

## RESEARCH ARTICLE

# Characterising multi-level effects of acute pressure exposure on a shallow-water invertebrate: insights into the kinetics and hierarchy of the stress response

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## ABSTRACT

Hydrostatic pressure is an important, ubiquitous, environmental variable of particular relevance in the marine environment. However, it is widely overlooked despite recent evidence that some marine ectotherms may be demonstrating climate-driven bathymetric range shifts. Wide-ranging effects of increased hydrostatic pressure have been observed from the molecular 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. Here, we quantify the transcription of a set of previously characterised genes during and after acute pressure exposure in adults of the shrimp *Palaemonetes varians*. Further, we perform respiratory rate and behavioural analysis over the same period. Increases in expression of genes associated with stress and metabolism were observed during and after high-pressure exposure. Respiratory rate increased during exposure and into the recovery period. Finally, differential behaviour was observed under elevated hydrostatic pressure in comparison to ambient pressure. Characterising generalised responses to acute elevated pressure is a vital precursor to longer-term, acclimation-based pressure studies. Results provide a novel insight into what we term the overall stress response (OSR) to elevated pressure; a concept that we suggest to be applicable to other environmental stressors. We highlight the importance of considering more than a single component of the stress response in physiological studies, particularly in an era where environmental multi-stressor studies are proliferating.

**KEY WORDS:** Stress, Cellular stress response, Behavioural homeostasis, Hydrostatic pressure, HSP70, Physiology

## 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 its ecology (Bjilmsma 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,

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 specific to the stressor or the scenario. 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 last, 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 in crustaceans 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). Furthermore, rapid muscle contraction, such as abdomen flicking, has been shown to induce a heat shock response (Paulsen et al., 2007). It is clear, then, that in order to understand the complete stress response repertoire of an organism, signatures of several major aspects of the response, and their relation to one another, need to be considered. Henceforth, the complete stress response of an organism, comprising the CSR, CHR, behavioural and metabolic responses will be termed the overall stress response (OSR).

Hydrostatic pressure represents the single largest continuous environmental gradient on the planet (MacDonald, 1997). As such, all biological processes are affected by hydrostatic pressure to varying degrees (Pradillon and Gaill, 2007). 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 hydrostatic pressure may be one of the most important (Brown and Thatje, 2014). Hydrostatic pressure 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 hydrostatic pressure has been shown to

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**List of symbols and abbreviations**

CHR	cellular homeostatic response
CSR	cellular stress response
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HPNS	high-pressure neurological syndrome
HSP70	70 kDa heat shock protein
IPOCAMP	Incubateur Pressurisé pour l'Observation et la Culture d'Animaux Marins Profonds
$\dot{M}_{O_2}$	oxygen consumption
NMDAR	N-methyl-D-aspartate receptor
NRQ	normalised relative quantity
OSR	overall stress response
RFC	relative fold change

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 hydrostatic pressure is important in order to gain a better understanding of the potential for marine organisms to respond to rapid anthropogenic-induced ocean warming. Such responses have already been shown to include bathymetric range shifts, such as the movement of ectotherms to deeper, relatively cooler waters so they remain within their thermal envelope (Perry et al., 2005; Weinberg, 2005; Dulvy et al., 2008).

Previous research has looked at the ability of shallow-water invertebrates to tolerate large changes in temperature and hydrostatic pressure in combination (Cottin et al., 2012; Morris et al., 2015). Yet, in order to fully 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 hydrostatic pressure during exposure and during recovery across organisational levels from the molecular level to overt behavioural responses. These results will help us to better understand the effects of hydrostatic pressure as component of multi-stressor scenarios, and to identify the hierarchy of those effects. The chosen study organism for this research is 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 is an established marine invertebrate research species for investigating hydrostatic pressure and temperature physiology, with several complementary studies conducted on it in recent years (Oliphant et al., 2011; Cottin et al., 2012; Ravaux et al., 2012). In addition, a recent study using the slope-depth king crab *Lithodes maja* showed comparable results at the gene expression level to previous responses observed in *P. varians* to elevated hydrostatic pressure, suggesting that *P. varians* can provide an insight into the responses of deeper-living species (Morris et al., 2015; Munro et al., 2015). A temperature of 15°C was chosen as it is within the optimal temperature envelope of the species, and has been previously shown to be a level at which the sub-lethal effects of elevated pressure can be observed (Oliphant et al., 2011; Morris et al., 2015).

For the molecular aspect of this study, genes were chosen that had been previously shown to exhibit differential expression in response to acute temperature and hydrostatic pressure exposures (Cottin et al., 2010, 2012; Morris et al., 2015). The *gapdh* gene and the two *hsp70* isoforms constitute part of the universal CSR (Kültz, 2005). The *narg* gene has been suggested as a proxy for the onset of

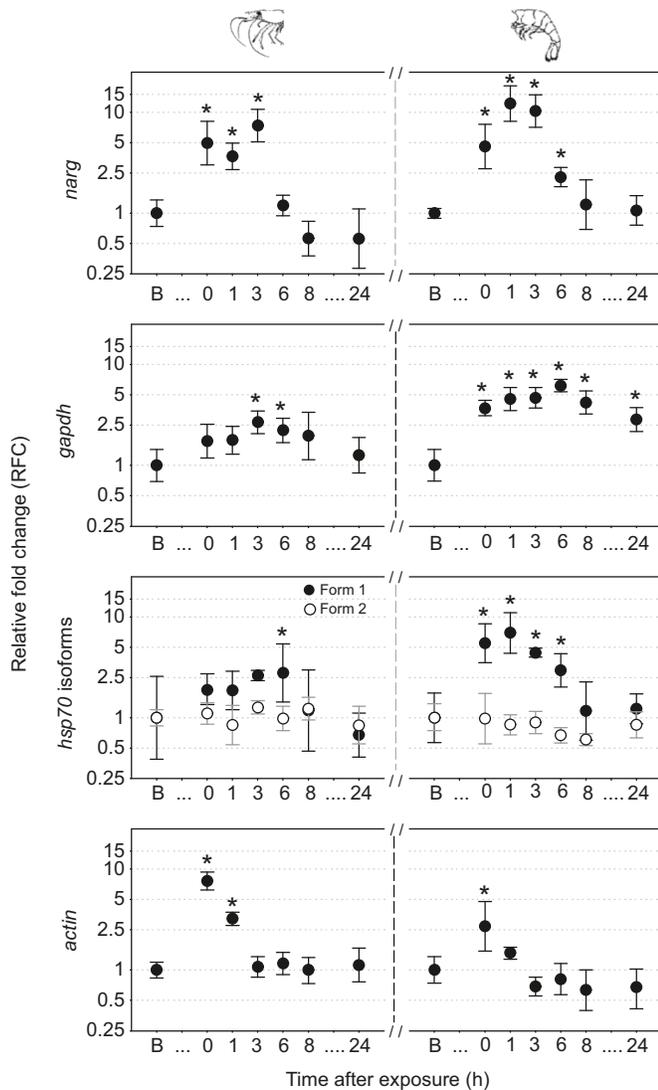
pressure-specific stress associated with neuronal pathologies characterised by high-pressure neurological syndrome (HPNS), and may represent a component of the CHR (Morris et al., 2015). The *actin* gene has been previously shown to be responsive to hydrostatic pressure increases, which, although not resolved, may be associated with loss of conformation and function of actin proteins under increased pressure scenarios (Somero, 1992), or associated with reflexive muscle contractions as observed in the behavioural analyses of this study. These genes were chosen because 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 stress exposure (Cottin et al., 2012; Morris et al., 2015). 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 examined metabolism via oxygen consumption 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. Although the exposure times tested in this study are not of direct ecological relevance, we hypothesise that any multi-level responses observed will be comparable to those observed in longer-term more ecologically relevant exposures. In summary, this research represents an important precursor to longer-term studies by providing an insight into the pathways and mechanism that are affected by elevated pressure, and their interconnected nature. Data presented provide a novel insight into the kinetics and hierarchy of responses to hydrostatic pressure stress, and are discussed in the context of generally applicable mechanisms of the stress response and recovery.

**RESULTS****Gene expression analysis**

The relative expression of five 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 an acute hydrostatic pressure treatment for 2 h at 10 MPa (Fig. 1). Expression of *narg* showed significant increases against baseline measurements from immediately after (0 h) up to 3 h after exposure in the head section and up to 6 h after exposure in the abdomen (Fig. 1). The maximal level of expression in the head was reached 3 h after exposure (RFC=7.4), whereas in the abdomen section, maximal expression was observed after 1 h (RFC=12.2). In the head section, there was no significant difference between samples taken 6, 8 or 24 h post exposure in comparison to the unstressed baseline measurements. Similarly, no significant difference was seen 8 h after exposure in the abdomen.

The *actin* gene showed significant, and maximal, expression immediately after experimental exposure in both the head (RFC=7.6) and the abdomen (RFC=2.7) (Fig. 1). The head also showed significant expression of *actin* 1 h after exposure, whereas the concomitant abdomen did not (Fig. 1). There was no significant difference in relative expression of *actin* 3 h after exposure in the head, and 1 h after exposure in the abdomen.

The *gapdh* gene showed significant increases in relative expression at all measured time points from 0 h to 24 h after exposure in the abdomen with maximal expression at 6 h post exposure (RFC=6.1) (Fig. 1). The head showed significant increases in relative expression of *gapdh* at 3 and 6 h post exposure (Fig. 1). However, no significant changes in relative expression were



**Fig. 1. Gene expression in head and abdomen sections of *Palaemonetes varians* after acute pressure exposure.** Change in expression of *narg*, *gapdh*, *hsp70* (form 1 and form 2) and *actin* at 0 h, 1 h, 3 h, 6 h, 8 h and 24 h after exposure to 10 MPa pressure for 2 h. Statistical comparisons were made against a baseline measurement (B) of each gene under non-stress conditions and graphs were scaled against the baseline measurements. \* $P < 0.05$  determined by a GLM and a *post hoc* Tukey-HSD test, calculated using R statistical software. Relative fold change and 95% confidence intervals are shown from five biological replicates.

observed at 0, 1, 8, or 24 h after exposure in comparison to the unstressed baseline measurements.

Two isoforms of HSP70 were tested. The *hsp70* (form 1) gene showed significant increases in the relative expression only at 6 h post exposure in the head (Fig. 1). In the abdomen significant increases in the relative expression of the *hsp70* (form 1) gene were observed between 0 and 6 h post exposure, with maximal expression seen at 1 h post exposure (RFC=6.9) (Fig. 1). The second isoform, *hsp70* (form 2), 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 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 ( $P < 0.025$ ). 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 h after exposure (Table 1). The *actin* gene showed significantly higher relative expression in the abdomen in comparison to the head immediately after exposure, and 1 h post exposure (Table 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 1). The *hsp70* (form 1) gene showed significantly higher expression in the abdomen than in the head up to 1 h post exposure, no significant difference in expression was seen between the anatomical sections at 3 and 6 h post exposure (Table 1). There was no significant difference in the relative expression of the *hsp70* (form 2) gene between the head and abdomen (Table 1).

### Respiration rate analysis

The acute 2 hour hydrostatic pressure exposure significantly ( $P < 0.05$ ) affected the respiratory rate, or oxygen consumption ( $\dot{M}_{O_2}$ ,  $\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ ) of the shrimp (Fig. 2). In comparison to unstressed baseline measurements, oxygen consumption was significantly higher during the exposure period, and also up to 3 h into the recovery period (Fig. 2). After 8 h of recovery, oxygen consumption had returned to the baseline measurement and after 26 h of recovery, oxygen consumption was significantly lower than all other measurement points, including the unstressed baseline measurement (Fig. 2).

### Behavioural analysis

Behavioural data were not analysed statistically because of 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 (Fig. 3) shows that the baseline measurement and recovery time periods have similar patterning. These distributions appear consistent throughout the 2 h analysis in each case. For the baseline measurement and the 1 h, 6 h and 24 h recovery measurements, the majority of shrimp occupied the bottom half of the chamber. Conversely, during the 2 h pressure exposure, the majority of shrimp occupied the top half of the chamber (Fig. 3). Again, this observation is consistent throughout the 2 h exposure period.

Video analysis of behavioural types throughout the experiment show similar patterns between the unstressed baseline measurement (Fig. 4A) and 6 and 24 recovery time periods (Fig. 4F,G). In these cases, the majority of shrimp remained motionless. During the pressure ramp (Fig. 4B) the shrimp showed no obvious changes in behavioural type until around 45 s into the pressure ramp at a pressure 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 pressure exposure (Fig. 4C) and the majority of shrimp showed active movement throughout the pressure exposure (Fig. 4C). During the 1 min 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

**Table 1. A nested comparison (time nested within abdominal section) between the relative fold change of each gene between head and abdomen sections of *Palaemonetes varians***

	<i>narg</i>	<i>gapdh</i>	<i>hsp70</i> (form 1)	<i>hsp70</i> (form 2)	<i>actin</i>
0 h	NS	Abdomen*	Abdomen**	–	Abdomen*
1 h	Abdomen**	Abdomen**	Abdomen*	–	Abdomen*
3 h	NS	Abdomen*	NS	–	–
6 h	NS	Abdomen**	NS	–	–
8 h	–	Abdomen*	–	–	–
24 h	–	Abdomen*	–	–	–
Pooled	NS	Abdomen*	Abdomen*	NS	Abdomen*

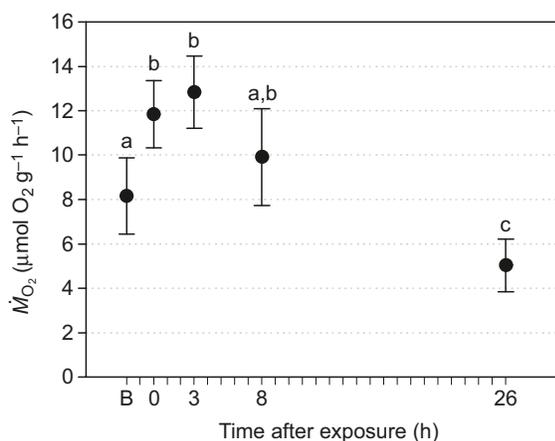
Comparisons were only made at time points that were significantly different from the control baseline treatment (–, no comparison made). Statistical significance was determined by GLM and *post hoc* Tukey HSD test: \* $P < 0.025$ , \*\* $P < 0.01$ ; NS, not significant.

at the end of the decompression (0.1 MPa) (Fig. 4D). A reduction in active movement was observed after 1 h of recovery compared with the exposure period (Fig. 4E) and active movement further decreased with recovery time (Fig. 4E–G).

## DISCUSSION

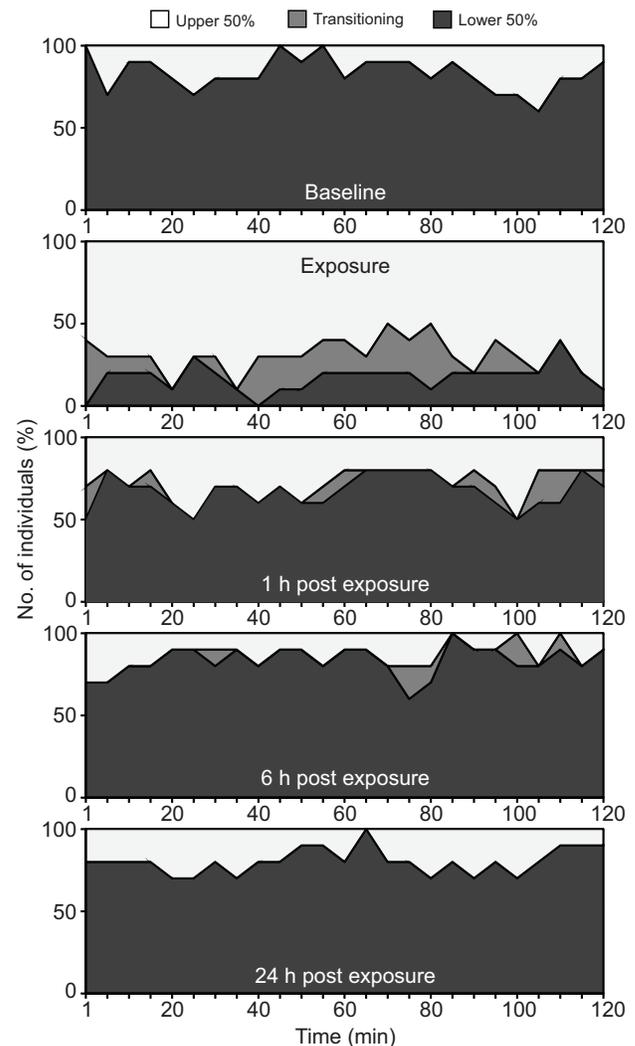
In order to understand the potential for shallow-water ectotherms to undergo climate-driven bathymetric range shifts, a holistic understanding of the wide-ranging and multilevel effects of increased hydrostatic pressure is first required. Therefore this study presents an analysis of the effects of a 2 h acute hydrostatic exposure in a shallow-water shrimp at multiple levels of biological organisation over the exposure period, and during the 26 h recovery phase. Although it is accepted that a 2 h rapid exposure to pressure equivalent to that at ~1000 m depth 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; *narg*, *gapdh*, *hsp70* (form 1) and *actin* were recorded in both the head and abdomen of *Palaemonetes varians* at various time points from 0 to 24 h following exposure to an acute 2 h hydrostatic pressure treatment. A second *hsp70* isoform, *hsp70* (form 2), 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. Furthermore, respiration rates of the shrimp

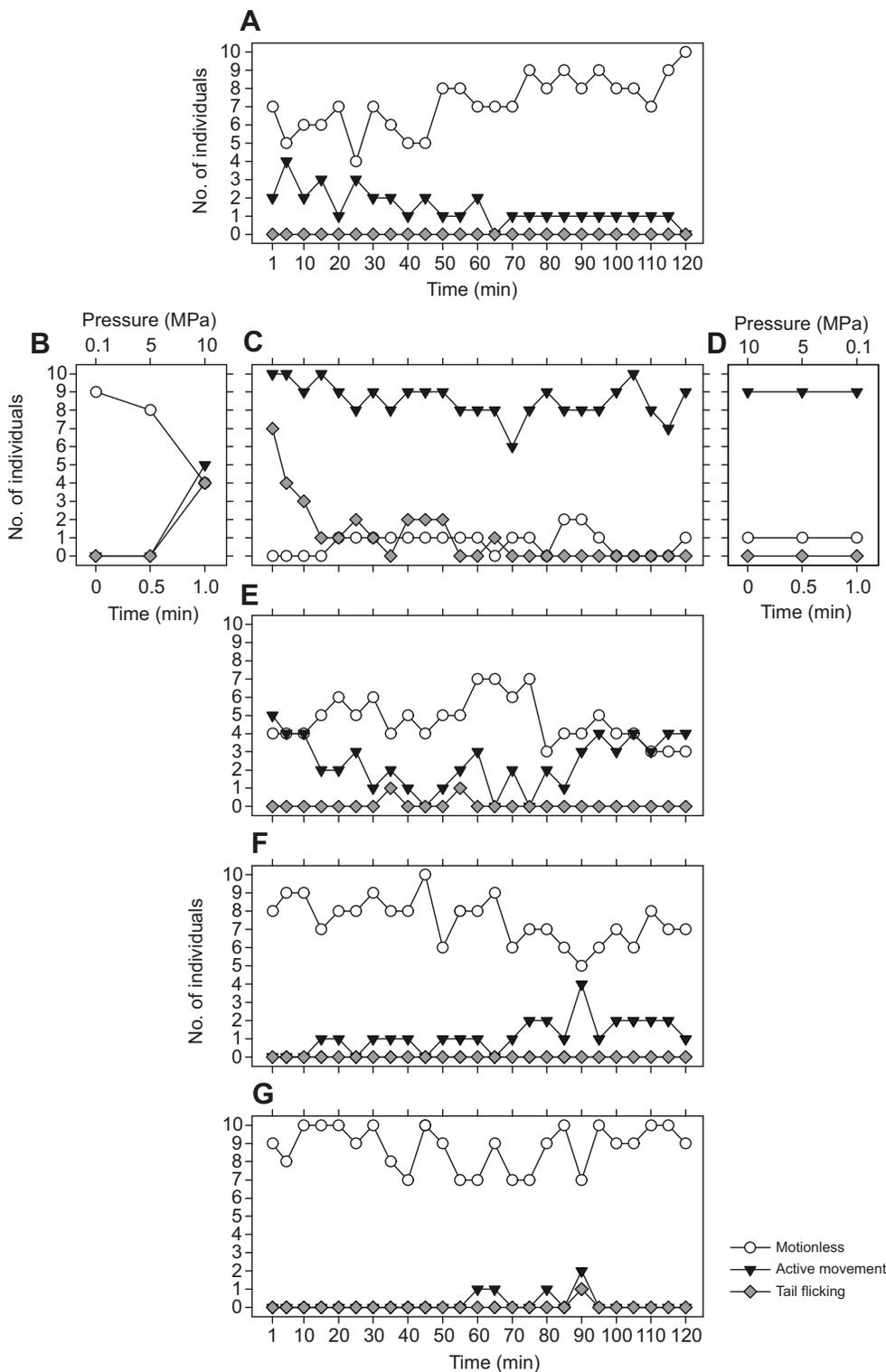


**Fig. 2. Oxygen consumption of *Palaemonetes varians* during and after acute pressure exposure.** Exposure and recovery data at 3, 8 and 26 h are presented alongside a 2 h unstressed baseline measurement of oxygen consumption (B). Statistical comparisons were made across all time periods, and statistically 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 deviation. Oxygen consumption is calculated as the mean and standard deviation of five biological replicates against the mean of three control measurements.

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 pressure



**Fig. 3. Changes in vertical distribution of *Palaemonetes varians* in the high-pressure (IPOCAMP) system during and after acute pressure exposure.** Shrimp were monitored during the 2 h 10 MPa pressure exposure (second panel) and during the recovery period (bottom three panels) and compared with behaviour during a 2 h unstressed (0.1 MPa) baseline observation (top). Vertical distributions are presented as a percentage 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.



**Fig. 4. Changes in behaviour in *Palaemonetes varians* in the high-pressure (IPOCAMP) during and after acute pressure exposure.** (A) Behaviour of 10 shrimp during a 2 h unstressed baseline measurement. (B) 1 min pressure ramp from atmospheric pressure (0.1 MPa) to 10 MPa (rate, 10 MPa min<sup>-1</sup>). (C) 2 h 10 MPa pressure exposure. (D) 1 min decompression from 10 MPa to atmospheric pressure (0.1 MPa). (E) 1 h post exposure. (F) 6 h post exposure. (G) 24 h post exposure. The 'movement' category was omitted to aid visualisation of the other categories, where measurements do not add up to  $N=10$ , 'movement' was observed in the remaining individuals. Specific definitions of each behavioural category can be found in the Materials and methods.

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 OSR upon exposure to a stressor, in this case an acute exposure to elevated pressure. Each distinct response has a differential temporal profile. These can be observed either as a transient contribution to the OSR (behavioural response), or a more enduring contribution (metabolic response). Yet, all are of

importance in understanding the effects of elevated pressure on physiology.

Up-regulation of *narg* with increased hydrostatic pressure has been suggested as a proxy for the onset of physiological pressure intolerances associated with high-pressure neurological syndrome (HPNS) (Morris et al., 2015). The *narg* gene encodes an NMDAR-regulated protein. NMDARs have been shown to become overactive in response to elevated hydrostatic pressure in non-adapted organisms (Rostain et al., 1986; Millan et al., 1989). Periods of

receptor overactivity may lead to the initiation of transcription of new receptors, and thus the up-regulation of associated genes, such as *narg* (Morris et al., 2015). No differences in the levels of relative expression of *narg* were seen between head and abdomen sections. This is unsurprising because 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 the head and abdomen regions of the organism (McLaughlin, 1983). We hypothesise that *narg* gene expression represents a component of the CHR. This represents a shift in the homeostatic point in nervous tissue (receptor overactivity) that may be counteracted by a cellular homeostatic-type response, such as an increase in the transcription of genes associated with NMDARs, in order to produce new receptors to offset existing receptor over-activity, thus restoring homeostasis.

HSP70 (70 kDa heat shock protein) is a commonly used marker of the CSR. An increase in expression of genes encoding 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). Results of this study confirm that the *hsp70* (form 1) gene is responsive to acute hydrostatic pressure stress. The relative fold change of the *hsp70* (form 1) gene shown in this study is lower, however, than observed in previous research in response to a large temperature shock (Cottin et al., 2010; Ravoux et al., 2012). The *hsp70* (form 2) gene showed no changes in expression at any time points, thus in response to pressure stress alone, the gene remains constitutively expressed. The *hsp70* (form 1) gene showed significantly higher expression in the abdomen than in the head. The abdomen was also shown to exhibit higher expression in post exposure samples in comparison to unstressed baseline measurements. Muscle-rich abdominal tissue may therefore exhibit a particularly high degree of intracellular macromolecular damage. Behavioural analysis showed an increase in violent muscle contractions (tail flicking) immediately upon exposure to elevated pressure. This type of muscle contraction is likely to cause muscle cell damage which would trigger the expression of heat shock proteins. In the hierarchy of responses then, a behavioural response, such as tail flicking, is followed by the heat shock response. The expression of HSP70 isoforms has been well documented as being a vital component of the CSR (Feder and Hofmann, 1999; Sørensen 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 (Sørensen 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 are occurring during an acute exposure to pressure, but also, and to a greater magnitude, during recovery from exposure.

The *gapdh* gene encodes an enzyme involved in glycolysis and its up-regulation may signify an increase in cellular metabolism. 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. As expected, up-regulation of *gapdh* occurs over time points covering the up-regulation of genes related to the other aspects of the OSR. Observed behavioural responses immediately upon exposure to elevated pressure will increase the organisms' metabolic demand, and may also play a part in the induction of a heat shock response. Both responses are, therefore, followed by a metabolic response, as seen by the induction of the *gapdh* gene. Although the behavioural response subsides rapidly following exposure, the heat shock response continues into the recovery period and therefore continues to incur a metabolic cost meaning that *gapdh* expression remains elevated.

The actin gene encodes an isoform of the actin protein family which was previously shown to be up-regulated under increased pressure exposures in *P. varians* (Morris et al., 2015). In this study, the actin gene showed the most transient response of all the genes tested, with up-regulation highest during the exposure. Actin isoforms may consist of skeletal muscle actins or cytoskeletal actins. Kim et al. (2009) reported 12 actin isoforms in the American lobster *Homarus americanus*, of which eight were skeletal, one was cardiac and three were cytoskeletal/cytoplasmic isoforms. Although the isoform identity of the actin sequence for *P. varians* is unresolved, it was found to be highly expressed in the muscle-rich tissue of the shrimp's abdominal section and was also expressed in the head section. Maximal expression coincided with high levels of active movement and reflexive muscle contractions during the elevated pressure exposure. Transcriptional regulation of the actin gene may be associated with rapid muscle contractions, such as tail-flicking behaviour. Alternatively, the actins of deep-sea-adapted fish have been shown to differ from shallow-water-adapted fish, with the deep-sea actins showing greater resistance to polymer dissociation under elevated pressure (Swezey and Somero, 1982). It has been hypothesised that increasing hydrostatic pressure causes a loss structure and function in non-pressure-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 increase in transcription. Hydrostatic pressure increases have wide-ranging 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 associated metabolic costs and there may be further-reaching implications on the ability of shallow-water organisms to tolerate and acclimate to deeper waters.

Oxygen consumption measurements show an increase in respiratory rate during pressure exposure and correlate well with the gene expression data, suggesting that the acute pressure 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 h 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 pressure exposure suggests that an oxygen debt is being accrued over the exposure period that is then being recompensed during the recovery phase. Interestingly, 8 h into the recovery period, the oxygen consumption of the shrimp had returned to a similar level to the unstressed baseline measurement, yet at 26 h after exposure, the 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 pressure

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 associated energy costs. Although the data presented here suggest that the oxygen debt accrued during the exposure was repaid within 8 h of recovery, the energy debt may not have been, and thus the drop in metabolic rate at 26 h 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 h exposure, behavioural observations show a clear increase in active movement during the acute exposure, throughout the 2 h period. Active movement remained high immediately following the exposure and slowly decreased over a 3 h 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 pressure ramp and over the first hour of the pressure exposure. This may be a reaction to the pressure increase itself rather than the high pressure or could be a response to pressure above a threshold (observed at around 7.5 MPa). The reduction in tail flicking behaviour may then be explained by the associated energy cost: 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, which was similar for all recovery time periods, but occupy the upper half of the chamber during exposure. This pattern of vertical distribution in response to the pressure exposure may represent a form of behavioural homeostasis (Johnson et al., 1992) in which the shrimp are actively seeking lower pressures (shallower waters) to counteract the negative physiological effects of the increased pressure exposure. Behavioural homeostasis has been shown in marine ectotherms in response to hypoxia (Gorr et al., 2010), whereby organisms actively seek cooler water where oxygen saturation is higher because of the temperature effect. Behavioural homeostasis is a whole-organism response to a stressor such as pressure, which, if successful, will have effects on other aspects of the stress response, thus negating the need for a pronounced CSR.

Our results show that although each quantified response is distinct, they are all inexorably linked. Upon exposure to acute elevated pressure, the first response observed was an increase in overt escape behaviour, such as active movement and tail flicking. Such behaviour is energetically costly and is followed by an increase in oxygen consumption and the transcription of metabolism-related genes. At the same time, elevated pressure 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 pressure 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 h acute elevated pressure exposure can be observed up to at least 26 h into recovery. This observed response will appear to be different depending on the time point at which it is observed, and the aspect of the OSR observed.

Thus, our results highlight the importance of considering a number of aspects of the OSR, as well as a number of time points during and following exposure, in order to properly assess the effects of a given stressor or stress scenario. Elevated pressure acts upon shallow-water-adapted organisms and produces responses from the transient universal CSR and the less-transient 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 we have termed them collectively the OSR. Biomarkers of stress are used exhaustively in studies of organism physiology but in most cases probably only capture one aspect of 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. As a result of the acute nature and high hydrostatic pressure of the exposures tested, we do not suggest that the results presented are of direct ecological relevance. However, we believe that the mechanistic and experimental understanding gained from this study can provide a foundation from which longer-term, more ecologically relevant exposures can be conducted. Furthermore, a recent study conducted on a eurybaric king crab species, *Lithodes maja*, demonstrated comparable gene expression results at pressures outside its natural depth distribution limits (Munro et al., 2015). The comparability of these results suggests that the responses of the shallow-water shrimp *P. varians* can provide a valuable insight into the effects of pressure on deeper-living marine ectotherms. The use of shallow-water species as potential proxies for deep-sea species in physiological studies is important because of the difficulty of collecting, maintaining and conducting experiments on deep-sea species. Future studies should aim to quantify various aspects of the stress response with regards to specific stressors or stress scenarios, and will thus provide a much greater insight into the impacts of single or multiple stressors on an organism.

## MATERIALS AND METHODS

### Maintenance of *Palaemonetes varians*

Adult male *Palaemonetes varians* Leach 1814 (total length, 4–6 cm) were net caught from Lymington salt marshes, UK (50°44′19.85″ N, 1°32′17.54″ W). The shrimp were acclimated to 15±0.5°C from the temperature at which they were caught, at a rate of 1°C per day. They were kept under a 12 h:12 h light:dark cycle in aerated filtered (1 µm) seawater (salinity, 32–34). The shrimp were fed with fish flakes *ad libitum* (TetraMin) three 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 experimental exposure.

### Experimental pressure exposure

The IPOCAMP (Incubateur Pressurisé pour l'Observation et la Culture d'Animaux Marins Profonds) pressurisation system (Autoclave, France) (Shillito et al., 2014) was used to conduct acute 2 h exposure to 10 MPa pressure (0.1 MPa=1 atm). Prior to the treatment, the IPOCAMP system was filled with aerated filtered seawater (salinity, 32–34) and acclimated to 15±0.1°C. Once acclimated, the shrimp were placed in the IPOCAMP, which was then sealed ready for pressurisation. The pressure of the system was ramped from ambient (0.1 MPa) to 10 MPa at 10 MPa min<sup>-1</sup>. The system was then held at 10 MPa and 15°C for 2 h followed by a 1 min depressurisation back to ambient.

### Gene expression analysis

Following experimental exposure, the shrimp were removed and placed back in their original aquarium at 15°C, and kept in the dark. Ten shrimp were randomly sampled at 0 h (within 5 min of the pressure exposure), 1 h, 3 h, 6 h, 8 h and 24 h post treatment. Ten shrimp were also randomly sampled prior to the experimental treatment for a baseline control group

comparison. Shrimp were decapitated: single clean cuts were made vertically from the dorsal extension of the carapace, separating the carapace and the first abdominal segment. Both portions were placed in individual 1.5 ml RNase- and DNase-free microcentrifuge tubes and snap-frozen. Samples were kept at  $-80^{\circ}\text{C}$  until further use.

Tissue samples were transferred to 3 ml of TRI-Reagent (Sigma-Aldrich) and homogenised. Total RNA was extracted according to the manufacturer's standard protocol. Total RNA purity was confirmed using a Nanodrop spectrophotometer (Thermo Fisher Scientific). RNA integrity ( $\text{RQI} \geq 7.5$ ) and concentration was assessed using Experion (Bio-Rad, UK). A volume containing 1.5  $\mu\text{g}$  of total RNA was treated with Promega RQ1 RNase-free DNase (Promega Corporation, Hants, UK) according to the manufacturer's protocol. Total RNA (0.68  $\mu\text{g}$ ) was reverse transcribed in a 20  $\mu\text{l}$  reaction using Superscript III (Invitrogen, UK) and oligo(dT)<sub>20</sub> primers.

Specific primers for qPCR for all genes were obtained from Morris et al. (2015); *narg* (accession number, FR667656), *gapdh* (GQ120565); the *hsp70* isoforms (FJ875280 and FJ875279) and *actin* (FJ654525). All primer-sets tested generated a single and discrete peak by melt curve analysis. Eight 10-fold serial dilutions were performed on a cDNA template to ensure that each primer-set had a qPCR reaction efficiency of between 90–105% and linearity greater than  $r^2=0.98$  across the predicted experimental cDNA concentration range, as set out by the MIQE guidelines (Bustin et al., 2009).

All qPCR reactions were performed on a Stratagene MxPro 3005 (Agilent, UK). Each 25  $\mu\text{l}$  reaction contained 12.5  $\mu\text{l}$  of Precision 2 $\times$  qPCR Master mix (Primer-Design, UK) with SYBR green, and 1  $\mu\text{l}$  of template cDNA. qPCR conditions were: 1 cycle of  $95^{\circ}\text{C}$  for 10 min followed by 40 cycles of [ $95^{\circ}\text{C}$  10 s,  $60^{\circ}\text{C}$  1 min]. 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.

In a pilot study, candidate reference genes were tested by geNorm analysis using qBase+ software (Biogazelle, UK). The combination of the *rps26* gene (Accession number - FR667658) and the *rpl8* gene (Accession number - GQ120564) was deemed to provide the best normalisation strategy for this study, both showing stable expression across experimental conditions. Thus, the geometric mean of the two reference genes was used to normalise gene of interest expression. Normalised relative quantities (NRQs) were calculated using qBase+ software. NRQs were then scaled to the baseline control measurement for each gene giving a relative fold change (RFC) value. 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 (Hothorn et al., 2008). For the anatomical section comparison, a nested GLM was performed followed by a Tukey-HSD test, and statistical significance was determined at a Bonferroni corrected  $P < 0.025$ . SigmaPlot v12.3 was used for graphics production (Systat Software). Non-scaled relative quantity values are available in supplementary material Table S1.

### Respiration rate analysis

Respiration analysis was conducted to quantify the effects of elevated pressure on whole organism metabolism, and validate the changes in the transcription of metabolism related genes. 5 shrimp were placed in sealed individual 55 ml falcon tubes containing 100% air saturated seawater and no air bubbles immediately prior to the experimental exposure and placed horizontally into the IPOCAMP system. Three control tubes containing no shrimp were also sealed to control for bacterial respiration. 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 min following exposure. The shrimp were then returned to 100% air saturated seawater. Further 2 h respiration measurements were taken at 1 h post-exposure, 6 h post-exposure, and 24 h post-exposure. Baseline respiration measurements were also taken by the same method using 5 shrimp and three control tubes not subjected to elevated pressure exposure. Oxygen saturation was determined using an oxygen micro-optode connected to a Microx TX3 array (PreSens, Germany), calibrated according to manufacturer's instructions. Oxygen consumption ( $M_{\text{O}_2}$ ,  $\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ ) was calculated, against controls, following standard methods for determining oxygen concentration in air-saturated seawater

(Benson and Krausse, 1984). 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.

### Behavioural analysis

Behavioural analysis was conducted in order to corroborate any observed changes in gene transcription and metabolism, and provide further insight into the effects of pressure at the whole organism level. Ten shrimp were placed into the IPOCAMP system inside a PVC viewing cage mounted inside the pressure chamber (see Ravaux et al., 2003 and Shillito et al., 2006 for schematic diagram and description). The IPOCAMP system was sealed and run at atmospheric pressure (0.1 MPa) for 1 h 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. Behaviour was recorded for 2 h prior to experimental exposure (baseline measurements); during the 1 min pressure ramp, two hour pressure exposure, and 1 min decompression; and then for 2 h periods at 1 h, 6 h and 24 h post exposure. Behaviour was measured and characterised for 30 s at 5 min 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 s analysis period. 'Active movement' was determined as a directed movement of greater than a single body length over the 30 s time periods. 'Movement' was determined as a visible movement, not necessarily directed, but not of greater than a single body length. '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 not observed in this experiment. The 'movement' category was not included in Fig. 4 to aid the visualisation of the other three behavioural categories. Where the total is less than 10 individuals, the remaining individuals showed movement of less than a full body length. Position of the shrimp vertically was also determined at 5 min intervals. Shrimp were noted as being either in the lower 50% of the chamber for more than 20 of the 30 s time period; in the upper 50% of the chamber for more than 20 of the 30 s time period; or spending less than 20 s either solely in the lower or upper 50% of the chamber, and thus deemed to be 'transitioning'.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

All authors contributed to the conception and design of the study. J.P.M. conducted all experimental work and analysis. The article was written by J.P.M. with input from authors during drafting and revision.

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### Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.125914/-DC1>

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