEDVLRRHPFYFKAAKCAIEVYIRLYDKPLKSE
Nasonia vitripennis     gi|345496372|ref|XP_001603208.2|      EIIEDQFDFHTYCMRKMTLRSYVGLLRLEDVLRSHPFYFKAAKCAIEVYLRLHDYPLPD-
Tribolium castaneum     gi|91081191|ref|XP_975602.1|          EIIEDQFDFHTYCMRKMTLRSYVGLLRLEDVLRGHPFYFKAAKCAIEVYLHLFDEPLKDE
Culex quinquefasciatus  gi|170034670|ref|XP_001845196.1|      EIIEDQFDFHTYCMRKMTLRSYVGLLRLEDVLRRHPFYYRAAKCAIEVYVRLFDKPLPAE
Anopheles gambiae       gi|158284805|ref|XP_307895.3|         EIIEDQFDFHTYCMRKMTLRSYVGLLRLEDVLRRHPFYFKAAKCAIEVYLRLFDKPLAAE
                                                              ********************:** ********* . **: **:
```

Arf gene

```
CLUSTAL O(1.2.0) multiple sequence alignment


Palaemonetes varians  gi|301016678|emb|FR667657.1|          ---IRPLWXHYFQNTSAIIFVVDSNDPQRLAEAKEELEILDEDKDLESCPLLIMANKQDL
Mus musculus          gi|81906189|sp|Q9DD04|Q9DD04_MOUSE     QDKIRPLWRHYFQNTQGLIFVVDSNDRERIQEGAAVLEKMLLEDELQDAVLLLFANKQDL
Bos taurus            gi|110278815|sp|Q3SZF2.3|ARF4_BOVIN    QDKIRPLWRHYFQNTQGLIFVVDSNDRERIQEGAEELQKMLQEDELRDAVLLLFANKQDL
Gallus gallus         gi|82197824|sp|Q5ZKR9|Q5ZKR9_CHICK     QDKIRPLWRHYFQNTQGLIFVVDSNDRERIEEAADELQKMLQEDELRDAVLLLFANKQDL
Xenopus laevis        gi|1703378|sp|P51644.2|ARF4_XENLA      QDKIRPLWRHYFQNTQGLIFVVDSNDRERIQEAAEELQKMLQEDELRDAVLLVFANKQDL
Monodelphis domestica gi|126340657|ref|XP_001366170.1|      QDKIRPLWRHYFQNTQGLIFVVDSNDRERVQESADELQKMLQEDELREAVLLVFANKQDM
                                                              ***** ******..:******** :*: *.   *: :  :.:*... **::*****:

gi|301016678|emb|FR667657.1|          PQAASPSFITNALNLRNLR-RTWFVQGTCAVNSTGIYKSLDWLAKEVS--
gi|81906189|sp|Q9DD04|Q9DD04_MOUSE    PNAMAISEMTDKLGLQSLRNRTWYVQATCATQGTGLYEGLDWLSHELSNV
gi|110278815|sp|Q3SZF2.3|ARF4_BOVIN   PNAMAISEMTDKLGLQSLRNRTWYVQATCATQGTGLYEGLDWLSNELSKR
gi|82197824|sp|Q5ZKR9|Q5ZKR9_CHICK    PNAMAISEMTDKLGLQSLRNRTWYVQATCATQGTGLYEGLDWLSNELSKR
gi|1703378|sp|P51644.2|ARF4_XENLA     PNAMAISEMTDKLTLQTLRNRTWYVQATCATQGTGLYEGLDWLSNELSKR
gi|126340657|ref|XP_001366170.1|      PNAMPVSELTDKLGLQNLRSRTWYVQATCATQGTGLYDGLDWLSHELSKR
                                      *:*   * :*: * *:.** ***:**.***.:.**:*..****:.*:*
```

Rps26 gene

```
CLUSTAL O(1.2.0) multiple sequence alignment


Palaemonetes varians   gi|301016607|emb|FR667658.1|          -----AIKKFQIRNIVEAAAVKDINEASVYQVYQLPKLYIKQHYCVSCAIHSKVVRNRSR
Argas monolakensis     gi|121952190|sp|Q09JL2|Q09JL2_9ACAR    -PKDKAIKKFVIRNIVEAAAVRDITVASVYEAYPLPKLYAKLHYCVSCAIHSKIVRNRSK
Ixodes scapularis      gi|241753583|ref|XP_002401133.1|       -PKDKSIKKFVIRNIVEAAAVRDITVASVYETYPLPKLYAKLHYCVSCAIHSKIVRNRSK
Nematostella vectensis gi|156407218|ref|XP_001641441.1|      -PKDKSIKKFVIRNIVEAAAVRDIADASVYEVYALPKLYVKLHYCVSCAIHSKVVRNRSK
Spodoptera frugiperda  gi|74844826|sp|Q962Q4|Q962Q4_SPOFR     -PKDKAIKKFVIRNIVEAAAVRDINEASVYASFQLPKLYAKLHYCVSCAIHSKVVRNRSK
Plutella xylostella    gi|74787518|sp|Q6F480|Q6F480_PLUXY     MPKDKAIKKFVIRNIVEAAAVRDINDASVYTSFQLPKLYAKLHYCVSCAIHSKVVRNRSK
                                                                :**** **********:**  ****  : ***** * ***********:*****:

gi|301016607|emb|FR667658.1|          RDRKIRTPPPR
gi|121952190|sp|Q09JL2|Q09JL2_9ACAR   QDRKDRAPPPR
gi|241753583|ref|XP_002401133.1|      EARKDRAPPPR
gi|156407218|ref|XP_001641441.1|      EDRKIRTPPPR
gi|74844826|sp|Q962Q4|Q962Q4_SPOFR    KDRRIRTPPKS
gi|74787518|sp|Q6F480|Q6F480_PLUXY    KDRRIRTPPQR
                                      . *: *:**
```

Cs gene

```
Clustal Omega Alignment

                                10        20        30        40        50        60
                       ....|....|....|....|....|....|....|....|....|....|....|....|
P. miliaris            DHEGGNVSAHTVHLVGSALSDPYLSFAAGMNGLAGPLHGLANQEVLVWLTKLQEKLGGAV
P varians              DHEGGNVSAHTTHLVGSALSDPYLSFAAGMNGLAGPLHGLANQEVLLWLTKLRSDIGDDV
Bombyx mori            DHEGGNVSAHTTHLVGSALSDPYLSFAAGLNGLAGPLHGLANQEVLVWLEKLRKQVGDNF
Camponotus flroidanus  DHEGGNVSAHTTHLVGSALSDPYLSFSAGMNGLAGPLHGLANQEVLVWLEKLRSQVGDSP
Clustal Consensus      ***********.**************:**:****************:** **:..:*

                                70        80        90        100       110       120
                       ....|....|....|....|....|....|....|....|....|....|....|....|
P. miliaris            SDAEMKEFIWNTLQGGQVVPGYGHAVLRKTDPRYECQRQFALKHLPDDPMFQLVSQIYKL
P varians              TEDQLKEFIWKTLKSGQVVPGYGHAVLRKTDPRYTCQREFALKHLPDDKMFKNWVR----
Bombyx mori            TEEQLKEFIWKTLKSGQVVPGYGHAVLRKTDPRYTCQREFALKHLPNDPLFKLVAAVYKV
Camponotus flroidanus  SDDKLKEFIWNTLKSGQVVPGYGHAVLRKTDPRYTCQREFALKHLPDDPLFKLVAQVYKV
Clustal Consensus      :: ::*****:**:.******************** ***:*******:* :*:
```

Ldh gene

```
Clustal Omega Alignment

                                10        20        30        40        50        60
                       ....|....|....|....|....|....|....|....|....|....|....|....|
Megachile rotunda      MACAFTLLTNNVSSEVALVDVMADKLKGEMMDLQHGSAFLKNAKINASTDYASTANSSLC
P. varians             MACAFSLLTQHICSELALVDVMADKLRGEMMDLQHGLTFLRNVKIEASTDFSVTAGSRVC
Halocaridina rubra     MACAFSLLTQHICSELALVDVVADKLRGEMMDLQHGLTFLRNVKIDASTDFSVTAGSRVC
Litopenaeus vannamei   MACAFSLLTQHICSELALVDVAADKLRGEMMDLQHGLTFLRNVKIDANTDYAVTAGSRVC
Clustal Consensus      *****:***:.:.**:***** ****:********* :**:*.**:*.**:: ** * :*

                                70        80        90        100       110       120
                       ....|....|....|....|....|....|....|....|....|....|....|....|
Megachile rotunda      IVTAGARQREGETRLDLVQRNADIFKGIIPQLVRYSPNTILLIVSNPVDILTYVAWKLSG
P. varians             IVTAGARQREGESRLSLVQRNVDIFKGIIPNLVKHSPNCILLVVSNPVDVLTYVAWKLSG
Halocaridina rubra     IVTAGARQREGESRLSLVQRNVDIFKGIIPNLVKHSPNCILLIVSNPVDVLTYVAWKLSG
Litopenaeus vannamei   IVTAGARQREGESRLSLVQRNVDIFKGMIPQLVKHSPNCILLIVSNPVDILTYVAWKLSG
Clustal Consensus      ************:**.*****.*****:**:**::*** ***:******:**********

                               130       140       150       160
                       ....|....|....|....|....|....|....|....|--
Megachile rotunda      LPKNRVIGSGTNLDSARFRFLLSQKLNVAPTSCHGWIIGEHG
P. varians             LPKHRVIGSGTNLDSARFRFHLSQKLNVAPSSTHGWIIGEHG
Halocaridina rubra     LPKHRVIGSGTNLDSARFRFHLSQKLNVAPASTHGWIVGEHG
Litopenaeus vannamei   LPKHHVIGSGTNLDSARFRFHLSQKLSVAPSSTHGWIIGEHG
Clustal Consensus      ***.:*************** *****.***:* ****:****
```

A1.2.2 *Lithodes maja*

Narg gene

Putative L.maja narg alignment

```
                          10         20         30         40         50         60         70         80         90        100
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
tr|Lmaja|Narg      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --CWHVXGLL
tr|F4WJY7|F4WJY7_ACREC MPSSNPLPPK ENALFKRILR CYEHKQYKNG LKFAKQILSN PKFSEHGETL AMKGLTLNCL GRKEEAYDHV RRGLRNDLQS HVCWHVYGLL
tr|E9IP68|E9IP68_SOLIN MPSSNPLPPK ENALFKRILR CYEHKQYKNG LKFAKQILSN PKFSEHGETL AMKGLTLNCL GRKEEAYDHV RRGLRNDLQS HVCWHVYGLL
tr|D3ZD89|D3ZD89_RAT  -MPAVSLPPK ENALFKRILR CYEHKQYRNG LKFCKQILSN PKFAEHGETL AMKGLTLNCL GKKEEAYELV RRGLRNDLKS HVCWHVYGLL
tr|Q58F05|Q58F05_HUMAN -MPAVSLPPK ENALFKRILR CYEHKQYRNG LKFCKQILSN PKFAEHGETL AMKGLTLNCL GKKEEAYELV RRGLRNDLKS HVCWHVYGLL
tr|C0H926|C0H926_SALSA -MPSITLPPK ENALFKRILR CYEHKQYRNG LKFCKQILSN PKFTEHGETL AMKGLTLNCL GKKEDAYELV RRGLRNDLKS HVCWHVYGLL
Clustal Consensus                                                                                                  ****

                         110        120        130        140        150        160        170        180        190        200
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
tr|Lmaja|Narg      QRSDKKYDEA IKCYRNALKW DRDNLQILRD LSLLQIQMRD LDGYKDTRYK LFELRPTQRA SWIGYAMSFH LLHNYDTALK ILEEFRKTIQ K--TTYDYEY SELLLYQNLV
tr|F4WJY7|F4WJY7_ACREC QRSDKKYDEA IKCYRNALKW DRDNIQILRD LSLLQIQMRD LEGYKDTRYQ LFMLRPTQRA SWIGFAISYH LLRDYEMALK ILDTFRNSPM I---CYDYEH SELLLYQNMV
tr|E9IP68|E9IP68_SOLIN QRSDKKYDEA IKCYRNALKW DRDNIQILRD LSLLQIQMRD LEGYKDTRYQ LFMLRPTQRA SWIGFAISYH LLRDYEMALK ILDTFRNSPM I---CYDYEH SELLLYQNMV
tr|D3ZD89|D3ZD89_RAT  QRSDKKYDEA IKCYRNALKW DRDNLQILRD LSLLQIQMRD LEGYRETRYQ LLQLRPAQRA SWIGYAIAYH LLEDYEMAAK ILEEFRKTQQ TSPDKVDYEY SELLLYQNQV
tr|Q58F05|Q58F05_HUMAN QRSDKKYDEA IKCYRNALKW DRDNLQILRD LSLLQIQMRD LEGYRETRYQ LLQLRPAQRA SWIGYAIAYH LLEDYEMAAK ILEEFRKTQQ TSPDKVDYEY SELLLYQNQV
tr|C0H926|C0H926_SALSA QRSDKKYDEA IKCYRNALKW DRDNLQILRD LSLLQIQMRD LEGYRETRYQ LLQLRPAQRA SWIGYAVAYH LLEDFEMAAK IVEEFRKTQQ TSPDKVDYEY SELLLYQNQV
Clustal Consensus  ********** ********** ********** ********** *:**::***: *: ***:*** ****:*:::* **.::: * * *:: **::         ***: ******** *

                         210        220        230        240        250        260        270        280        290        300
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
tr|Lmaja|Narg      QRSDKKYDEA IRESRNLLEA LKHLDTYEAN ICDKVTLQEL RGELLMKLGR REAASEVYRG LIKRNPENRD YYLKLEDALA LNTVHQRLHL YDELKEKFPR AQVPKRLPLN
tr|F4WJY7|F4WJY7_ACREC QRSDKKYDEA IQESGECEQA LKHLDKYSDQ ICDKVTVKET YGKLRLQLKQ YAEAEQVYKE LLDINPENTT YYTRLAEAER HSSPSETLQM LQRYEELFPR ALAPRRLQLN
tr|E9IP68|E9IP68_SOLIN QRSDKKYDEA IQESGECEQA LKHLDKYSDQ ICDKVTVKET YGKLRLQLKQ YAEAEQVYKE LLDINPENTT YYTRLAEAEK HSSPSETLHM LQRYEELFPR ALAPRRLQLN
tr|D3ZD89|D3ZD89_RAT  QRSDKKYDEA LREAGLYREA LEHLCTYEKQ ICDKLAVEET KGELLLQLCR LEDAADVYRG LQERNPENWA YYKGLEKALK PANMLERLKI YEEAWTKFPR GLVPRRLPLN
tr|Q58F05|Q58F05_HUMAN QRSDKKYDEA LREAGLYREA LEHLCTYEKQ ICDKLAVEET KGELLLQLCR LEDAADVYRG LQERNPENWA YYKGLEKALK PANMLERLKI YEEAWTKYPR GLVPRRLPLN
tr|C0H926|C0H926_SALSA QRSDKKYDEA LREAGLFREA LEHLITYEKQ ICDKLAVEET RGELLLQLDR SEEATEVYRQ LQERNPENWA YYQGLEKALK PVSIEERQKL YEEAWVKYPK GLVPRRLPLT
Clustal Consensus  ********** ::*:    :* *:** .*. : ****::::*   *:* ::* :    * :**:  * . ****   **  * .*     .  :  ::  :.    :*: . .*:** *.

                         310        320        330        340        350        360        370        380        390        400
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
tr|Lmaja|Narg      QRSDKKYDEA YAHGPELESL LDPYLRAALQ KGVPPLFTDI QSLYTEPDRI LIVQNLMERY LKNLEETGHL CQS--DNEYK EPASVLFVLM FLAQHHSYLG DTEKALSLIE
tr|F4WJY7|F4WJY7_ACREC QRSDKKYDEA YATGDEFKVL VDRYLRRGLH KGVPPLFVNL RSLYTDKEKV EIISSLLVQY KEALKLHDHF SDQEKDNPRE PASALLWTYY YLAQHYDYLG QTEKALIEID
tr|E9IP68|E9IP68_SOLIN QRSDKKYDEA YATGDEFKAL VDRYLRRGLH KGVPPLFVNL RSLYTDKEKV EIISSLLVQY KEALKLHGHF SDQEKDNPRE PASALLWTYY YLAQHYDYLG QTEKALIEID
tr|D3ZD89|D3ZD89_RAT  QRSDKKYDEA FLSGEKFKEC LDRFLRMNFS KGCPPVFNTL RSLYRDKEKV AIVEELVVGY ETSLKSCRLF NP-NDDGKEE PPTTLLWVQY YLAQHYDKIG QPSIALEYIN
tr|Q58F05|Q58F05_HUMAN QRSDKKYDEA FLSGEKFKEC LDKFLRMNFS KGCPPVFNTL RSLYKDKEKV AIIEELVVGY ETSLKSCRLF NP-NDDGKEE PPTTLLWVQY YLAQHYDKIG QPSIALEYIN
tr|C0H926|C0H926_SALSA QRSDKKYDEA FLTGEKFREC LDCYLRTNFS KGCPPVFTTL KSLYHDKEKV SIVEELVVGY ETCLKSCRMF NQ-NDGGKEE PPTTLLWVQY YLAQHFDHVG QQTLALDYIN
Clustal Consensus  ********** :  * ::.   :* :**  :  ** **:*  : :*** : :::  *:..*:  *    *:    :          :   :::*:.   :****.. :* :   **  *:

                         410        420        430        440        450        460        470        480
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
tr|Lmaja|Narg      QRSDKKYDEA RALEHTPTLI ELYIVKGKIL KHAGDYVGAY EALQEAQALD TADRYVNSKA ASYLIKANLV RQAEEMCSKF TREGVSAMEN LNEMQCMW
tr|F4WJY7|F4WJY7_ACREC QRSDKKYDEA AAIDHTPTLI ELFVTKGRIY KHAGNVQEAY KWLDEAQGLD TADRYINSKC AKYMLRANLI KEAEETCGKF TREGVLAMEN LNEMQCMW
tr|E9IP68|E9IP68_SOLIN QRSDKKYDEA AAIDHTPTLI ELFVTKGRIY KHAGNVQEAY KWLDEAQGLD TADRYINSKC AKYMLRANLI KEAEETCGKF TREGVLAMEN LNEMQCMW
tr|D3ZD89|D3ZD89_RAT  QRSDKKYDEA TAIESTPTLI ELFLVKAKIY KHAGNIKEAA RWMDEAQALD TADRFINSKC AKYMLKANLI KEAEEMCSKF TREGTSAVEN LNEMQCMW
tr|Q58F05|Q58F05_HUMAN QRSDKKYDEA TAIESTPTLI ELFLVKAKIY KHAGNIKEAA RWMDEAQALD TADRFINSKC AKYMLKANLI KEAEEMCSKF TREGTSAVEN LNEMQCMW
tr|C0H926|C0H926_SALSA QRSDKKYDEA TAIESTPTLI ELFLIKAKIY KHAGNIKEAA RWMDEAQALD TADRFINSKC AKYMLKAGLV KEAEEMCSKF TREGASAVEN LNEMQCMW
Clustal Consensus  ********** *:: ***** **:: *.:*  ****:   *  . ::***.** ****::***. *.*:::* *: ::*** *.** ****. *:** ********
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Hsp70a

HSP70a Protein alignment

```
                                         10         20         30         40         50         60
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tr|HSP70a|L_maja                GDHHANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVFDAKRLIGRKFEDHHVQS
tr|A0T3E6|HSP70kDa|P_japonicus  ----ANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVFDAKRLIGRKFDDHTVQS
tr|B8YEL0|HSP70kDa|P_trituberc  ----ANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVFDAKRLIGRKFTDHHVQS
tr|T2C8Q7|HSP70kDa|P_transvers  ----ANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVFDAKRLIGRKFNDHNVQS
tr|M4PVJ8|HSP70kDa|G_cruentata  ----ANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVFDAKRLIGRKFNDHNVQS
tr|Q3S349|HSP70kDa|P_marmoratu  ----ANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVFDAKRLIGRKFNDHNVQS
Clustal Consensus                   ************************************************** ** ***

                                         70         80         90         100        110        120
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|
tr|HSP70a|L_maja                DMKHWPFDVINDSTKPKIQVEYKGEKKSFYPEEISSMVLIKMKETAEAYLGSIVKDAVIT
tr|A0T3E6|HSP70kDa|P_japonicus  DMKHWPFEIINESTKPKIQVEYKGDKKTFYPEEISSMVLIKMKETAEAYLGSTVKDAVVT
tr|B8YEL0|HSP70kDa|P_trituberc  DMKHWPFEVIEDSTKPKIRVEYKGEKKSFYPEEISSMVLMKMKETAEAYLGAAVKDAVIT
tr|T2C8Q7|HSP70kDa|P_transvers  DMKHWPFDVIDDNTKPKIKVEYKGEAKSFYPEEISSMVLIKMKETAEAYLGSIVKDAVIT
tr|M4PVJ8|HSP70kDa|G_cruentata  DMKHWPFDVIDDNTKPKIKVEYKGEAKSFYPEEISSMVLIKMKETAEAYLGSIVKDAVIT
tr|Q3S349|HSP70kDa|P_marmoratu  DMKHWPFDVIDDNTKPKIKVEYKGEAKSFYPEEISSMVLIKMKETAEAYLGSVVKDAVVT
Clustal Consensus               *******::*::.*****:*****: *:************:***********: *****:*

                                        130        140        150        160        170
                                ....|....|....|....|....|....|....|....|....|....|....|
tr|HSP70a|L_maja                VPAYFNDSQRQATKDRGITSEFAAACRS-------TIWESSQRVGCIA--
tr|A0T3E6|HSP70kDa|P_japonicus  VPAYFNDSQRQATKDAGTISGLNVLRIINEPTAATIAYGLDKKVGGERNV
tr|B8YEL0|HSP70kDa|P_trituberc  VPAYFNDSQRQATKDAGTISGVNVLRIINEPTAAAIAYGLDKKVGGERNV
tr|T2C8Q7|HSP70kDa|P_transvers  VPAYFNDSQRQATKDAGTISGLNVLRIINEPTAAAIAYGLDKKVGGERNV
tr|M4PVJ8|HSP70kDa|G_cruentata  VPAYFNDSQRQATKDAGTISGLNVLRIINEPTAAAIAYGLDKKVGGERNV
tr|Q3S349|HSP70kDa|P_marmoratu  VPAYFNDSQRQATKDAGTISGLNVLRIINEPTAAAIAYGLDKKVGGERNV
Clustal Consensus               *************** *  * . .             :  .::**
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Hsp70 b

HSP70b protein alignment

```
                                         10         20         30         40         50         60
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|
trHSP70bL_maja|                 GDHHANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVFDAKRLIGRKFEDTTVQG
tr|H2B5Z8|HSP70kDa|C_quadricar  ----ANDQGNRTTPSYVAFTDAERLIGDAAKNQVAMNPNNTVFDAKRLIGRKFNDSTVQA
tr|B7ZEC1|HSP70kDa|C_pagurus    ----ANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVFDAKRLIGRKFEDATVQS
tr|A0A024A413|HSC70kDa|M_ensis  ----ANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVFDAKRLIGRKFDDATVQS
tr|E2JE32|HSP70kDa|P_carinicau  ----ANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTVFDAKRLIGRKFEDHTVQS
Clustal Consensus                   *****************:*********************************:* ***.

                                         70         80         90         100        110        120
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|
trHSP70bL_maja|                 GMKHWPFTVVNEGGKPKIVVEYKGENKKFFPEEISSMVLIKMKETAEAYLGISVKDAVVT
tr|H2B5Z8|HSP70kDa|C_quadricar  DMKHWPFMVINDGGKPKIQIEYKGEMKSFYPEEISSMVLIKMKETAEAYLGATVKDAVIT
tr|B7ZEC1|HSP70kDa|C_pagurus    DMKQWPFTVISDGGKPKITVDHKGETKKFFPEEISSMVLIKMKETAEAYLGSTVKDAVVT
tr|A0A024A413|HSC70kDa|M_ensis  DMKHWPFTIVNESTKPKIQVEYKGDKKTFYPEEISSMVLIKMKETAEAYLGATVKDAVVT
tr|E2JE32|HSP70kDa|P_carinicau  DMKHWPFTIINESTKPKIQVEYKGDKKTFYPEEISSMVLIKMKETAEAYLGSTVKDAVVT
Clustal Consensus                **:*** ::.:. **** :::**: *.*:********************** :*****:*

                                        130        140        150        160        170
                                ....|....|....|....|....|....|....|....|....|....|....|
trHSP70bL_maja|                 VPAYFNDSQRQATKDRGITSEFAAACRS-------TIWESSQRVGCIA--
tr|H2B5Z8|HSP70kDa|C_quadricar  VPAYFNDSQRQATKDAGTISGMNVLRIINEPTAAAIAYGLDKKVGGERNV
tr|B7ZEC1|HSP70kDa|C_pagurus    VPAYFNDSQRQATKDAGTISGMNVLRIINEPTAAAIAYGLDKKIGGERNV
tr|A0A024A413|HSC70kDa|M_ensis  VPAYFNDSQRQATKDAGTISGLNVLRIINEPTAAAIAYGLDKKVGGERNV
tr|E2JE32|HSP70kDa|P_carinicau  VPAYFNDSQRQATKDAGTISGLNVLRIINEPTAAAIAYGLDKKVGGERNV
Clustal Consensus               *************** *  * : .             :  .:::*
```

Cs gene

CitS protein alignment

```
                                          10         20         30         40         50         60
                                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tr|CitS|L_maja                   DHEGXNVSAHTTHLVGSALSDPYLSFAAGMNGLAGPLHGLANQEVLMWVTRLRSEIGDEV
tr|A0A023GE10|CitS|A_triste      DHEGGNVSAHTTHLVGSALSDPYLSLAAGMNGLAGPLHGLANQEVLVWLTKMQKELGGEV
tr|A0A026X2M5|CitS|C_biroi       DHEGGNVSAHTTHLVGSALSDPYLSFAAGMNGLAGPLHGLANQEVLVWLEKLRGQVGDNP
tr|J3JV43|CitS|D_ponderosae      DHEGGNVSAHTTHLVGSALSDPYLSFAAGLNGLAGPLHGLANQEVLLWIQKLRQQVGDKY
tr|U5EUY0|CitS|C_appendiculata   DHEGGNVSAHTVHLVGSALSDPYLSFAAGMNGLAGPLHGLANQEVLVWLQKLRKELGDNA
Clustal Consensus                **** ******.**************:***:*****************:*: ::: ::* :

                                          70         80         90         100        110        120
                                 ....|....|....|....|....|....|....|....|....|....|....|....|....|
tr|CitS|L_maja                   TEDQLKEFIWQTLKSGQVVPGYGHAVLRKTDPRYTCQREFALKHLPDDKLFKLVAKLYNV
tr|A0A023GE10|CitS|A_triste      SDEQLKEFVWKTLKSGQVVPGYGHAVLRKTDPRYTCQREFALKHLPQDPMFKLVSQLYTV
tr|A0A026X2M5|CitS|C_biroi       SDDKLKEFIWNTLKSGQVVPGYGHAVLRKTDPRYTCQREFALKHLPDDPLFKLVAQVYKV
tr|J3JV43|CitS|D_ponderosae      TEEQLKEFIWKTLKSGQVVPGYGHAVLRKTDPRYTCQREFALKHLPEDPLFKLVSDIYKV
tr|U5EUY0|CitS|C_appendiculata   GEEKVKEFIWKTLKSGQVVPGYGHAVLRKTDPRYTCQREFALKHLPDDPLFKLVSNIYKV
Clustal Consensus                 ::::***:*:*************************************:* :****:.:*.*

                                          130        140
                                 ....|....|....|....|...
tr|CitS|L_maja                   VPPILTELGKVKNPWPNVDA---
tr|A0A023GE10|CitS|A_triste      VPPILLELGKVKNPWPNVDAHSG
tr|A0A026X2M5|CitS|C_biroi       VPPILLETGKVKNPWPNVDAHSG
tr|J3JV43|CitS|D_ponderosae      VPPILLELGKVKNPWPNVDAHSG
tr|U5EUY0|CitS|C_appendiculata   VPPILTELGKVKNPWPNVDAHSG
Clustal Consensus                ***** * ************
```

Eef1a gene

EEF1a protein alignment

```
                                          10         20         30         40         50         60         70         80         90
                                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tr|EEF1a|L_maja                  GEFEAGISKNGQTREHVLLCFTLGVKQLIVAVNKMDSTEPKFSEARFQEIKKELTTYVKKVGYNPTIVPILPISGFNGDNMLEKSDHMPW
tr|B9ZZQ1|EEF1a|P_japonicus      GEFEAGISKNGQTREHVLLCFTLGVKQLIVAVNKMDSTEPKYSEERFKGIHKEVSAYVKKVGYNPAIVPIIPISGFNGDNMLEKSENMGW
tr|A8R5V2|EEF1a|U_major          GEFEAGISKNGQTREHVLLCFTLGVKQLIVAVNKMDSTEPKYSSARYEEIKKELTAYVKKVGYNPAIVPILPISGFNGDNMLEKSENMTW
tr|I6UGH0|EEF1a|S_paramamosain   GEFEAGISKNGQTREHVLLCFTLGVKQLIVAVNKMDSTEPKYSEARFNEIKKELTAYVKKVGYNPTIVPILPISGFNGDNMLEKSDNMSW
tr|D9ZNA9|EEF1a|C_borealis       GEFEAGISKNGQTREHVLLCFTLGVKQLIVAVNKMDSTEPKYSEARFNEIKKELTVYVKKVGYNPTIVPILPISGFNGDNMLEKSDNMTW
Clustal Consensus                ****************************************:*. *:: *:**::.*********:****:**************:.* *

                                          100        110        120        130        140        150        160        170        180
                                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tr|EEF1a|L_maja                  WTKAKIERKSGSYEFTTLFEALDNIEPPSRPVDKALRLPLQDVYKIGGIGTVPVGRVETGILKPGMVVNFAPTGPTTEVKSVEMHHEALT
tr|B9ZZQ1|EEF1a|P_japonicus      WKKQKISRKSDNYEFETLFDALDNIEPPTRPLDKALRLPLQDVYKIGGIGTVPVGRVETGILKPGMVVNFAPTGPTTEVKSVEMHHEALT
tr|A8R5V2|EEF1a|U_major          WTKQKIERKSGNYEYLTLFDALDNIEPPSRPVDKPLRLPLQDVYKIGGIGTVPVGRVETGILKPGMVVNFAPTGPTTEVKSVEMHHEALT
tr|I6UGH0|EEF1a|S_paramamosain   WSKQKIERKSGSYEYITLFDALDNIEPPSRPVDKPLRLPLQDVYKIGGIGTVPVGRVETGILKPGMVVNFAPTGPTTEVKSVEMHHEALT
tr|D9ZNA9|EEF1a|C_borealis       WGKTKIERKNGSYEFTTLFEALDNIDPPSRPLDKPLRLPLQDVYKIGGIGTVPVGRVETGILKPGMVVNFAPTGPTTEVKSVEMHHEALV
Clustal Consensus                * * **.**. .**: ***:*****:**:**:** ************************************************.

                                          190        200        210        220        230        240        250        260        270
                                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tr|EEF1a|L_maja                  EACPGDNVGFNVKNVAVKDLKRGFVASDSKNDPAREAGDFTAQVIVLNHPGQIQPGYSPVLDCHTAHIACKFAELIQKIDRRTGKEIEAN
tr|B9ZZQ1|EEF1a|P_japonicus      EAVPGDNVGFNVKNVSVKDLKRGFVASDSKNDPAKEAADFTAQVIVLNHPGQIQAGYSPVLDCHTAHIACKFAELLTKIDRRTGKELEAG
tr|A8R5V2|EEF1a|U_major          EAQPGDNVGFNVKNVSVKDLKRGFVASDSKNDPAREAADFTAQVIVLNHPGQVQAGYTPVLDCHTAHIACKFAELIQKIDRRTGKEIEAS
tr|I6UGH0|EEF1a|S_paramamosain   EAVPGDNVGFNVKNVSVKELKRGFVASDSKNDPAKEAGDFTAQVIVLNHPGQIQAGYSPVLDCHTAHIACKFAELIQKIDRRTGKEIEAN
tr|D9ZNA9|EEF1a|C_borealis       EANPGDNVGFNVKNVSVKELKRGFVASDSKNDPAKEAGDFTAQVIVLNHPGQIQAGYSPVLDCHTAHIACKFAELIQKIDRRTGKEIESN
Clustal Consensus                ** ************:**:***************:**.***************:* **:*****************: *********:*:

                                          280        290        300        310
                                 ....|....|....|....|....|....|....|....|
tr|EEF1a|L_maja                  PKVIKSGDSCIVKMIPSKPMCVETFQKYAPLGRFAVRDMKQTVAV
tr|B9ZZQ1|EEF1a|P_japonicus      PKHVKSGDSCIVKMVPSKPMCVETFQQYAPLGRFAVRDMKQTVAV
tr|A8R5V2|EEF1a|U_major          PKHIKSGDSCIVKMVPSKPMCVETFQKYAPLGRFAVRDMKQTVAV
tr|I6UGH0|EEF1a|S_paramamosain   PKQIKSGDSCIVKMVPSKPMCVETFQKYAPLGRFAVRDMKQTVAV
tr|D9ZNA9|EEF1a|C_borealis       PKQIKSGDSCIVKMVPSKPMCVETFQKYAPLGRFAVRDMKQTVAV
Clustal Consensus                ** :**********:***********:******************
```

Rpl8 gene

RPL8 protein alignment

```
                                10        20        30        40        50        60
                                |---|---|---|---|---|---|---|---|---|---|---|---|
tr|RPL8|L_maja                 ------------------------------------------------------------
tr|E2B849|RPL8|H_saltator      MGRVIRAQRKGAGSVFRSHTKRRKGAPKLRSLDFSERHGYIKGVVKDIIHDPGRGAPLAV
tr|Q56FH5|RPL8|L_testaceipes   MGRVIRAQRKGAGSVFRAHTKKRKGAPKLRSLDFSERHGYIKGVVRDIIHDPGRGAPLAV
tr|C5MKI0|RPL8|P_varians       ------------------------------------RHGYIKGIVKTMIHDPGRGAPIAE
tr|Q2I3E8|RPL8|L_vannamei     MGRVIRAQRKGRGSVFKSHTHTRKGKPALRAIDYAERHGYLRGIIKQIIHDPGRGAPLAI
Clustal Consensus

                                        70        80        90        100       110       120
                                |---|---|---|---|---|---|---|---|---|---|---|---|
tr|RPL8|L_maja                 VHFSDPYRYKTRKELFLAAEGMYTGQFIYCGKKANLDVGNVMPIGTLPEGTVICNLEEKT
tr|E2B849|RPL8|H_saltator      VHFRDPYKFKTRKELFIAPEGMYTGQFLYCGKKANLQIGNVMPVGTMPEGTIVCNLEEKT
tr|Q56FH5|RPL8|L_testaceipes   VHFRDPYKFKTRKELFIAPEGMYTGQFLYCGKKANLQIGNVMPVGTMPEGTIVCNLEEKT
tr|C5MKI0|RPL8|P_varians       VHFRDPYRYKTRKELFIAAEGTYTGQFIYCGKKANLDVGNVLPIGTLPEGTIVCNLEEKT
tr|Q2I3E8|RPL8|L_vannamei     VHFRDPYRYKIRKEIFLAAEGMYSGQFVYCGKKANLDVGNVIPIGNLPEGTIICNLEEKT
Clustal Consensus              *** ***::* ***:*:* ** *:***:********::***:*:*.:****::*******

                                        130       140       150       160       170       180
                                |---|---|---|---|---|---|---|---|---|---|---|---|
tr|RPL8|L_maja                 GDRGRIARGSGNYAQVIAHNPETKKTRVKLPSGAKKVLPSANRAMIGIVAGGGRIDKPIL
tr|E2B849|RPL8|H_saltator      GDRGRLARASGNYATVIAHNPDTKKTRVKLPSGAKKVIPSNNRAMVGIVAGGGRIDKPIL
tr|Q56FH5|RPL8|L_testaceipes   GDRGRLARASGNYATVIAHNPDSKKTRVKLPSGAKKVIPSNNRAMVGIVAGGGRIDKPIL
tr|C5MKI0|RPL8|P_varians       GDRGRLARGSGNYAQIIAHNPDTRRTRVKLPSGAKKVLPSANRAMIGIVAGGGRIDKPIL
tr|Q2I3E8|RPL8|L_vannamei     GDRGRIARGSGNYAQVIAHNPETKKTRVKLPSGAKKVLPSANRAMIGIVAGGGRIDKPIL
Clustal Consensus              *****:**.***** :*****::::*************:** ****:***************

                                        190       200       210
                                |---|---|---|---|---|---|...
tr|RPL8|L_maja                 KAGRAYHKYRVKRNSWPKVRGVAMNPXEH----
tr|E2B849|RPL8|H_saltator      KAGRAYHKYKAKRNCWPKVRGVAMNPVEHPHGG
tr|Q56FH5|RPL8|L_testaceipes   KAGRAYHKYKAKRNCWPKVRGVAMNPVEHPHGG
tr|C5MKI0|RPL8|P_varians       KAGRAYHKYRVKRNSWPKVRGVAMNPVEHPHGG
tr|Q2I3E8|RPL8|L_vannamei     KAGRAYHKYRVKRNCWPKVRGVAMNPVEHPHGG
Clustal Consensus              **********:.***.*********** **
```

A1.3 *Hsp70* isoform identity

A1.3.1 *Hsp70* isoform, protein alignment

L.maja putative HSP70 isoform protein alignment

```
                                10          20          30          40          50          60
                                |---|---| ----|----| ----|----| ----|----| ----|----| ----|----|
HSP70a isoform 3'-5' Frame 3   GDHHANDQGN RTTPSYVAFT DTERLIGDAA KNQVAMNPNN TVFDAKRLIG RKFEDHHVQS
HSP70b isoform 3'-5' Frame 3   GDHHANDQGN RTTPSYVAFT DTERLIGDAA KNQVAMNPNN TVFDAKRLIG RKFEDTTVQG
Clustal Consensus              ********** ********** ********** ********** ********** *****  **.

                                70          80          90          100         110         120
                                |---|---| ----|----| ----|----| ----|----| ----|----| ----|----|
HSP70a isoform 3'-5' Frame 3   DMKHWPFDVI NDSTKPKIQV EYKGEKKSFY PEEISSMVLI KMKETAEAYL GSIVKDAVIT
HSP70b isoform 3'-5' Frame 3   GMKHWPFTVV NEGGKPKIVV EYKGENKKFF PEEISSMVLI KMKETAEAYL GISVKDAVVT
Clustal Consensus               ****** *: *:. **** * *****:*.*: ********** ********** *  *****:*

                                130         140         150         160
                                |---|---| ----|----| ----|----| ----|----| .
HSP70a isoform 3'-5' Frame 3   VPAYFNDSQR QATKDRGITS EFAAACRSTI WESSQRVGCI A
HSP70b isoform 3'-5' Frame 3   VPAYFNDSQR QATKDRGITS EFAAACRSTI WESSQRVGCI A
Clustal Consensus              ********** ********** ********** ********** *
```

A1.3.2 *Hsp70* isoform, nucleotide alignment

Putative HSP70 isoform DNA alignment

```
                                10         20         30         40         50         60         70
                        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Putative HSP70a isoform AGCTATGCAT CCAACGCGTT GGGAGCTCTC CCATATGGTC GACCTGCAGG CGGCCGCGAA TTCACTAGTG
Putative HSP70b isoform AGCTATGCAT CCAACGCGTT GGGAGCTCTC CCATATGGTC GACCTGCAGG CGGCCGCGAA TTCACTAGTG
Clustal Consensus       ********** ********** ********** ********** ********** ********** **********

                                80         90         100        110        120        130        140
                        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Putative HSP70a isoform ATTCCCCGAT CTTTGGTGGC TTGACGCTGG GAGTCGTTGA AGTAGGCTGG GACGGTGATC ACAGCATCTT
Putative HSP70b isoform ATTCCCCGGT CTTTAGTTGC CTGTCGCTGG GAGTCATTAA AATATGCTGG CACTGTGACC ACAGCATCCT
Clustal Consensus       ******** * **** ** **  ** ****** ***** ** * * ** *****  ** **** * ******** *

                                150        160        170        180        190        200        210
                        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Putative HSP70a isoform TGACTATGGA CCCAAGGTAG GCCTCTGCTG TTTCCTTCAT CTTGATTAGC ACCATGGATG AGATTTCCTC
Putative HSP70b isoform TCACAGATAT GCCTAAGTAC GCCTCGGCCG TTTCCTTCAT CTTTATAAGC ACCATGGATG AAATCTCTTC
Clustal Consensus       * **        ** * ***  ***** ** * ********** *** ** *** ********** * ** ** **

                                220        230        240        250        260        270        280
                        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Putative HSP70a isoform TGGGTAAAAT GATTTCTTCT CTCCCTTGTA CTCTACTTGA ATCTTTGGTT TTGTGCTGTC ATTAATTACA
Putative HSP70b isoform GGGAAAGAAC TTTTTATTCT CTCCCTTATA CTCCACAACG ATCTTTGGCT TGCCACCTTC ATTGACGACA
Clustal Consensus        **  * **    *** **** ******* ** *** **     ******** * *    *  ** *** *  ***

                                290        300        310        320        330        340        350
                        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Putative HSP70a isoform TCGAAAGGCC AGTGCTTCAT GTCACTCTGA ACGTGGTGGT CCTCAAATTT GCGGCCAATC AGTCTCTTGG
Putative HSP70b isoform GTGAATGGCC AGTGCTTCAT GCCACCTTGC ACCGTTGTGT CTTCGAACTT GCGGCCGATA AGTCTCTTGG
Clustal Consensus         *** **** ********** * ***  **  **      ** * ** ** ** ****** **  **********

                                360        370        380        390        400        410        420
                        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Putative HSP70a isoform CATCAAAGAC AGTGTTGTTG GGGTTCATGG CTACTTGGTT CTTGGCAGCA TCTCCAATGA GACGCTCTGT
Putative HSP70b isoform CATCAAAGAC AGTGTTGTTG GGATTCATGG CCACCTGGTT CTTGGCGGCG TCCCCAATGA GACGCTCGGT
Clustal Consensus       ********** ********** ** ******* * ** ***** ****** **  ** ******* ******* **

                                430        440        450        460        470        480
                        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|
Putative HSP70a isoform GTCTGTGAAG GCCACATAGG AAGGAGTGGT GCGGTTTCCC TGATCGTTGG CATGATGATC TCCAC
Putative HSP70b isoform GTCGGTGAAG GCCACATAGG AGGGCGTGGT GCGGTTACCC TGATCATTGG CATGATGATC TCCAC
Clustal Consensus       *** ****** ********** * ** ***** ****** *** ***** **** ********** *****
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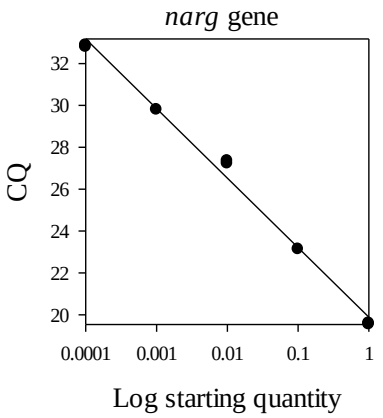
A1.4 Standard curves

Standard curves are produced from qPCR cDNA dilution series for all genes studied. Graphs presented alongside; optimised primer concentrations (nM); dynamic linear range of the standard curve; linearity represented by an R^2 value; and the amplification efficiency across the linear dynamic range.

A1.4.1 *Palaemonetes varians*

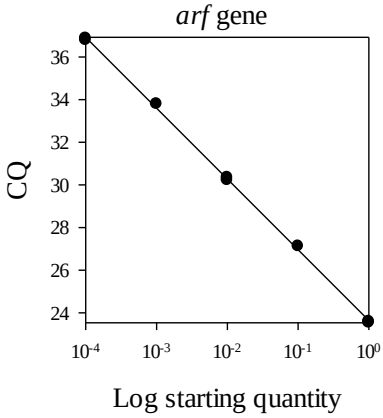


Primer mix – 300FWD, 300REV

Dilution – 5 orders of magnitude

R^2 – 0.99203

Efficiency – 1.00

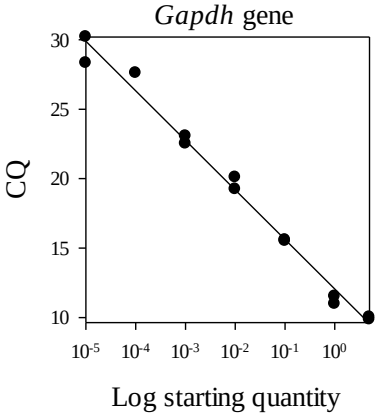


Primer mix – 300FWD, 900REV

Dilution – 5 orders of magnitude

R^2 – 0.99401

Efficiency – 1.00



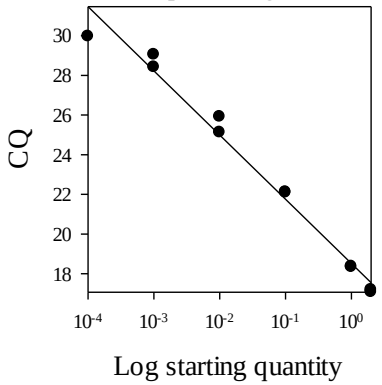
Primer mix – 300FWD, 300REV

Dilution – 6.5 orders of magnitude

R^2 – 0.98892

Efficiency – 0.91

Hsp70 F1 gene



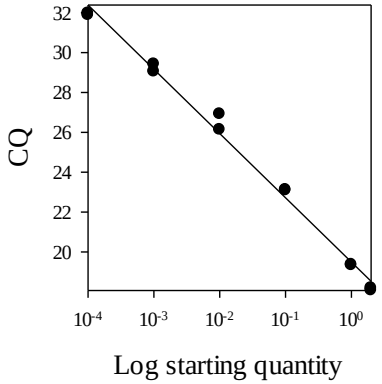
Primer mix – 50FWD, 50REV

Dilution – 5 orders of magnitude

R^2 – 0.98151

Efficiency – 1.04

Hsp70 F2 gene



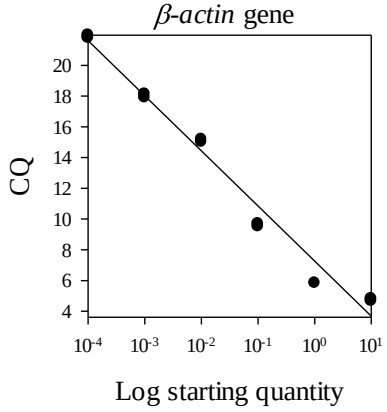
Primer mix – 300FWD, 900REV

Dilution – 6 orders of magnitude

R^2 – 0.9813

Efficiency – 0.90

β-actin gene



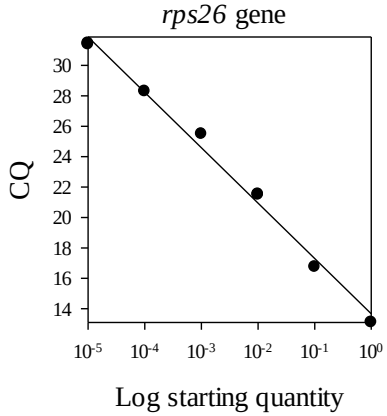
Primer mix – 300FWD, 300REV

Dilution – 6 orders of magnitude

R^2 – 0.98006

Efficiency – 0.90

rps26 gene

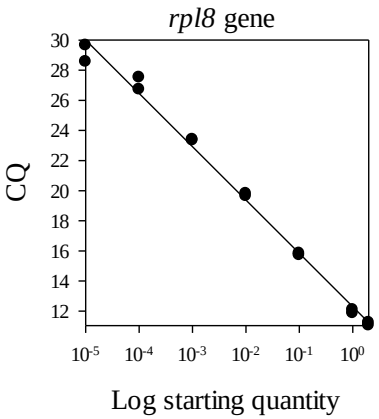


Primer mix – 300FWD, 900REV

Dilution – 6 orders of magnitude

R^2 – 0.99204

Efficiency – 0.90

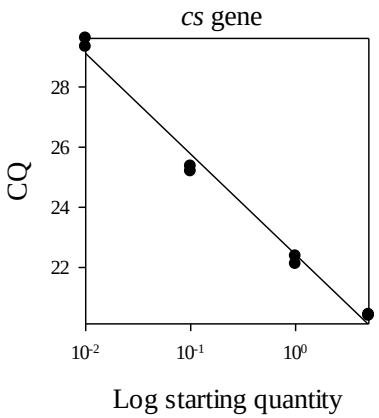


Primer mix – 900FWD, 900REV

Dilution – 6 orders of magnitude

R^2 – 0.99293

Efficiency – 0.92

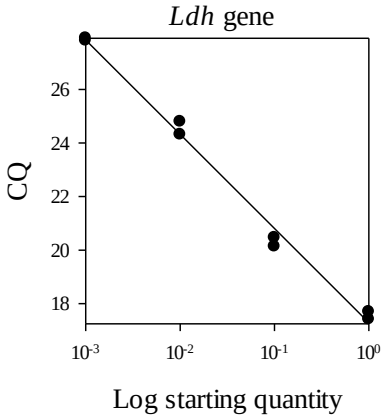


Primer mix – 300FWD, 300REV

Dilution – 3.5 orders of magnitude

R^2 – 0.9882

Efficiency – 0.99



Primer mix – 300FWD, 900REV

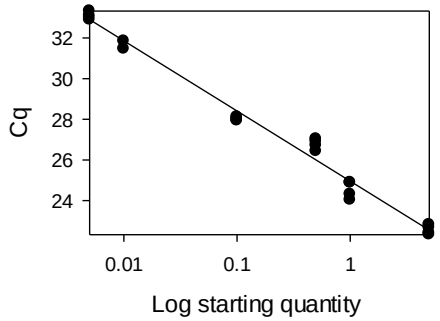
Dilution – 4 orders of magnitude

R^2 – 0.9806

Efficiency – 0.92

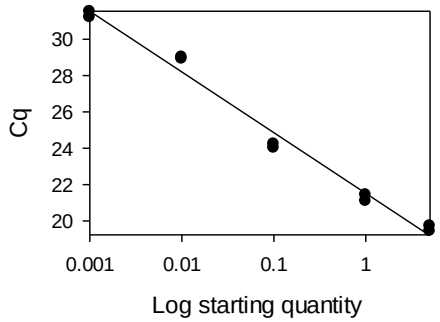
A1.4.2 *Lithodes maja*

Narg gene



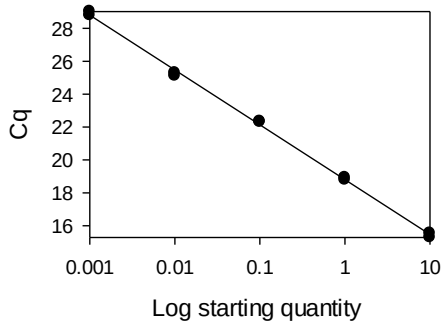
Primer conc. = 300nM FWD & REV
R^2 = 0.97
Efficiency = 97%
Linear dynamic range = 3 orders of magnitude

Cs gene



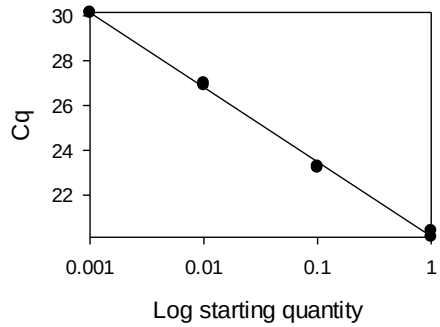
Primer conc. = 300nM FWD, 900nM REV
R^2 = 0.98
Efficiency = 99.2%
Linear dynamic range = 4.5 orders of magnitude

Hsp70a gene



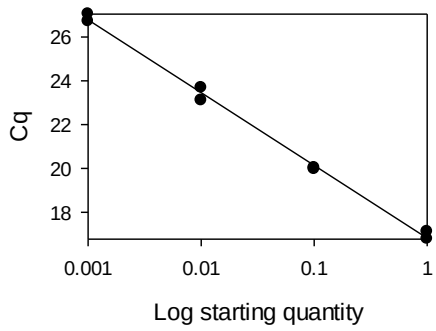
Primer conc. = 300nM FWD & REV
R^2 = 0.9985
Efficiency = 99%
Linear dynamic range = 5 orders of magnitude

Hsp70b gene



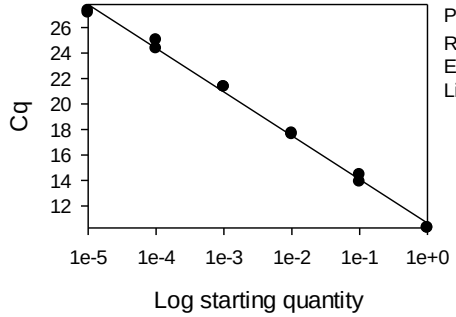
Primer conc. = 300nM FWD & REV
R^2 = 0.99
Efficiency = 99.5%
Linear dynamic range = 4 orders of magnitude

eef1α gene



Primer conc. = 300nM FWD & REV
R^2 = 1.0
Efficiency = 100%
Linear dynamic range = 4 orders of magnitude

Rpl8 gene



Primer conc. = 300nM FWD & REV
R^2 = 0.99
Efficiency = 96%
Linear dynamic range = 6 orders of magnitude

A2. APPENDIX FOR CHAPTER 5

A2.1 Behavioural analysis from Cottin et al. (2012)

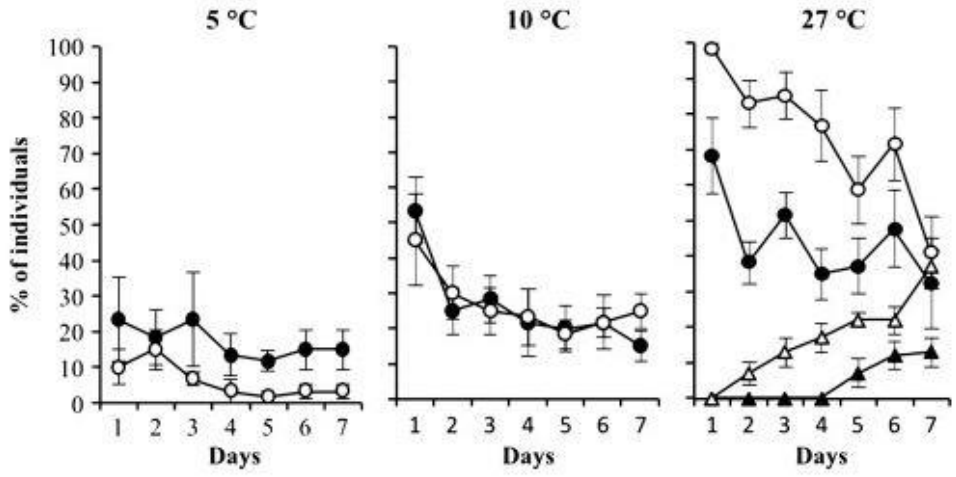


Figure A2.1 The effect of temperature and HP on the behavioural response of adult *Palaemonetes varians* over a 7-day exposure period to 0.1 MPa (black markers) and 10 MPa (white makers) at 5°C, 10°C, and 27°C. Active movement is represented by circles in each treatment, and cannibalism is represented by triangles in each treatment. The exposures were performed in triplicate with n=10 in each exposure. Where cannibalism was noted, "active movement" is expressed as a percentage of living individuals. Graphs and analysis from Cottin et al. (2012).

A3. APPENDIX FOR CHAPTER 6

A3.1 Larval respiratory rate measurements performed by Alastair Brown

Respiration rate analysis was conducted. Vials (2.8 ml) containing individuals were gently inverted 3 times to ensure homogeneity of seawater oxygen within the vial. The vial lid was then removed, and the oxygen saturation of the water was determined using an oxygen microoptode connected to a Microx TX3 array (PreSens, Germany), calibrated according to Section 2.3. The animal was then removed from the vial and gently blotted on tissue paper, transferred to a pre-weighed tin capsule, and frozen at -80°C for subsequent biomass (dry mass) determination. Respiration was calculated from the difference between the mean oxygen saturation in the control vials and the oxygen saturation in the treatment vials following established methods for determining oxygen concentration in air-saturated seawater (Section 2.3). Respiration rates were normalised according to biomass (dry mass), determined by freeze drying and subsequently weighing the samples. The respiratory responses of each ontogenetic stage to each pressure treatment were evaluated independently as fewer treatments were carried out for crab I compared to the other stages. The data were analysed using a one-way ANOVA, as they were normally distributed and homoscedastic (Shapiro-Wilk and Levene's test, respectively).

A3.2 Larval respiratory rate results

HP significantly affected respiratory rate (MO_2, μmol O_2 $mg^{-1}h^{-1}$), for all ontogenetic stages (Figure 6.1). The HP at which significant decreases in respiration rate were first observed shifted with increasing ontogenetic age, with significant decrease at 30.0 MPa in zoea I stage, at 20.0 MPa in the megalopa stage, and at 15 MPa in the crab I stage (Figure A2.1).

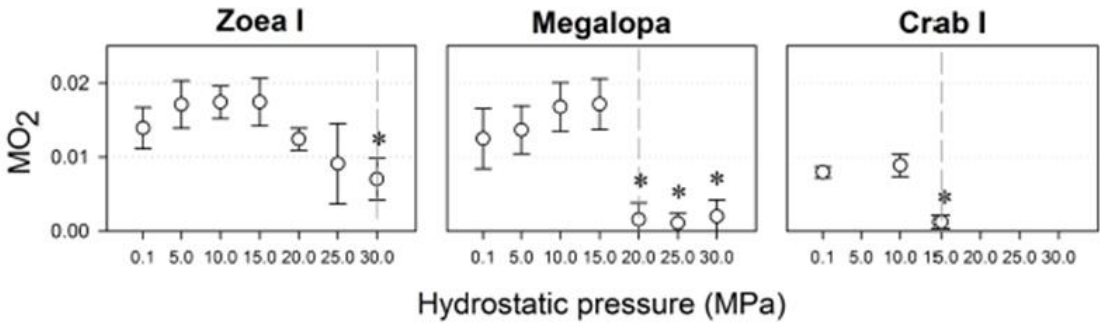


Figure A3.1 The rate of oxygen consumption with SD (MO_2, μmol O_2 $mg^{-1}h^{-1}$) across three early ontogenetic life stages at hydrostatic pressures ranging from 0.1 MPa (atmospheric control) to 30 MPa (equivalent of 3000 m water depth). Statistical significance is displayed as: * = $p<0.05$, calculated from 5 biological replicates. Vertical dashed lines visually emphasise the hydrostatic pressure at which significant differences are first observed

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