The effect of high hydrostatic pressure acclimation on acute temperature tolerance and phospholipid fatty acid composition in the shallow-water shrimp *Palaemon varians*

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ABSTRACT

Extant deep-sea fauna, including hydrothermal vent endemics such as bresiliid shrimp, are descended from shallow-water ancestors. Previous studies have demonstrated the capacity of shallow-water shrimp to acclimate to hydrostatic pressure representative of the vent environment. It has been proposed that this hyperbaric acclimation depends in part on shifts in phospholipid fatty acid composition to maintain biomembrane function. These shifts are also predicted to reduce critical temperature tolerance, potentially limiting the possibility of direct colonisation of the hydrothermal vent environment. Here, we present evidence that acclimation to high hydrostatic pressure (10 MPa ≈ 1000 m water depth) decreases acute temperature tolerance from 30.2 °C to 27.1 °C in the shallow-water shrimp *Palaemon varians* acclimated to 10 °C. Statistically significant shifts in phospholipid fatty acid composition occurred during exposure to high hydrostatic pressure, suggesting that homeoviscous modifications support shifts in environmental tolerances during hyperbaric acclimation. Despite the reduction in temperature tolerance, *P. varians* retains sufficient thermal scope to tolerate the thermal regime in the hydrothermal vent environment, allowing for the possibility of direct deep-sea hydrothermal vent colonisation by shallow-water shrimp.

1. Introduction

Extant deep-sea faunas are descended from shallow-water ancestors (see Brown and Thatje, 2014). Understanding the physiological adaptations that allowed shallow-water organisms to repeatedly colonise diverse deep-sea environments and establish the present high biodiversity is of fundamental interest to deep-sea biologists and ecologists. Deep-sea hydrothermal vents are of particular interest due to putatively extreme environment conditions present (high hydrostatic pressure, high temperature variability, high concentrations of potentially toxic chemical species). Alvinocarid hydrothermal vent shrimp represent key elements of hydrothermal vent communities in the Mid-Atlantic Ridge, and in the Pacific and Indian Oceans (Lunina and Vereshchaka, 2014). These shrimp appear to have originated in the Late Eocene/Early Oligocene (~35 Ma), arising from non-hydrothermal vent deep-sea shrimp, which themselves arose from shallow-water shrimp (Sun et al., 2018b). This shallow-water ancestry suggests that alvinocarid shrimp and shallow-water shrimp are appropriate for comparative studies, through which valuable understanding can be gained of physiological

adaptations to deep-sea hydrothermal vent conditions.

Alvinocarid shrimp demonstrate varying degrees of adaptation to hydrothermal conditions with adaptations ranging from few and inconspicuous, to numerous and prominent (Lunina and Vereshchaka, 2014). For example, *Alvinocaris* spp. appear similar to non-vent deep-sea shrimp. In contrast, *Mirocaris* spp. and *Rimicaris* spp. display conspicuous morphological and ecological adaptations (Lunina and Vereshchaka, 2014), such as the dorsal thoracic modified compound eye in *Rimicaris* spp. which putatively detects light emitted by hydrothermal vents (O'Neill et al., 1995). Hydrothermal vent taxa are typically considered models of extreme temperature adaptation. For example, the thermal optimum of the Pompeii worm *Alvinella pompejana* exceeds 42 °C (Ravaux et al., 2013). However, hydrothermal vent shrimp do not appear to demonstrate higher maximum critical temperatures than shallow-water shrimp (Shillito et al., 2006; Mestre et al., 2015) despite occupying what is putatively the most extreme thermal marine environment. For example, the critical thermal maximum reported for *M. fortunata* matches the modelled critical thermal maximum of shallow-water shrimp acclimated to 19 °C (Mestre et al., 2015),

M. fortunata's preferred temperature (Smith et al., 2013). Yet comparisons between critical thermal tolerance of deep-sea and shallow-water shrimp must be treated with caution. Assessments of both hydrothermal-vent and shallow-water shrimp species temperature tolerances have been made at native hydrostatic pressures only. Thus, the potential influence of hydrostatic pressure adaptation on temperature tolerance remains unexplored.

The physiological effects of high hydrostatic pressure present a significant impediment to colonisation of the deep sea by shallow-water crustaceans (Brown and Thatje, 2014, 2015). Deep-sea hydrothermal-vent shrimp appear adapted to high hydrostatic pressure. The bathymetric distribution of hydrothermal-vent shrimp extends beyond the hydrostatic pressure tolerance of shallow-water shrimp at *in situ* temperatures (Oliphant et al., 2011; New et al., 2014) and some hydrothermal-vent shrimp species are thought to be excluded from shallow vent fields by relatively low hydrostatic pressure (Yahagi et al., 2015). Even deep-sea hydrothermal-vent shrimp that survive at surface pressures may be adversely affected by these conditions. For example, metabolic rate in *M. fortunata* is depressed following depressurisation of shrimp sampled at 1617 m depth (Shillito et al., 2006). Pressure-related shifts in *M. fortunata*'s metabolic rate are not transient or overcome by acclimation to surface pressure: metabolic rate in *M. fortunata* declines further with sustained exposure to surface pressure [cf. Shillito et al., 2006, Smith et al., 2013). The effects of sustained low pressure exposure may even contribute to apparent metabolic adaptations to the acute spatial and temporal scale of temperature changes inherent in the vent environment such as reduced metabolic sensitivity to temperature reported in *M. fortunata* (Smith et al., 2013).

In contrast, acclimation to high hydrostatic pressure increases acute hydrostatic pressure tolerance in the shallow-water shrimp *Palaemon varians* (New et al., 2014). New et al. (2014) suggest that this may be due to homeoviscous modifications in cell membrane lipid bilayers. Adjustment to cell membrane phospholipid fatty acid composition (homeoviscous acclimation) is crucial to maintaining the function of cell membranes: phospholipid fatty acid composition modulates membrane fluidity, determining membrane permeability and affecting mobility and function of cell membrane proteins (Guschina and Harwood, 2006). Both hydrostatic pressure and temperature affect the fluidity of cell membranes and thus membrane functioning (Hazel and Williams, 1990; Hazel, 1995). Typical responses to increased pressure or decreased temperature include increasing the proportion of unsaturated fatty acids in cell membranes, which maintains membrane fluidity (Guschina and Harwood, 2006; Hazel and Williams, 1990; Hazel, 1995). Such modifications do not occur in the very short term during cold acclimation (e.g. 24 h; Rongers et al., 2012), but can occur over slightly longer time scales (e.g. 72 h; Waagner et al., 2013). Unsaturation of phospholipid-derived fatty acids in response to high hydrostatic pressure may result in reduced acute thermal tolerance, but whether this is the case, and whether shifts in environmental tolerances during hydrostatic pressure acclimation are dependent on homeoviscous acclimation has yet to be studied. Identifying the effects of acclimation to high hydrostatic pressure on acute temperature tolerance and phospholipid fatty acid composition in a shallow-water shrimp may resolve this uncertainty, whilst also offering insight into the possibility of direct colonisation of deep-sea hydrothermal vents by shallow-water shrimp.

The shallow-water species *Palaemon varians* has been used as a comparator for deep-living shrimps (Gonzalez-Rey et al., 2006, 2007; Smith et al., 2013; Brown et al., 2017a). *P. varians* (recently identified as the senior synonym of *Palaemonetes varians*; De Grave and Ashelby, 2013), a temperate brackish-water shrimp, has emerged as a model species for hyperbaric and thermal stress physiology and ecotoxicology through experiments assessing behavioural, respiratory, and molecular responses to acute and sustained temperature, hydrostatic pressure, and toxic metal exposures (Cottin et al., 2010; Oliphant et al., 2011; Cottin et al., 2012; Ravaux et al., 2012; Smith et al., 2013; New et al., 2014; Morris et al., 2015a,b,c; Brown et al., 2017a; Brown and Hauton, 2018).

Experimentation has revealed that temperature regimes tolerated by *P. varians* are comparable to those of hydrothermal vent shrimp, and the acute temperature tolerance of palaemonid (including *P. varians*) and hydrothermal vent shrimp are similar at their native hydrostatic pressures (Ravaux et al., 2003; Shillito et al., 2006; Oliphant et al., 2011; Ravaux et al., 2012; Mestre et al., 2015). Further, *P. varians*' sustained hyperbaric tolerance extends to the depth of some hydrothermal vents inhabited by vent shrimp (New et al., 2014; Cottin et al., 2012; Morris et al., 2015a). *P. varians* therefore appears a suitable comparator for hydrothermal vent shrimp. Here, we report on the effects of high hydrostatic pressure acclimation on critical thermal tolerance and cell membrane lipid bilayer composition in the shallow-water palaemonid shrimp *Palaemon varians*.

2. Materials and methods

2.1. Capture and maintenance of Palaemon varians

Approximately 130 adult *Palaemon varians* were collected from Lymington salt marshes (Hampshire, UK) on the 4th of February 2014. Sampling was carried out using a hand net towed near ditch margins. Shrimp were transferred to the National Oceanographic Centre Southampton (NOCS) in 10 l buckets containing water from the collection site. At the NOCS, shrimp were transferred to 10 l aquaria containing 8.5 °C and 25 salinity seawater aerated via bubble stones attached to air pumps. Aquaria were then placed in temperature-controlled conditions at 10.0 °C with 12:12 photoperiod. Shrimp were fed with Tetra Goldfish flakes to excess three times per week. Water changes were conducted three times per week on alternate days to feeding (50% water change; 10.0 °C, 32.4 salinity, 1 μm-, UV-filtered seawater). Animals were maintained in captivity for at least 4 weeks before experimental exposures.

2.2. Effect of pressure acclimation on critical thermal maximum (CT_{max})

Both thermal and hyperbaric tolerance typically decrease during ontogeny (Mestre et al., 2013), including in crustaceans (e.g., Munro et al., 2015; Brown et al., 2017b). Consequently, experimental approaches focused on adults. To reduce potential variability in response resulting from differences in digestive state, shrimp were starved for 3 days before experimentation.

To determine critical thermal maximum (CT_{max}) three replicate treatments were performed. In each replicate treatment ten shrimp were transferred from aquaria in the temperature-controlled room into a PVC cage with an inclined lid within the high pressure flow-through IPOCAMP system run at experimental temperature for at least 1.5 h (Ravaux et al., 2003; Shillito et al., 2001, 2006). Shrimp were then maintained in the IPOCAMP for 1 h before initiating treatments to allow shrimp to recover from handling stress.

Temperature and pressure within the IPOCAMP system were recorded throughout treatments using a temperature/pressure data logger (SP2T4000, NKE instrumentation). For control treatments, the shrimp were maintained within the IPOCAMP system at atmospheric pressure (0.1 MPa) for 7 days (168 h) before assessment of CT_{max}. For experimental treatments, pressure was increased from 0.1 MPa to 10 MPa stepwise by 1 MPa 5 min^{-1} and then maintained for 7 days (168 h) before assessment of CT_{max}. On days 3 and 5 of the treatment a 10 l water change (10.0 °C, 32.4 salinity, 1 μm-, UV-filtered seawater) of the unpressurised 40 l reservoir tank was performed, without affecting hydrostatic pressure within the pressurised component of the system. No mortalities were observed during the 168-h acclimation period in control or experimental treatments. In both control and experimental treatments, temperature within the IPOCAMP system was increased at a constant rate by 0.26 °C min^{-1} following the 168-h acclimation period.

A video camera attached to an endoscope and connected to a digital video recorder was used to record shrimp behaviour. The endoscope

was inserted into the central viewing port in the IPOCAMP lid. Illumination was provided by cold lights inserted into other viewing ports in the IPOCAMP lid. Video recording began 15 min before the start of the temperature increase. The behaviour of each individual shrimp was assessed though video-analysis for the 15 s before and after the temperature within the system reached each 1 °C increment. The behaviour of each individual shrimp was categorised (following Ravaux et al., 2003; Shillito et al., 2006; Oliphant et al., 2011; New et al., 2014; Morris et al., 2015c; Pallareti et al., 2018) as:

1. Movement – detectable movement of any kind except Active Movement (see below): pereopod or pleopod movements; scaphognathite beating; antennal lateral sweeping; cleaning of mouth parts by rubbing them along each other.
2. Active Movement (AM) – walking or swimming a distance greater than own body length in less than 30s.
3. Motionless – no detectable movement at normal tape-reading speed.
4. Loss of Equilibrium (LoE) – resting on the bottom in either a 'sideways' or 'upside-down' position for > 2 s.

LoE could co-occur with any other behaviour, but Movement, Active Movement, and Motionless were mutually exclusive. LoE was interpreted as an indicator behaviour of loss of function and AM was interpreted as an indicator behaviour of stress (following Ravaux et al., 2003; Shillito et al., 2006; Oliphant et al., 2011; Morris et al., 2015c; Pallareti et al., 2018).

2.2.1. Statistical analysis

A logistic regression was used to model the LoE response to increasing temperature and predict CT_{max}, assuming a logistic distribution based on the sigmoidal data pattern (Fig. 1). Statistical analysis was performed using R and RStudio software (v. 3.0.2 and v. 0.98.501; R Core Team, 2013). CT_{max} was assessed as the temperature at which 50% of shrimp experienced LoE.

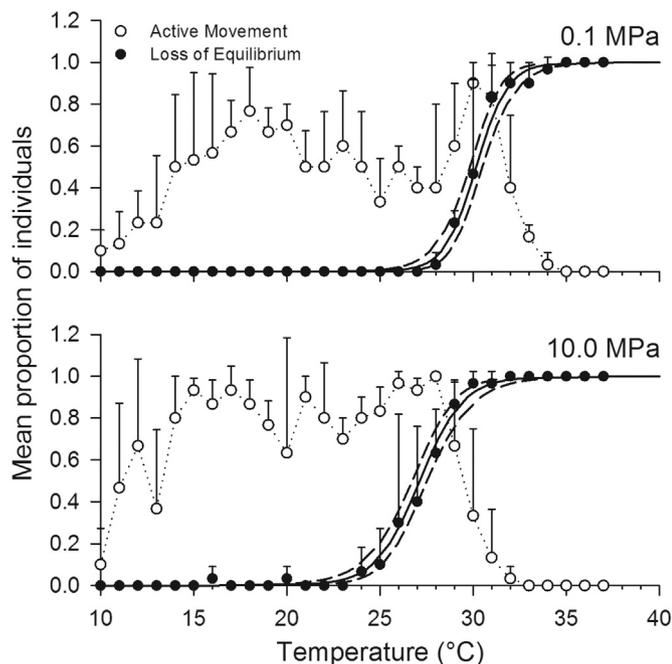The effect of pressure acclimation on Active Movement was

Fig. 1. Temperature tolerance of *Palaemon varians* after acclimation to different pressures (0.1 MPa and 10.0 MPa) for 1 week at 10 °C. Open and closed circles respectively represent Active Movement and Loss of Equilibrium (mean + SD for clarity; *n* = 3) at different temperatures during a thermal ramp. The solid line and dashed lines represent LoE and 95% confidence intervals modelled using probit analysis, assuming a logistic distribution.

analysed using a General Linear Model (GLM) Analysis of Variance (ANOVA) comparing AM at each temperature. Data were proportional and therefore were arcsine square root transformed prior to statistical analysis to achieve homogeneity of variance. The *post-hoc* multiple pairwise comparisons Šidak Simultaneous test was used to determine which treatments were significantly different in both GLM ANOVA analyses.

2.3. Phospholipid fatty acid composition

The effect of hyperbaric acclimation on phospholipid fatty acid composition in *P. varians* was assessed in separate treatments. 10 adult shrimp were exposed to each of the 168-h control and experimental acclimations described above. No mortalities were observed during the acclimation period in control or experimental treatments. Subsequent to the acclimation period shrimp were not exposed to the thermal ramp used to determine CT_{max}, but were removed from the IPOCAMP instead (Cottin et al., 2012). Depressurisation in the experimental treatment was acute (few seconds). Shrimp were rapidly decapitated: single clean cuts were made vertically from the dorsal extension of the carapace, separating the carapace and the first abdominal segment. The abdomen sections were placed individually in 1.5 ml centrifuge tubes, frozen in liquid nitrogen, and stored at −80 °C for subsequent analysis. Abdomen sections were selected for subsequent analysis because previous study identified greater responses to high hydrostatic pressure in the abdomen of *P. varians* than in its head (Morris et al., 2015c). Both temperature and pressure within the IPOCAMP system were recorded throughout the treatments using a temperature/pressure data logger (SP2T4000, NKE instrumentation).

1.5 ml centrifuge tubes containing individual abdomen sections (*n* = 3 per treatment selected at random) were removed from −80 °C storage and individual tissue samples were homogenised (using an IKA T10 Basic Ultra-Turrax homogenizer) in chloroform:methanol (2:1, *v/v*). Total lipid was extracted according to (Folch et al., 1957). Samples were thoroughly vortexed following the addition of aqueous potassium chloride (0.88%, *w/v*), then centrifuged for 5 min at 1500 rpm to establish a biphasic system. The upper phase (non-lipid substances in aqueous methanol) was removed via pipetting and discarded. The lower phase (lipid in chloroform) was filtered through a Whatman No.1 filter paper, pre-washed with chloroform:methanol 2:1 v/v, into pre-weighed vials. Samples were then dried down under N_2 gas using an N-EVAP system (Organomation), placed in a desiccator for 1 h, weighed to establish total lipid mass, and redissolved in chloroform to a final concentration of 1 mg ml^{-1}. Samples were stored at −20 °C.

Phospholipids were purified by thin layer chromatography (TLC). A 1 mg aliquot of total lipid was run on 2 mm, 20 × 20 cm silica gel plates in a hexane:diethyl ether:acetic acid (80:20:2 v/v/v) solvent system. To enable lipid visualisation under ultraviolet light, plates were sprayed with 2′,7'dichloro-fluorescein in methanol (0.1%, w/v). Phospholipids were scraped off plates into vials in which trans-methylation reactions were established following Christie (1982). Scrapings were dissolved in 1% sulphuric acid in methanol and toluene (1:2 v/v) and incubated at 50 °C for 16 h. Subsequently, equal volumes of hexane:diethyl ether (1:1 v/v) and deionised milli-Q water were added and samples were mixed thoroughly by vortexing. A biphasic system was established though centrifuging for 2 min at 1500 rpm. The upper phase was removed into a vial and aqueous sodium bicarbonate (2% w/v) was added. Samples were mixed thoroughly by vortexing. A biphasic system was established through centrifuging for 2 min at 1500 rpm. The upper solvent fraction was removed into vials and dried down under N_2 gas. This purified phospholipid fraction was redissolved in ~0.1 ml hexane. The solution was run through a TRACE2000, Thermo Electron, Gas Chromatograph (Thermo Scientific, UK) equipped with a Restek Stabilwax column (0.32 mm i.d. × 30 m). Hydrogen was used as the carrier gas (Pond et al., 2014).

Table 1

Critical temperature maximum (CT_{max}) loss of equilibrium (LoE) model parameters, with values for critical thresholds.

Acclimation	a	b	Goodness of fit (Deviance P)	CT_{max} (°C)
10 °C, 0.1 MPa	− 35.7609	1.1854	0.999	30.2
10 °C, 10.0 MPa	− 23.2365	0.8564	0.737	27.1

Models were derived using probit analysis of LoE data, assuming a logistic distribution: $\ln\left[\frac{Y}{1-Y}\right] = a + bX$, where X is the exposure temperature or pressure and Y is the proportion of individuals demonstrating LoE (following New et al., 2014).

2.3.1. Statistical analysis

Phospholipid fatty acid data were expressed as percent composition of total fatty acids. Thus phospholipid fatty acid data were proportional and were therefore arcsine-square-root transformed prior to statistical analysis. Statistically significant differences within each exposure duration treatment were assessed by one-way ANOVA, with hydrostatic pressure as the fixed factor ($\alpha = 0.05$).

3. Results

3.1. Effect of pressure acclimation on critical thermal maximum (CT_{max})

Loss of Equilibrium models did not differ significantly from observed LoE data (Goodness-of-fit Deviance $p > .05$). LoE was significantly affected by acclimation to 10 MPa ($p < .001$) (Fig. 1, Table 1). Temperature tolerance (CT_{max}) was significantly lower at 10 MPa (27.1 °C) than at 0.1 MPa (30.2 °C). Further, the gradient of the modelled LoE response was significantly lower at 10 MPa than at 0.1 MPa, indicating a less acute temperature effect ($p = .026$) (Fig. 1, Table 1).

The effect of temperature on Active Movement was affected by acclimation pressure ($F_{27,112} = 3.024$, $p < .001$) (Fig. 1). Temperatures resulting in significant differences were determined using the post-hoc multiple pairwise comparisons Holm-Sidak test. AM initially increased with increasing temperature at both acclimation pressures, but significant increase in AM occurred at lower temperature at 10.0 MPa (15 °C) than at 0.1 MPa (17 °C). AM decreased steeply at the onset of LoE.

3.2. Phospholipid fatty acid composition

Phospholipid fatty acid composition was significantly affected by acclimation to high hydrostatic pressure (Fig. 2).

4. Discussion

4.1. Hyperbaric acclimation effects on critical thermal maximum (CT_{max}) and phospholipid fatty acid composition

The CT_{max} of shrimp is known to depend on acclimation temperature (Ravaux et al., 2016). The CT_{max} of *P. varians* acclimated to surface hydrostatic pressure (0.1 MPa) at 10 °C in this study was similar to that of a previous study: respectively, 30.2 °C and 30.9 °C (Oliphant et al., 2011). Although CT_{max} differs by 0.7 °C between studies, this difference is likely due to differing acclimation periods. CT_{max} decreases rapidly during the first days of acclimation to low temperature, but thermal acclimation continues for 20+ days and CT_{max} decreases further during this period (New et al., 2014). Whereas shrimp in this study were acclimated to 10 °C for at least 5 weeks prior to assessment of thermal tolerance, shrimp in Oliphant et al.'s (2011) study were acclimated shrimp to 10 °C for only 3 days prior to assessment of thermal tolerance.

Acclimation for 7 days to high hydrostatic pressure (10 MPa) at 10 °C reduced the acute temperature tolerance of adult shallow-water shrimp *Palaemon varians* by 3.1 °C, from 30.2 °C to 27.1 °C. Identical acclimation treatment increased acute hydrostatic pressure tolerance in *P. varians* by 2.1 MPa (New et al., 2014), suggesting that the decrease in acute temperature tolerance is not an artefact of increased stress at high hydrostatic pressure. Both CT_{max} observed for *P. varians* acclimated to 10 °C at 0.1 and at 10 MPa are lower than that observed for *M. fortunata* at its native pressure (36 ± 1 °C; Shillito et al., 2006), demonstrating that the hydrothermal vent shrimp is physiologically adapted to the high hydrostatic pressure and thermally variable conditions of hydrothermal vent environments.

Homeoviscous adaptations to cell membrane bilayer have been demonstrated for organisms adapted to extreme cold and high pressure and have been posited as a mechanism of acclimation to low temperature and high hydrostatic pressure (Hazel and Williams, 1990). Adaptations comprise shifts in the composition of cell lipid bilayers towards increased proportions of mono- and poly-unsaturated fatty acids and decreased saturated fatty acids under conditions of low temperature and high hydrostatic pressure, maintaining membrane fluidity and therefore function under these conditions. The changes in acute temperature tolerance and acute hyperbaric tolerance demonstrated for *P. varians* after acclimation to 10 MPa occur alongside small but significant shifts in phospholipid fatty acid composition. Although there was no significant change in total monounsaturated, total poly-unsaturated, or total unsaturated phospholipid fatty acids, there were significant changes in some individual phospholipid fatty acids. Some shifts in individual phospholipid fatty acids were consistent with the prediction based on the homeoviscous acclimation hypothesis that the proportion of unsaturated fatty acids in cell membranes increases in response to high hydrostatic pressure: 16:1n-7 (palmitoleic acid), 20:4n-6 (arachidonic acid), 22:5n-3 (docosapentaenoic acid), 16:2 (hexadecadienoic acid), and 16:4n-1 (hexadecatetraenoic acid) all increased significantly. Increases in unsaturated fatty acids may result in increased membrane fluidity (Hazel and Williams, 1990; Hazel, 1995). For example, significant increase in palmitoleic acid has been reported to contribute to maintaining membrane fluidity in amphipods in response to decreased temperature (e.g. Lahdes et al., 2000). Whilst maintaining membrane fluidity contributes to maintaining essential membrane function at low temperature or high pressure, increased membrane unsaturation also results in greater leakage across membranes (Hazel and Williams, 1990; Hazel, 1995). Proton leakage across the inner mitochondrial membrane is proposed to contribute to limiting critical thermal tolerance by increasing oxygen demand beyond oxygen supply capacity as temperature increases (Pörtner, 2001). Thus increased proton leakage across the inner mitochondrial membrane resulting from unsaturation of the membrane may contribute to the observed reduction in critical thermal tolerance observed with acclimation to high hydrostatic pressure. Indeed, positive selection analysis indicates adaptive change in the mitogenome of the alvinocarid shrimp *Shinkaicaris leurokolos* related to the electron transport chain (Sun et al., 2018a) which is coupled with proton transfer and may thus affect critical temperature tolerance.

The reason that only few unsaturated phospholipid fatty acids were observed to increase significantly may also be related to their specific functions. For example, docosapentaenoic acid is thought to have a role in attenuating decrease in long-term potentiation in synapse (Kaur et al., 2011). Thus increase in docosapentaenoic acid may counteract adverse effects of high hydrostatic pressure on the function of crustacean neuromuscular synapse (Sébert, 2010; Brown et al., 2017b), which have been reported to demonstrate long-term potentiation (Baxter et al., 1985). In contrast, specific functions of hexadecadienoic and hexadecatetraenoic acids are uncertain. Despite this, the increase in hexadecatetraenoic acid in *P. varians* in response to high hydrostatic pressure may be particularly interesting. Hexadecatetraenoic acid is an unusual fatty acid which has only been reported in marine phytoplankton, amoeba, and the krill *Euphausia superba*, and it has been unclear whether hexadecatetraenioc acid is synthesised in *E. pacifica* or
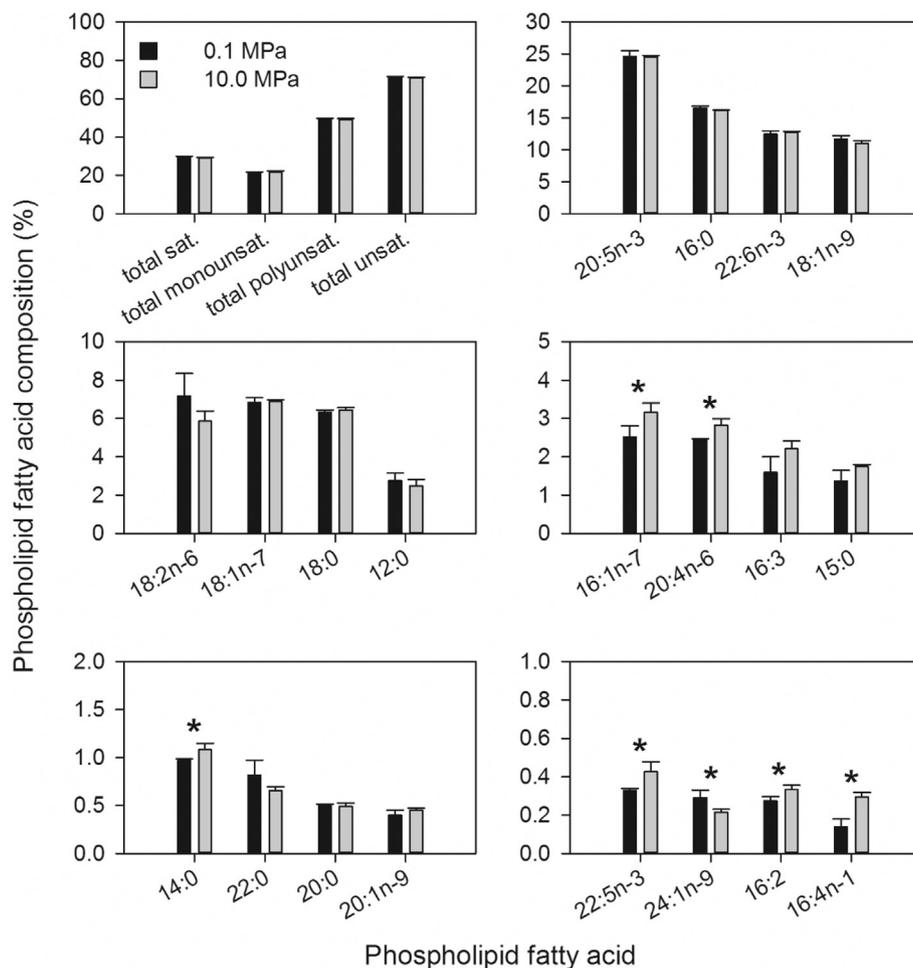
Fig. 2. Phospholipid fatty acid composition of *Palaemon varians* acclimated to different pressures (0.1 MPa and 10.0 MPa) for 1 week at 10 °C. Bars and error bars represent mean + SD; $n = 3$. Asterisks indicate significant differences in the contribution of individual phospholipid fatty acids to phospholipid acid composition in shrimp acclimated to different pressures.

derived from phytoplankton (Yamada et al., 2017). Results presented here suggest that starved *P. varians* is capable of synthesising hexadecatetraenoic acid in response to increased hydrostatic pressure, raising the possibility that the presence of hexadecatetraenoic acid in *E. pacifica* may relate to diurnal vertical migration of up to 400 m in this species (Endo and Yamano, 2006).

The observed shifts in fatty acid composition in *P. varians* also suggest that responses to high hydrostatic pressure are more complex than simply increasing the proportion of unsaturated fatty acids in cell membranes. For example, the significant increase in the saturated fatty acid 14:0 (myristic acid) may be unexpected. However, myristic acid has a specific role in acylation of membrane proteins and anchoring those proteins to the cell membrane (Calder, 2015). Thus, the increase in myristic acid may reflect increased protein concentration in the cell membrane in response to the impaired function of membrane proteins such as Na$^+$ K$^+$ ATPase (Gibbs and Somero, 1989) at high hydrostatic pressure. Na$^+$ K$^+$ ATPase is important in maintaining Ca^{2+} homeostasis in crustaceans (Freire et al., 2008) and is crucial to maintaining membrane potential required to generate action potential in cells regulating cardiac function (Lodish et al., 2003).

Similarly, although not statistically significant, the apparent reduction in polyunsaturated 18:2n-6 (linoleic acid) is unexpected. Linoleic acid is an essential fatty acid that cannot be synthesised from other dietary components and therefore the proportion of linoleic acid in cell membranes will decrease if linoleic acid is metabolised. Linoleic acid is a precursor to arachidonic acid (20:4n-6) (Calder, 2015), which increased significantly in response to high hydrostatic pressure.

Arachidonic acid levels play a role in determining eicanosoid production: arachidonic acid is the precursor to a range of eicanosoids such as prostaglandins, thromboxanes, and leukotrienes that have many regulatory roles in cell growth and in the central nervous system (Needleman et al., 1988, Calder, 2015). Both cell growth and central nervous system function are known to be adversely affected by high hydrostatic pressure (Sébert, 2010; Brown et al., 2017b). Arachidonic acid also has a role in cell signalling: arachidonic acid is also known to interact with voltage- and ligand-gated ion channel functions (Antollini and Barrantes, 2016). For example, arachidonic acid synthesis is linked to NMDA receptor activity (Dumuis et al., 1988). NMDA receptor activity is reported to increase in crustaceans including *P. varians* in response to high hydrostatic pressure (Morris et al., 2015a,b,c; Munro et al., 2015; Brown et al., 2017b; Brown and Thatje, 2018). The significant decrease in 24:1n-9 (nervonic acid) may also be related to the effects of high hydrostatic pressure on central nervous system function. Nervonic acid is incorporated in the myelin sheath of nerve fibres during nerve biosynthesis (Stoffel and Bosio, 1997), which may be a response to the adverse effects of high hydrostatic pressure.

Although observed changes in phospholipid fatty acids suggest that homeoviscous acclimation may contribute to adjustments in critical thresholds, further experimentation with greater replication is required to confirm this, and other acclimation mechanisms may also contribute to shifts in environmental tolerances. For example, cytoprotective molecules such as the osmolyte trimethylamine N-oxide (TMAO) counteract the inhibitory effects of pressure on the stability and function of many proteins (Yancey and Siebenaller, 2015) and may be involved in

acclimation to high hydrostatic pressure in crustaceans: TMAO is reported to increase with depth in amphipods (Downing et al., 2018). Similarities in the effects of low temperature and high hydrostatic pressure have prompted the suggestion that increases in mitochondrial densities and changes in mitochondrial functional properties that are observed during cold acclimation (Pörtner, 2002) may also occur during acclimation to high hydrostatic pressure (New et al., 2014). No data are yet available to assess these possibilities.

Remodelling membrane composition at high hydrostatic pressure may be more important in hyperbaric acclimation than suggested by phospholipid fatty acid data presented here. Membrane phospholipid fatty acid remodelling may occur over longer timescales or may depend on dietary assimilation of phospholipids. Studies that have assessed the effect of acclimation to high hydrostatic pressure have not explored the impact of feeding on acute environmental tolerances (e.g., New et al., 2014; Brown et al., 2017b) due to the technological difficulties in introducing food into hyperbaric aquaria. Similarly, *P. varians* were not fed during the 7 day treatments within the hyperbaric aquaria. Hypothetically, assimilation of appropriate dietary phospholipids and subsequent bilayer remodelling could increase tolerance of high hydrostatic pressure in shallow-water organisms. Studies on the effects of feeding during hyperbaric exposures are necessary to understand better acclimation to high hydrostatic pressure.

4.2. Ecological implications of hyperbaric acclimation effects on CT_{max}

Critical thermal tolerance in *P. varians* following 7-day acclimation to 5 °C at 0.1 MPa is 27.3 °C (New et al., 2014), similar to the 27.1 °C observed here following 7-day acclimation to 10.0 MPa at 10 °C. The effects of low temperature and high hydrostatic pressure acclimation are typically cumulative or synergistic, suggesting that acclimatisation to the environmental conditions in the non-hydrothermal vent deep sea environment (low temperature 5 °C and high hydrostatic pressure 10.0 MPa) would result in CT_{max} reduction below ~24 °C. Nonetheless, CT_{max} would remain sufficient in *P. varians* to allow occupation of known hydrothermal vent habitats.

Although *P. varians* inhabits a similar thermal range to hydrothermal vent shrimp the spatial/temporal scale in temperature variation differs significantly. Temperatures experienced by *P. varians* in salt marsh habitat range from 0 °C in winter to 33 °C in summer, but with no > 6 °C variation in a 12 h period (Pallareti et al., 2018). In contrast, hydrothermal vent shrimp such as *M. fortunata* occur in high abundances within 1 m of hot fluid outflows in hydrothermal vent fields (Marcon et al., 2013), with thermal distribution ranging from 4 to 24.6 °C (Desbruyeres et al., 2001). Although *M. fortunata* preferentially selects a mean temperature < 1 °C greater than *P. varians* (respectively 19.2 °C and 18.3 °C at 0.1 MPa; Smith et al., 2013), *M. fortunata* appears to have evolved reduced metabolic sensitivity in response to the acute spatial and temporal scale of temperature changes in the hydrothermal vent environment (Smith et al., 2013). Nonetheless, it is likely that *P. varians* acclimated to high hydrostatic pressure and low temperature retain capacity to tolerate the thermal regime in the hydrothermal vent environment. Given that related extant shallow-water and hydrothermal-vent shrimp are physiologically capable of coping with the thermal and hyperbaric conditions of the hydrothermal vent environment, the common ancestor of these lineages may have possessed similar capability, allowing for the possibility of direct hydrothermal vent colonisation by shallow-water shrimp.

In contrast, the chemical composition of the hydrothermal environment is likely to have represented a barrier to direct vent colonisation by shallow-water shrimp. Recent ecotoxicological assessments suggest that the hydrothermal vent environment would prove toxic to *P. varians* (Brown et al., 2017a). Whilst the hydrothermal vent shrimp appear to have evolved physiological strategies to mitigate metal-rich vent seawater (Auguste et al., 2016), the common ancestor of hydrothermal vent and shallow-water shrimp is likely to have lacked

adaptations to tolerate deep-sea hydrothermal vent seawater chemistry. Direct colonisation of the deep-sea hydrothermal vent environment may therefore be unlikely. Indeed, Alvinocarididae hydrothermal vent shrimp share closer affinities with other deep-water shrimp families and non-hydrothermal vent shrimp families with both shallow-water and deep-water representatives, than with exclusively shallow-water shrimp families (Bracken et al., 2009; Li et al., 2011; Aznar-Cormano et al., 2015; Hernández-Ávila et al., 2015; Liao et al., 2017; Davis et al., 2018; Sun et al., 2018b). This suggests that shrimp colonised the wider deep-sea environment before colonising hydrothermal vents.

4.3. Conclusion

Acclimation to high hydrostatic pressure reduces temperature tolerance in the shallow-water palaemonid shrimp *Palaemon varians*. Such physiological plasticity may have been an important factor in the colonisation of deep-sea environments by shallow-water shrimp, but *P. varians* acclimated to high hydrostatic pressure and low temperature likely retain sufficient thermal scope to tolerate the hydrothermal vent environment. Nonetheless, it appears shrimp colonised the wider deep sea before colonising deep-sea hydrothermal vents. Shifts in phospholipid fatty acid composition during hyperbaric exposure suggest that homeoviscous modifications in the cell lipid bilayer contribute to observed shifts in critical temperature and critical pressure tolerances during hyperbaric acclimation.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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