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The role of ontogeny in physiological tolerance: decreasing hydrostatic pressure tolerance with development in the northern stone crab *Lithodes maja*

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Extant deep-sea invertebrate fauna represent both ancient and recent invasions from shallow-water habitats. Hydrostatic pressure may present a significant physiological challenge to organisms seeking to colonize deeper waters or migrate ontogenetically. Pressure may be a key factor contributing to bottlenecks in the radiation of taxa and potentially drive speciation. Here, we assess shifts in the tolerance of hydrostatic pressure through early ontogeny of the northern stone crab *Lithodes maja*, which occupies a depth range of 4–790 m in the North Atlantic. The zoea I, megalopa and crab I stages were exposed to hydrostatic pressures up to 30.0 MPa (equivalent of 3000 m depth), and the relative fold change of genes putatively coding for the *N*-methyl-D-aspartate receptor-regulated protein 1 (*narg* gene), two heat-shock protein 70 kDa (HSP70) isoforms and mitochondrial Citrate Synthase (*CS* gene) were measured. This study finds a significant increase in the relative expression of the *CS* and *hsp70a* genes with increased hydrostatic pressure in the zoea I stage, and an increase in the relative expression of all genes with increased hydrostatic pressure in the megalopa and crab I stages. Transcriptional responses are corroborated by patterns in respiratory rates in response to hydrostatic pressure in all stages. These results suggest a decrease in the acute high-pressure tolerance limit as ontogeny advances, as reflected by a shift in the hydrostatic pressure at which significant differences are observed.

1. Introduction

Many deep-sea invertebrates are thought to have evolved in shallow habitats and radiated into deep waters (defined as depths below 200 m) [1], although re-emergence into shallow waters is also reported [2,3]. Periods of climate-driven extinction and replacement are thought to define evolution in the deep sea, with evidence for successive cycles of shallow-water taxa invading the deep sea throughout the Phanerozoic [4–6]. Evolutionary patterns may be influenced by global anoxic and dysoxic conditions driving extinction events in this environment [6,7], with the most severe extinction events occurring in the mid-Cretaceous, and at the Permian–Triassic and Ordovician–Silurian boundaries [4]. Some deep-sea taxa appear to have escaped extinction, undergoing extensive *in situ* radiation and diversification [8,9]. Extant deep-sea taxa are therefore thought to consist of both ancient and recent lineages with shallow-water origins.

Benthic deep-sea fauna show distinct zonation patterns, with the highest levels of species turnover found at the upper slope boundary (approx. 1000 m) and at the transition zone between the slope and the abyssal plain (2000–3000 m) [10], indicating biodiversity bottlenecks [11]. Depth is an important predictor of diversity, and unimodal trends in biodiversity at bathyal depths suggest that these depths are the primary site of speciation in the deep sea [12]. The depth-differentiation hypothesis proposes that speciation is encouraged by the large temporal and spatial environmental heterogeneity found at bathyal

depths [13]. While distributional limits may be attributed to a number of biotic and abiotic factors, including competition, topographic complexity and population isolation due to oxygen minimum zones, the physiological effects of low temperature and high pressure are also suggested to play an important role in determining vertical structure, limiting gene flow and driving speciation by promoting a stress-evolution mechanism [5,6,10,12–15]. Studies testing the hyperbaric tolerance of shallow-water invertebrates at ecologically relevant temperatures consistently find evidence for a common hydrostatic pressure threshold at bathyal depths [14].

Hydrostatic pressure affects many levels of biological organization, including behavioural, cellular and molecular responses [14,16]. At a molecular and structural level, pressure is known to affect lipid bilayer fluidity, enzyme processes and protein folding in shallow-water adapted organisms [17–19]. High pressures and low temperatures both cause membrane lipids to become more ordered, moving from a fluid phase to a gel phase [20]. Such changes have knock-on effects at other levels of biological hierarchy. For example, changes in membrane fluidity affect a number of membrane-associated processes, including those involved in cellular signalling [21].

Invertebrate and vertebrate species share a common, generic stress response to multiple potential environmental stressors, known as the cellular stress response (CSR), which includes the activation and upregulation of a set of highly conserved stress proteins that assess and counteract macromolecular damage [22]. In marine systems, transcriptome-based approaches are increasingly being applied to monitor the CSR and apply thresholds for phenotypic plasticity, to assess the capacity for organisms to modify their phenotype to adapt to new environmental conditions [23–25]. Previous investigations of molecular physiological responses to temperature and pressure in invertebrates have focused on generic markers of stress and the CSR members of the heat-shock protein 70 kDa family (HSP70) [26–28], but more recently, this has been extended to novel pressure-specific stress markers [29].

Anomuran crabs of the family Lithodidae represent a case of speciation and range expansion through global cold waters. Lithodids are thought to have evolved in the northeast Pacific from a shallow-water anomuran population in the Cenozoic era (approx. 13–25 Mya), and consist of two subfamilies: the Haplogastrinae and Lithodinae [2,30]. The Haplogastrinae and some Lithodinae radiated within the shallow north Pacific; however, three lithodine genera (*Lithodes*, *Neolithodes* and *Paralomis*) colonized deep water before radiating and extending their distribution into other ocean basins [2]. Bottlenecks in the radiation of the Lithodidae have so far been assigned to lineage-specific thermal tolerances and differences in early life histories among genera [2].

Lithodes maja and *Lithodes santolla*, currently found in the Atlantic, are thought to have originated from among the earliest lithodids to leave the north Pacific [31]. *L. maja* occupies 'intermediate' depths relative to its confamilials (4–790 m), and appears to have extended its bathymetric distribution in order to colonize the north Atlantic [31,32]. As with other lithodines, the larval development of *L. maja* is lecithotrophic and involves morphological and metabolic reorganization during early development, passing through three putatively demersal planktonic zoeal stages and a megalopa stage, which settles and metamorphoses into the juvenile crab I stage (the first feeding stage) [32–35].

This paper investigates the effects of hydrostatic pressure on respiration rate, and the transcription of four genes in three distinct early ontogenetic stages (zoea I, megalopa, crab I) of the northern stone crab, *L. maja*. This study uses traditional markers of the CSR (HSP70 isoforms, *hsp70a*, *hsp70b* genes), a marker of aerobic capacity (Citrate synthase, *CS* gene), and a potential pressure-specific marker (*narg* gene, putatively coding for *N*-methyl-D-aspartate receptor-regulated protein 1) to test the hypothesis that hydrostatic pressure tolerance decreases with increasing ontogenetic age. Members of the HSP70 family are not only involved in protein folding/unfolding, assembly/disassembly and the degradation of misfolded proteins under 'normal' non-stress conditions, but also play an important role in stress resistance [36]. The nuclear *CS* gene encodes the citrate synthase enzyme; CS enzyme activity is considered a correlate of aerobic capacity at a mitochondrial level and is used as an indicator of aerobic metabolism [37–41]. *N*-methyl-D-aspartate (NMDA) receptors are thought to play an important role in the onset of high-pressure neurological syndrome (HPNS) in vertebrates, which is characterized by a series of consistent pathologies, including convulsions [42–44]. HPNS occurs as a result of hyper-excitability of the central nervous system, and studies in rats and the frog *Xenopus laevis* support the hypothesis that it is characterized in part by an increase in the sensitivity of the NMDA receptor [45,46]. By quantifying expression of these genes, we are able to examine the molecular physiological constraints acting on the early life stages of *L. maja*, and the degree to which acute changes in hydrostatic pressure might constrain bathymetric distributions at each ontogenetic stage.

2. Material and methods

(a) Adult sampling and maintenance

Adult specimens of *L. 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). Before relocation to the National Oceanography Centre Southampton (NOCS), UK, the animals were maintained for between 2 and 6 weeks in an open aquarium system (temperature approx. 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 h. 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 h darkness, atmospheric pressure) at NOCS. The maintenance temperature was chosen to match the temperature in the field.

(b) Larval rearing

Larvae were obtained from two gravid females. Individuals used for gene expression studies were obtained from a single female (pre-moult carapace 63.4 mm; post-moult carapace length 70.1 mm) that moulted during the night of 3 April 2012 and oviposited the following day. 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 logistical limit on the number of larvae from different parents that can be reared and maintained simultaneously. The eggs were fertilized by a male with a carapace length of 83.2 mm and were brooded for 328 days. Hatching began in late February 2013 and was extended

over 24 days (2005 larvae total, 83.6 ± 183.4 larvae per day). Individuals used for respiration studies were obtained from a different female (pre-moult carapace length 73.4 mm; post-moult carapace length 81.3 mm) that moulted during the night of 19 June 2012 and oviposited the following day. The eggs were fertilized by a male with carapace length 96.4 mm and were brooded for 327 days. Hatching began in mid-May 2013 and was extended over 18 days (2903 larvae total, 161.3 ± 365.9 larvae per day). Freshly hatched larvae were collected in filters placed within the outflow of the aquarium where the gravid female was isolated. Larvae hatched predominantly at night and consequently larvae were removed each morning, ensuring larval age varied by no more than 24 h. Larvae were isolated individually within 100 ml rearing cups (temperature 6°C, 24 h darkness, atmospheric pressure). Larvae were inspected daily and water changes were made every other day. Morphological changes and the presence of exuvia were used to determine larval stage [32]. The larval development of *L. maja* is lecithotrophic [32] 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 were starved for 3 days prior to starting pressure treatments to avoid molecular contamination from gut contents.

(c) Pressure treatments

Acute pressure exposures were performed on individuals from the zoea I, megalopa and crab I stages, and across a range of different hydrostatic pressures: 0.1–30.0 MPa. Five individuals were used for most pressure treatments, except where numbers were limiting and only four individuals were used—these are highlighted in the results. In separate, yet comparable treatments, oxygen consumption rates (MO_2, μmol O_2 mg^{-1} h^{-1}) were measured to assess respiratory response to acute increases in hydrostatic pressure. Oxygen consumption rates were assessed following an adaptation of established protocols [47,48]. Owing to larval mortality, fewer crab I were available for these treatments; therefore, fewer pressure treatments were carried out: 0.1, 10.0 and 15.0 MPa. Three vials without individuals were used for each pressure treatment to control for microbial respiration within the seawater.

For all treatments, individuals were placed within 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 (Stauff, Germany), which was filled with fresh water [49]. Both the water and the pressure vessel were pre-incubated for 24 h at 6°C. The vessel was then pressurized to the selected pressure using a Maximator M72 manual hydraulic pump: experimental pressure was reached within 10 s [49]. The vessel was kept at constant temperature (6°C) and pressure for 4 h. 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 min of instantaneous depressurization. Samples were stored at −80°C until further analysis. For respiration rate analysis, vials were gently inverted three 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 micro-optode connected to a Microx TX3 array (PreSens, Germany), calibrated according to manufacturer's instructions. 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.

(d) Respiratory rate measurements and statistical analysis

Oxygen consumption 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 [50]. Respiration rates were normalized according to biomass (dry mass), determined by freeze drying and subsequently weighing the samples.

The respiratory responses of each ontogenetic stage to pressure treatments were evaluated independently, as fewer treatments were carried out for crab I compared with 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; $p > 0.05$).

(e) RNA extraction and reverse transcription

Whole individuals were transferred from −80°C storage to liquid nitrogen and then to a 5 ml tube containing 2.2 ml TRI-reagent (Sigma-Aldrich, UK), were homogenized, and total RNA was extracted following the manufacturer's protocol. Total RNA was assessed for purity and integrity using a Nanodrop spectrophotometer (Thermo Fischer Scientific) and Experion (Bio-Rad), respectively. Subsequently, total RNA was DNase-treated using Promega RQ1 RNase-free DNase (Promega, UK) following the manufacturer's protocol, and 0.68 μl of DNase-treated total RNA was reverse-transcribed in a 20 μl reaction using Superscript III (Invitrogen, UK) and oligo $(dT)_{23}$ primers according to the manufacturer's protocol.

(f) Primer design

Degenerate PCR-based gene hunting techniques were used to generate *L. maja*-specific gene sequences for seven genes: the *narg* gene, *CS* gene, two *hsp70* isoforms, and three candidate reference genes, *tubulin α1*, *eef1a* and *rpl8*. Reference genes are used as internal controls to correct for differences in starting cDNA template quantities in quantitative PCR (qPCR) [51]. 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 ClustalW v. 2.0.12 [52]. Regions of the putative target genes were amplified by PCR reactions using the degenerate primers and *L. maja* template cDNA. The resulting amplicons were gel extracted and cloned using the pGem-T easy vector kit (Promega, UK). Colonies with the appropriate insert were grown, plasmids were extracted and sequences were determined using conventional dideoxy-termination sequencing (Source BioScience, UK). Primers for use in qPCR were designed from the determined *L. maja* gene sequences (electronic supplementary material S1) using standard criteria and Primer Express software (Applied Biosystems, USA).

(g) Quantitative PCR

Quantitative PCR (qPCR) combines simultaneous amplification and fluorescent detection, enabling accurate quantification of expression levels within the samples. To ensure primer specificity and efficiency, each qPCR primer pair was optimized in order to determine the optimum primer concentrations and reaction efficiencies, ensuring 90–105% efficiency across the predicted cDNA concentration range (electronic supplementary material S2) [51,53]. A melt curve analysis was also applied to ensure that all samples produced a single distinct product.

All reactions were carried out on the Mx3005P qPCR system (Agilent, UK). Each 25 μl reaction contained 12.5 μl Precision 2× qPCR Master mix (Primer-Design, UK) containing SYBR green fluorescent dye, 1 μl (approx. 34 ng) of template cDNA and 0.5 μl ROX Reference Dye (Invitrogen, UK). Two technical replicates were run for each sample, in addition to negative controls for every gene. Standard qPCR conditions were applied (electronic supplementary material S1).

Quantification cycle (Cq) values were analysed using qBase+ software (Biogazelle, UK [38]). The Cq values for each of the genes of interest (*narg*, *CS*, *hsp70a* and *hsp70b* genes) were normalized against the best reference genes (*eef1a* and *rpl8* genes, determined using the geNorm function within the qBase+ software [54]). These normalized relative quantities were scaled relative to the 0.1 MPa atmospheric control treatment for each gene and are therefore presented as relative fold change (RFC) values. Comparisons between 0.1, 10.0, 20.0, 25.0 and 30.0 MPa treatments within each life stage were made by one-way ANOVA in conjunction with a *post hoc* Tukey test.

3. Results

(a) Sequences identified by gene hunting

Six consensus sequence fragments were determined in *L. maja* by degenerate PCR-based gene hunting, producing significant BLAST hits. These sequences were deposited in the EMBL-EBI database. A 947 bp fragment (accession no. LN713467) was translated as a putative elongation factor 1-alpha (*eef1α* gene) that shared a 96% amino acid identity (query coverage = 99%, *E*-value = 0.0) with the crab *Macrophthalmus japonicas* (electronic supplementary material S3*a*). A 449 bp fragment (LN713464) was translated as a putative ribosomal protein L8 (*rpl8* gene), and shared a 94% amino acid identity (query coverage = 99%, *E*-value = 4×10^{-98}) with the shrimp *Litopenaeus vannamei* (electronic supplementary material S3*b*). A 1206 bp fragment (LN713465) was translated as a putative NMDAR-regulated protein 1 (NARP 1) (*narg*) that shared a 62% amino acid identity (query coverage = 100%, *E*-value = 1×10^{-179}) with the termite *Zootermopsis nevadensis* (electronic supplementary material S3*c*). This amino acid sequence was also identified as containing a predicted NARP 1 domain (Pfam-A domain prediction). Two fragments, both 485 bp in length, were translated as putative 70 kDa heat-shock protein (*hsp70*) isoforms. The first fragment (LN713468), named *hsp70a*, shared a 93% amino acid identity (query coverage = 84%, *E*-value = 1×10^{-86}) with the crab *Goniopsis cruentata* (electronic supplementary material S3*d*). The second fragment (LN713469), named *hsp70b*, shared an 88% amino acid identity (query coverage = 84%, *E*-value = 2×10^{-77}) with the crab *Cancer pagurus* (electronic supplementary material S3*e*). The nucleotide and translated amino acid sequences for both putative *hsp70* isoforms were aligned and showed distinctiveness (electronic supplementary material S4). Their identity as distinct isoforms of the *hsp70* gene was further confirmed by expression analysis, where each isoform showed differential expression patterns (described below). Finally, a 423 bp fragment (accession no. LN713466) was translated as a putative citrate synthase protein (*CS*) that shared 86% amino acid identity (coverage = 99%, *E*-value = 8×10^{-84}) with the beetle *Dendroctonus ponderosae* (electronic supplementary material S3*f*).

(b) Pressure effect on respiration rate

Hydrostatic pressure significantly affected respiratory rate (MO_2, μmol O_2 mg^{-1} h^{-1}), for all ontogenetic stages (figure 1). The hydrostatic pressure at which significant decreases in oxygen consumption were first observed shifted with increasing ontogenetic age, with significant decrease at 30.0 MPa in zoea I stage ($F_{6,28} = 8.555$, $p < 0.001$), at 20.0 MPa in the megalopa stage ($F_{6,28} = 31.157$, $p < 0.001$) and at 15 MPa in the crab I stage ($F_{2,12} = 72.431$, $p < 0.001$).

(c) Pressure effect on relative gene expression

The effect of hydrostatic pressure on the relative transcription of all genes was dependent on ontogenetic stage (figure 1). In the zoea I stage, there were no significant RFCs of the *narg* gene or the *hsp70b* gene, but both the *CS* gene and *hsp70a* gene showed a significant fold increase at 30.0 MPa in the zoea I stage (RFC 3.3, $p < 0.05$; 16.4, $p < 0.01$). In the megalopa stage, significant differences were seen at 25.0 and 30.0 MPa in the *narg* gene (RFC 5.6 and 4.6, respectively, $p < 0.001$), the CS gene (RFC 2.4 and 3.8, respectively, $p < 0.05$) and the *hsp70a* gene (RFC 3.1 and 12.8, respectively, $p < 0.01$); there was also a significant fold increase of the *hsp70b* gene at 30.0 MPa (RFC 7.0, $p < 0.01$). Finally, in the crab I stage, significant fold increases were seen at 25.0 and 30.0 MPa in the *narg* gene (RFC 2.8 and 3.6, $p < 0.01$) and the *hsp70b* gene (RFC 4.3 and 8.7, $p < 0.01$), and increases at 20.0, 25.0 and 30.0 MPa in the *CS* gene (RFC 3.8, 6.1 and 8.0, respectively, $p < 0.05$) and the *hsp70a* gene (RFC 7.7, 59.7 and 73.1, respectively, $p < 0.01$).

4. Discussion

This study is the first to provide patterns in transcriptional responses to hydrostatic pressure in a continental slope-depth-adapted species, demonstrating a decrease in the acute pressure tolerance limit as ontogeny advances. This presents 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, and may also be useful for the reconstruction of physiological bottlenecks in the radiation history of species.

In this study, all three early ontogenetic stages of *L. maja* are able to tolerate exposure to pressures beyond that of their adult depth distribution (4–790 m); however, the pressure at which respiratory and transcriptional pressure effects are first observed decreases between the zoea I, megalopa and crab I stages. These results are consistent with a number of studies that have examined the effects of pressure on the embryonic and larval forms of several shallow-water benthic invertebrates. These studies reveal some general trends in ontogenetic pressure tolerance shifts: initial increases in tolerance from early embryonic to early larval forms are followed by subsequent decreases in tolerance in later life-history stages [55]. Without exception, all stages tolerated pressures greater than those found in the adult depth range [14].

Respiratory responses observed in this study support the observation of an ontogenetic shift in the upper hydrostatic pressure tolerance in *L. maja*. Increases in pressure above acclimation pressure typically result in an increase in oxygen consumption, followed by a subsequent decline or inhibition of oxygen consumption [11,47,56–58]. As with aerobic thermal windows, acute external increases in hydrostatic pressure beyond the 'optimum range' are proposed to lead to increases in homeostatic effort, and a corresponding increase in mitochondrial activity [11,58,59]. In the absence of adaptations to increased hydrostatic pressure, impaired membrane and enzyme functionality at critical upper

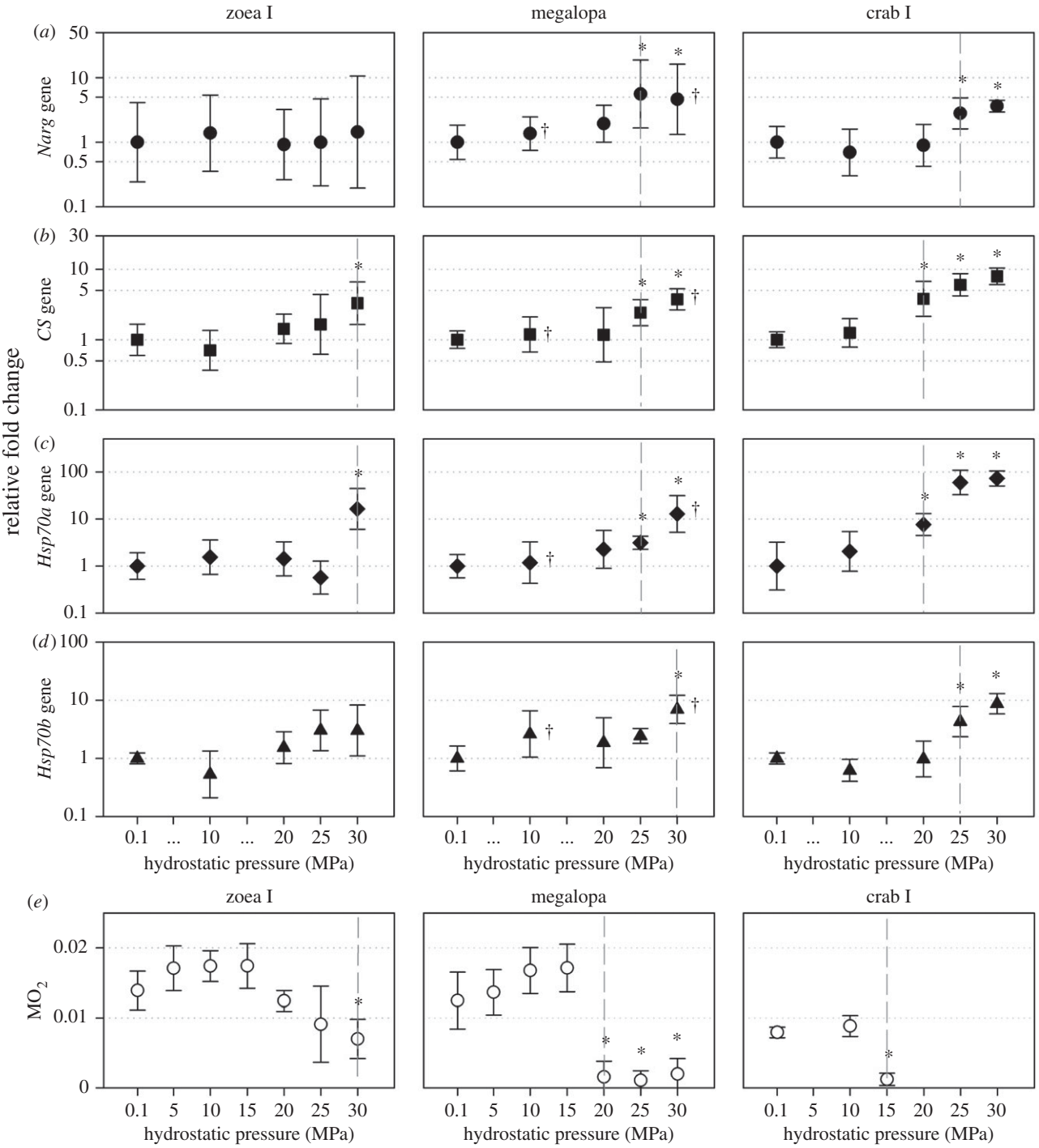

Figure 1. The northern stone crab *Lithodes maja*. Relative fold changes (RFCs) and 95% CIs of four genes. (*a*) The *narg* gene (circles); (*b*) the *CS* gene (squares); (*c*) the *hsp70a* gene (diamonds); (*d*) the *hsp70b* gene (triangles); and (*e*) the rate of oxygen consumption with SD (MO_2, μmol 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). RFCs scaled to the 0.1 MPa control treatment. Statistical significance is displayed as $*p < 0.05$, calculated from five biological replicates unless otherwise stated. †Four biological replicates. Vertical dashed lines visually emphasize the hydrostatic pressure at which significant differences are first observed.

pressures may lead to reduced metabolism, and a mismatch between oxygen demand and oxygen supply, resulting in a shift from aerobic to anaerobic metabolism [11,57,59,60]. While tolerable in the short term, long-term survival in this state is not possible [61]. This change in mitochondrial activity in response to hydrostatic pressure may be reflected in the expression of the citrate synthase (*CS*) gene.

Acute exposure to elevated hydrostatic pressure also affected transcription of two known members of the CSR, 70 kDa heat-shock protein isoforms *hsp70a* and *hsp70b* [62]. These results are consistent with previous studies demonstrating increased transcription of genes coding for heat-shock proteins in response to the effects of high hydrostatic pressure and/or low temperature [26,27,29]. Increases in hydrostatic pressure have long been known to affect protein folding, impacting quaternary, tertiary and secondary structure [63]. The upregulation of two HSP70 isoforms provides additional evidence for macromolecular damage in *L. maja* in response to elevated hydrostatic pressure.

Further evidence of cellular damage may be inferred by the upregulation of the *narg* gene, putatively coding for NMDA receptor-regulated protein 1. It is proposed that

when approaching critical pressure limits, neuronal cell death leads to an upregulation of genes associated with NMDA receptor activity [29]. Significant upregulation of the *narg* gene in response to increases in hydrostatic pressure in the temperate shallow-water shrimp *Palaemonetes varians* may be an indicator for the onset of pressure intolerances associated with neuronal tissue sensitivity [29]. This upregulation is proposed to be a precursor to behavioural signs of pressure intolerance such as 'loss of equilibrium' (where the shrimp are observed lying motionless on their side or dorsal surface for more than 2 s) [29,57]. However, the sequence of transcriptional and respiratory responses identified in *L. maja* in all stages (significant decrease in respiration rate prior to *narg* transcriptional response) contrasts with the sequence in the shallow-water shrimp *P. varians* at acclimation temperature, where the *narg* transcriptional response in stage 3 juveniles precedes the significant decrease in respiration rate observed in adult shrimp (cf. [29,57]). Although this contrast suggests functional nervous pressure adaptation in *L. maja*, the evolutionary and ecological distances between these taxa prevent definitive conclusion. Regardless, this study supports the hypothesis that hydrostatic pressure affects critical biological processes, and potentially imposes a lower bathymetric limit beyond which adaptations are required [11,26,27,29,57,58,64–66].

Aerobic thermal tolerance windows have been shown to change throughout ontogeny in fish. Tolerance windows initially increase from the early embryonic stages to the juvenile stage, as diffusion across the cell surface is replaced by a ventilation and circulatory system [67]. Subsequently, thermal tolerance windows narrow with increasing age and metabolic demands [67]. Theories of thermal tolerance are often extended to hydrostatic pressure tolerance, because the effects of high pressure and low temperature are thought to be analogous [11,64]. A narrowing of pressure tolerance through ontogeny in *L. maja* may be associated with a number of morphological and functional changes. Many crustacean larvae experience a shift in cardiac function through early development. For example, in the shrimp *Metapenaeus ensis* and isopod *Ligia oceanica*, a transition from myogenic to neurogenic cardiac control has been observed [68,69]. In the king crab *L. santolla*, the cardiac region becomes delineated from the megalopa to the juvenile [70], which may coincide with a shift in cardiac function [71]. The gastric region also becomes delineated from the megalopa to the juvenile in *L. santolla* [70], reflecting the transition from endotrophy to exotrophy, which is associated with an increase in the activities of digestive enzymes [72]. In *L. maja*, there are significant differences in biomass, oxygen consumption and chemical composition between the zoea, megalopa and juvenile stages [32].

The three deep-sea lithodid genera have distinct generalized lower depth limits: *Lithodes* has the shallowest distribution (approx. 200–1250 m, 2.1–12.6 MPa), *Paralomis* occupies intermediate depths (approx. 200–2000 m, 2.1–20.2 MPa) and *Neolithodes* has the deepest distribution (approx. 2000–3500 m, 20.2–35.2 MPa) [31]. In this study, all three life stages of *L. maja* were able to tolerate acute pressure conditions beyond the equivalent pressures of the known adult distribution (4–790 m, approx. 0.14–8.0 MPa). Respiration rate measurements for the crab I stage indicate that oxygen consumption is significantly reduced at 15 MPa (equivalent to approx. 1500 m), the first of the examined pressures to fall beyond the maximum observed depth of the genus *Lithodes* (approx. 1250 m, 12.6 MPa) [31]. While these data are an important first step towards fully understanding the role of pressure tolerance in the ecology and evolution of marine taxa, it is not possible to fully determine the role of pressure limitation in *L. maja* without knowledge of adult tolerance limits, and without a sustained pressure treatment to assess the long-term effects of pressure and potential acclimation. To date, such long-term studies are completely lacking from the literature, mostly because of the lack of adequate technology. Available studies have largely focused on acute pressure exposures ranging from seconds to few hours, and rarely extend to more than a few days (reviewed in [14]). This study provides an important bridge for longer-term exposure studies in adults, and provides an indication of the short-term role of hydrostatic pressure limitation in early ontogenetic stages.

Hydrostatic pressure tolerance may affect ontogenetic migration patterns in lithodid crabs. While little is known about the ecology of the early ontogeny of *L. maja*, there is evidence of ontogenetic migration in *Lithodes ferox*. There is almost no overlap between the bathymetric distribution of adults and juveniles, and juvenile *L. ferox* are almost exclusively found in deeper waters than adults [73]. Although the sub-Antarctic king crab *L. santolla* displays the opposite bathymetric distribution, this pattern may relate to the specific behavioural ecology of this species: juveniles form dense aggregations around shallow kelp holdfasts at depths of 3–5 m [74]. In *Paralithodes camtschaticus*, seasonal migration into shallow waters has been observed and is related to larval release, a migratory process likely to be driven by planktotrophic larval development [75].

In summary, we demonstrate a shift in the short-term, acute high-pressure tolerance limit between the zoea I, megalopa and crab I stages of *L. maja* as evidenced from changes in gene expression and aerobic metabolism. While further studies on the effects of pressure at every level of biological organization, and through the full life cycle, are necessary to understand better the mechanisms underlying these physiological limits, this study provides the first indication of the molecular response to hydrostatic pressure in the early ontogeny of a continental slope-depth-adapted species, and indicates that bathymetric distribution may become more constrained as ontogeny advances.

Data accessibility. DNA sequences: EMBL-EBI/GenBank accessions LN713464 to LN713469. Sequence alignments, qPCR assay efficiencies, and primer sequences for each gene listed in this article have been uploaded as part of electronic supplementary material. Clustal Omega alignment files are available from the Dryad data repository system (http://dx.doi.org/10.5061/dryad.d6t40).

Authors' contributions. J.P.M., S.T., A.B. and C.H. conceived the work; C.M. and A.B. reared the larvae; C.M. and J.P.M. carried out the molecular laboratory work; A.B. carried out respiratory rate measurements; C.M. drafted the manuscript; all authors contributed to manuscript writing.

Competing interests. We declare we have no competing interests.

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