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RESEARCH ARTICLE

Pressure tolerance of the shallow-water caridean shrimp *Palaemonetes varians* across its thermal tolerance window

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SUMMARY

To date, no published study has assessed the full physiological scope of a marine invertebrate species with respect to both temperature and hydrostatic pressure. In this study, adult specimens of the shallow-water shrimp species *Palaemonetes varians* were subjected to a temperature/pressure regime from 5 to 30°C and from 0.1 to 30 MPa. The rate of oxygen consumption and behaviour in response to varying temperature/pressure combinations were assessed. Rates of oxygen consumption were primarily affected by temperature. Low rates of oxygen consumption were observed at 5 and 10°C across all pressures and were not statistically distinct (P=0.639). From 10 to 30°C, the rate of oxygen consumption increased with temperature; this increase was statistically significant (P<0.001). *Palaemonetes varians* showed an increasing sensitivity to pressure with decreasing temperature; however, shrimp were capable of tolerating hydrostatic pressures found outside their normal bathymetric distribution at all temperatures. 'Loss of equilibrium' (LOE) in ≥50% of individuals was observed at 11 MPa at 5°C, 15 MPa at 10°C, 20 MPa at 20°C and 21 MPa at 30°C. From 5 to 20°C, mean levels of LOE decreased with temperature; this was significant (P<0.001). Low mean levels of LOE were observed at 20 and 30°C and were not distinct (P=0.985). The physiological capability of *P. varians* to tolerate a wide range of temperatures and significant hydrostatic pressure is discussed.

Key words: Caridea, physiology, evolution, deep sea, biogeography, bathymetric distribution.

INTRODUCTION

Shallow-water fauna are known to have given rise to deep-sea species through colonisation of bathyal and abyssal depths. Though the origin and antiquity of extant deep-sea fauna is uncertain, it is generally considered that climate-driven extinction events in the deep sea, and subsequent re-colonisation of this realm, took place during many geological ages (Jablonski et al., 1983; Horne, 1999; Wilson, 1999; Aquino-Souza et al., 2008). As such, extant deep-sea fauna are considered to comprise both ancient and more recent shallow-water lineages (Horne, 1999; Wilson, 1999). Certainly, some deep-sea and shallow-water species are closely related, sharing common ancestors. Tokuda and colleagues found that deep-sea bresiliid shrimp have a close taxonomic relationship with shallow-water palaemonid shrimp (Tokuda et al., 2006). Similarly, Distel and colleagues identified a close taxonomic relationship between hydrothermal vent and cold seep Bathymodiolinae mussels from the deep sea and Mytilinae mussels from shallow water (Distel et al., 2000). Close taxonomic relationships have also been demonstrated between deep-sea and shallow-water isopods within the Asellota (Raupach et al., 2009).

Shallow-water fauna are adapted to the relatively warm conditions that, except at high latitudes, predominate in the upper oceans. Consequently, the frigid temperatures, which presently prevail in the deep sea, are thought to limit the vertical migration of shallow-water fauna. Recent colonisation of the deep sea may thus have occurred only through an isothermal water column, limited either to periods during the Mesozoic and early Cenozoic eras or, more recently, to high latitudes (Young et al., 1997; Tyler and Young, 1998; Tyler et al., 2000; Thatje et al., 2005; Mestre et al., 2009). Colonisation during the Mesozoic and early Cenozoic eras, when deep-sea temperatures were warmer, required gradual adaptation to cold temperatures as conditions in the deep sea evolved to their present psychrospheric state (Clarke, 1983; Tyler et al., 2000). Alternatively, high latitude species, being mostly cold stenothermal and pre-adapted to life in the deep sea, may have colonised bathyal and abyssal depths through regions of deep-water formation (Tyler and Young, 1998; Tyler et al., 2000; Villalobos et al., 2006).

Recently, increasing significance has been given to studies examining temperature and pressure tolerance in embryonic and larval stages of shallow-water species with phylogenetic links to deep-sea species (Young et al., 1995; Young et al., 1996; Young et al., 1997; Tyler et al., 2000; Villalobos et al., 2006; Aquino-Souza et al., 2008). Predominantly focused on echinoderms, these studies have demonstrated impressive pressure tolerance; in all cases larvae were able to tolerate pressures found far outside their normal adult distribution. Indeed, juveniles of several echinoderm species are known to settle outside their normal adult depth range; however, these individuals do not survive to adulthood (Gage and Tyler, 1981a; Gage and Tyler, 1981b; Sumida et al., 2001; Howell et al., 2002). The potential for depth range extension and colonisation of the deep sea may, therefore, be genuine for some shallow-water species. There is evidence that the echinoid *Echinus acutus* has extended its bathymetric range, indicating that migrations to the deep sea are still occurring (Tyler and Young, 1998). Nevertheless,

generalised assumptions across all shallow-water fauna should be discouraged given the limited diversity of the animals studied (echinoderms predominate) and the relatively modest amount of data available.

Comparisons of temperature and pressure tolerance between shallow-water and deep-sea fauna offer insight into, and understanding of, predispositions and evolutionary adaptations for life in the deep sea (Childress and Fischer, 1992; Somero, 1992; Mestre et al., 2009). Baro-physiological studies involving animals adapted to 0.1 MPa (atmospheric pressure) have demonstrated the pressure sensitivity of enzyme activity, protein structure and plasma membrane fluidity (for reviews, see Somero, 1992; Pradillon and Gaill, 2007). Under pressure, enzyme reactions associated with an increased system volume during catalysis are inhibited. Pressure may also disrupt protein structures, causing denaturation, and the viscosity of plasma membranes is increased through the ordering of lipid molecules within the membrane. Deep-living organisms exhibit enzymatic adaptations that reduce volume change during catalysis, pressure-stable protein structures and homeoviscous adaptations within plasma membranes that maintain fluidity under ambient pressure (MacDonald, 1984; Somero, 1992; Airriess and Childress, 1994) [see also Pradillon and Gaill (Pradillon and Gaill, 2007) and references therein].

Our study investigated the effects of various pressure and temperature combinations on adult specimens of the variable shrimp *Palaemonetes varians*, Leach 1814. Our aim was to test the pressure tolerance of *P. varians* across its thermal tolerance window and in doing so establish this species' thermal and baro-physiological thresholds. *Palaemonetes varians* is a shallow-water brackish shrimp (Decapoda, Caridea) native to Western Europe. Physiological studies have found that this species is highly tolerant of hypoxia, and temperature and salinity fluctuations, and it has recently received attention as a potential aquaculture crop (e.g. Palma et al., 2008; Palma et al., 2009). Furthermore, *P. varians*' close taxonomic relationship to the Atlantic hydrothermal vent shrimp species *Rimicaris exoculata*, *Chorocaris chacei* and *Mirocaris fortunata* (Tokuda et al., 2006) makes this species an excellent case for comparison with these deep-living shrimps, and it has previously been used as such (Gonzalez-Rey et al., 2007; Gonzalez-Rey et al., 2008).

MATERIALS AND METHODS

Sampling and acclimation

Adult specimens of *P. varians* were collected from Lymington salt marshes (Hants, UK) using a hand-held trawling net in October and November 2009. Animals were maintained in a running seawater system in the aquarium of the National Oceanography Centre (Southampton, UK) where experimental work was conducted in October and November 2009. Prior to experimental treatments, animals were acclimated stepwise to the desired experimental temperatures (5, 10, 20, 30°C) for a period of 3 days, using temperature-controlled incubators with a photoperiod of 12 h:12 h light:dark – shrimp were not fed during this period. Shrimp were acclimated in filtered seawater (1 µm filtered) with a salinity of 32.7.

For a preliminary assessment of the critical thermal maximum (CT_{max}), experiments were conducted at the UPMC (Paris, France), and the procedures for animal sampling and acclimation thus differ slightly. The *P. varians* specimens were collected in October 2009 from Bay of Mont Saint Michel (France) using a hand-held trawling net. The shrimp were transferred to a 140 l aquarium with constant aeration, and the water temperature was controlled by a programmable unit (HC-1000A, Aquavie, Paris, France). The water temperature was gradually decreased from 17°C (field temperature at the time of collection) to 10°C, at a rate of 1°C per week. The salinity of the water during acclimation was 30. The photoperiod was set to 12 h:12 h light:dark and the animals were regularly fed during the 3 months prior to the CT_{max} experiments.

Preliminary assessment of CT_{max} and low temperature tolerance

Preliminary CT_{max} experiments used shrimp sampled and acclimated in France. Experiments were carried out at 0.1 MPa. *Palaemonetes varians* specimens of similar size (carapace length 10.6±1.1 mm, N=20) were placed individually in a beaker filled with 80 ml of seawater and capped with a transparent lid to allow observation throughout the experiment. The beaker was placed in a water bath (Polystat 22, Bioblock Scientific, Illkirch, France), which regulated the temperature of the surrounding water. The water temperature was monitored in the beaker using an electronic thermometer (Codiam Scientific, Gonesse, France). The initial temperature was 13°C, and this was increased at a constant rate of 1°C min^{-1}. CT_{max} was defined as the temperature at which coordinated movements were lost. This was apparent either as spasmodic motions ('spasm' response; vibrations of the pleopods and/or sudden contraction of the abdomen) or as loss of equilibrium (LOE; shrimp on the bottom of the beaker either on its side or on its back for more than 2 s). The experiment ended when the shrimp lay on the bottom of the beaker for more than 30 s. The trial was repeated 20 times, which corresponds to a total of 20 individual shrimp (N=20).

A preliminary experiment for low temperature tolerance determination was conducted at the National Oceanography Centre, Southampton, using shrimp sampled and acclimated in the UK. Fifty adult *P. varians*, acclimated to 5°C (see above), were transferred to aquaria in a temperature-controlled open water bath and the temperature reduced, stepwise by 1°C day^{-1}, to 1°C. After a period of 3 days (72 h), the temperature was further lowered to 0°C. As a result of high survival, after a period of 21 days (504 h) the temperature was further reduced to –1°C until 100% mortality was observed [after 27 days (648 h)]. During the experimental period, food was offered; however, no feeding was observed.

Behavioural analysis

Behavioural analysis was carried out on shrimp sampled and acclimated in the UK. Behavioural observations were conducted using the IPOCAMP™ pressurised incubator previously described by Shillito et al. (Shillito et al., 2001; Shillito et al., 2006). Importantly, all air is vented from the IPOCAMP™ system prior to pressurisation. This ensures that no air is forced into solution by pressurisation within the IPOCAMP™ system; consequently, the partial pressure of dissolved gases remains unaltered at pressure.

The IPOCAMP™ was set to run for a 2 h period prior to the start of each experiment to ensure the whole system was maintained at the desired experimental temperature. Water temperature was monitored at the inlet and outlet to the IPOCAMP™ pressurised chamber; the water temperature within the chamber was also measured using a thermometer prior to experimentation. Ten shrimp were placed inside a PVC cage with an inclined lid [see Ravaux et al. and Shillito et al. for descriptions and schematic diagram (Ravaux et al., 2003; Shillito et al., 2006)], which was mounted on a tripod platform inside the pressure chamber. The platform elevated the cage, allowing a clear view *via* an endoscope inserted into a central viewing port in the lid of the IPOCAMP™ (Shillito et al., 2006). The system was run at atmospheric pressure (0.1 MPa) for

1 h prior to pressurisation to allow acclimation and recovery from handling stress. The endoscope was connected to a video recorder and TV monitor. The video recorder was set to record 5 min before the end of the acclimation period. Pressurisation was achieved in a stepwise manner allowing observation of behaviour at each pressure increment. Pressure was increased by 1 MPa every 5 min up to 30 MPa. Pressure was then decreased in the same stepwise method of 1 MPa every 5 min. Once back at atmospheric pressure the system was left to run for 1 h to allow continued observations. Once removed from the IPOCAMP^{TM}, shrimp were maintained in aquaria at experimental temperature for 3 days to monitor post-experiment mortality. Three replicate experiments were carried out at each temperature (5, 10, 20, 30°C).

Behaviour in response to pressure was determined for the final 30 s at each pressure; this allowed animals to recover from any possible shock initiated by the previous pressure increase or decrease. Individuals were identified and their behaviour classified into four categories according to previous studies (Ravaux et al., 2003; Shillito et al., 2006), as follows.

'Motionless'; no movement detected at normal tape-reading speed; this category was also designated when an individual's movement seemed to be the result of neighbouring shrimp 'pushing', with no apparent reaction of the individual.

'Movement'; any kind of detectable movement except for active walking or swimming (see below): pereopod or pleopod movements, scaphognathite beating, antennal lateral sweeping on the dorsal side, and cleaning of mouth parts by rubbing them along each other.

'Active movement'; when the shrimp moved (walked or swam) a distance exceeding their own length in less than 30 s.

'Loss of equilibrium' (LOE); when a given shrimp rested on the bottom in either an 'upside-down' or a 'sideways' position for more than 2 s. LOE could co-occur with any of the other three categories.

The occurrence of spasms was not taken into account in this analysis because they were too difficult to monitor in this small species with the method of observation used (i.e. an endoscope inserted into a viewing port in the lid of the IPOCAMP^{TM}).

Respiration rates

Respiration rate experiments used shrimp sampled and acclimated in the UK. Individual animals were transferred to 33 ml plastic vials filled with filtered seawater (1 µm filtered) acclimated to the respective experimental temperature; they were sealed underwater to ensure no air was trapped inside. Importantly, as air was excluded from within the vial, no air could be forced into solution by pressurisation; consequently, the partial pressure of dissolved gases remained unaltered by pressure. A single vial was placed inside a pressure vessel (Mestre et al., 2009). The pressure vessel was filled with freshwater; both the pressure vessel and the freshwater were pre-incubated at the experimental temperature. Pressurisation to the experimental pressures (0.1, 5, 10, 15, 20, 25, 30 MPa) was continuous and was achieved using a Maximator model manual hydraulic pump (see Mestre et al., 2009). The pressure intervals selected reflect the bathymetric range over which *P. varians* (<1 MPa) and hydrothermal vent bresiliid shrimp (≤30 MPa) occur. Pressurisation was acute, taking approximately 30 s or less. Pressure vessels were placed in a temperature-controlled open water bath during the experimental period. Experiments at 5 and 10°C were conducted within a temperature-controlled room. Vial volume was kept constant at all temperatures. Isolation periods were reduced at higher temperatures as a result of increased metabolic rate and hence greater rates of oxygen consumption. Adjustments were made to ensure that the final oxygen concentration within the vial did not fall below 50% of initial concentrations. Animals were isolated for 90 min at 5°C, 60 min at 10°C, 45 min at 20°C and 20 min at 30°C. After the isolation period, vessels were immediately depressurised and the vial removed. The oxygen concentration of water inside the vial was measured using a temperature-adjusted oxygen meter and microoptode (Microx TX 3, PreSens, Regensburg, Germany; accuracy ±0.4% O_2 at 20.9% O_2, ±0.05% O_2 at 0.2% O_2). These measurements were calibrated with fully aerated seawater that had been left to settle for 30 min (100% O_2 saturation) and seawater deoxygenated by over-saturation with sodium sulphite (0% O_2 saturation) (Thatje et al., 2010). These calibration solutions were incubated at the experimental temperatures prior to use. The oxygen concentration of 100% oxygen-saturated seawater was calculated according to Benson and Krause (Benson and Krause, 1984). Control vials containing no animals were subjected to experimental treatments. Five replicates and one control were run at each temperature/pressure combination. Oxygen consumption was calculated from the difference in final oxygen concentration between the control and experimental vials as described by Thatje et al. (Thatje et al., 2010). This method discounted any oxygen consumption by microbial activity. After oxygen measurements were performed, animals were removed from their vials, blotted to remove excess water, then placed in 12 ml plastic vials and frozen at –80°C until deceased. Animals were defrosted, blotted again to remove excess water and weighed to obtain their fresh mass in mg.

Statistics

Respiration data were subject to analysis by general linear model (GLM) ANOVA; *post hoc*, multiple comparisons of factors, temperature (°C) and pressure (MPa), were carried out using the Holm–Šidák method. Behavioural data, as proportional data, were $\arcsin(\sqrt{N})$ transformed and subjected to analysis by GLM ANOVA; *post hoc*, multiple comparisons of factors, temperature (°C) and pressure (MPa), were carried out using the Holm– Šidák method.

RESULTS

Preliminary assessment of CT_{max} and low temperature tolerance

Preliminary CT_{max} experiments used shrimp sampled and acclimated in France. Enhanced activity of shrimp specimens was observed when the temperature increased. Indeed, from 20.6±0.21°C onwards, more than 50% of the shrimp were observed actively moving (Active movement, Fig. 1). The peak of this activity, where 100% of the shrimp were actively moving, corresponds to approximately 31.3±0.19°C and was followed by a fast decrease in activity from 32.4±0.12°C until the end of the experiment. An apparent loss of locomotory coordination, expressed as spasmodic movements of the pleopods and/or abdomen with no resulting displacement (spasm response), was first observed at 30.9±1.0°C. This disorder of locomotor activity was also observed as the shrimp lost their balance (LOE response) upon reaching 30.9±1.3°C.

The preliminary experiment for low temperature tolerance determination used shrimp sampled and acclimated in the UK. During the experiment, mortality at 1°C was 0% and at 0°C mortality was low (<6%). The first mortality occurred on the 8th day of the experiment, after 3 days at 0°C. Subsequent mortalities at 0°C occurred on the 18th and 19th day of the experiment (after 13 and 14 days at 0°C, respectively); survival remained constant until the temperature was lowered to –1°C. Only when the temperature was –1°C did mortality rates increase and reach 100% (after 27 days at –1°C, day 53 of the experiment).

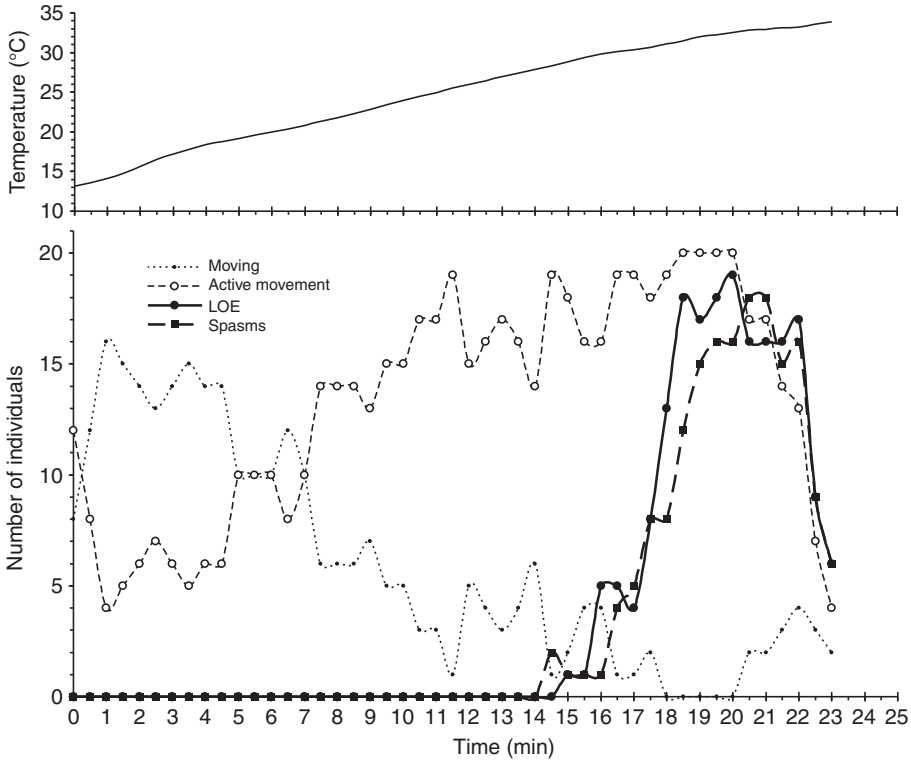

Fig. 1. Determination of the critical thermal maximum (CT_{max}) of *Palaemonetes varians* at 0.1 MPa pressure (shrimp sampled and acclimated in France). Distribution of behavioural categories of *N*=20 shrimp (lower graph; totals for behavioural categories of 20 trials) related to mean temperature (upper graph; temperature averaged across all 20 trials) throughout exposure to an increasing water temperature. LOE, loss of equilibrium. For an explanation of the different behaviours, see Materials and methods. Each behavioural point represents the observations for a 30 s period. For each observation time, the maximal error with regard to the corresponding temperature is approximately ±0.5°C.

Effect of temperature and pressure on behaviour

Behavioural analysis used shrimp sampled and acclimated in the UK. For analysis of behavioural observations (recorded as the number of individuals showing a particular behaviour), the behavioural classes defined above were categorised as follows: total movement (i.e. movement + active movement = 10 – motionless), active movement and LOE. In this analysis, active movement and LOE are considered to be the important indicators of pressure tolerance.

Effect of temperature

When levels of active movement for a single temperature were averaged across all pressures, greater mean levels of active movement were observed at higher temperatures. The lowest mean level of active movement occurred at 5°C (1.3±1.8 individuals); the highest occurred at 30°C (6.0±3.5 individuals). At 10 and 20°C, mean levels of active movement were 3.6±3.4 and 4.8±3.6 individuals, respectively. From 5 to 30°C, mean levels of active movement increased in an approximately linear pattern; the differences in mean levels of active movement between temperatures were significant (P<0.001).

Levels of LOE for a single temperature were also averaged across all pressures; lower mean levels of LOE were observed at higher temperatures. The highest mean level of LOE occurred at 5°C (4.8±3.6 individuals), the lowest occurred at 30°C (2.2±2.5 individuals). At 10 and 20°C, mean levels of LOE were 3.8±3.7 and 3.0±3.6 individuals, respectively. The differences in mean levels of LOE between temperatures were statistically significant (P<0.001) except for those between 20 and 30°C (P=0.985).

The effect of temperature on the behavioural categories active movement and LOE of *P. varians* was significant (P<0.001 and P<0.001, respectively).

Effects of pressure

Levels of active movement at a single pressure, averaged across all temperatures, generally decreased with increasing pressure. The highest mean levels of active movement occurred at 7, 8, 9 and 11 MPa (7.6±2.9, 7.6±3.0, 7.6±3.0 and 7.6±2.6 individuals, respectively); the lowest occurred at 29 and 30 MPa (0.0±0.0 and 0.0±0.0 individuals, respectively); this difference was statistically significant (P<0.001). Increasing mean active movement occurred between 1 and 7 MPa (4.1±2.0 and 7.6±2.9 individuals, respectively) and was significant (P<0.001).

Levels of LOE at a single pressure, averaged across all temperatures, increased with pressure. The lowest mean levels of LOE occurred between 0.1 and 6 MPa (0.0±0.0 individuals for all), the highest mean LOE occurred at 30 MPa (9.5±0.9 individuals); this increase was significant (P<0.001).

The effect of pressure on the behavioural categories active movement and LOE of *P. varians* was significant (P<0.001 and P<0.001, respectively).

Combined effects of temperature and pressure

Temperature influenced the effect of pressure on the behaviour of *P. varians* (Fig. 2). At 5°C, as pressure was increased, total movement was generally <10 individuals up to 12 MPa, indicating that some shrimp were motionless (Fig. 2A). At 12 MPa, total movement reached 10±0 individuals and remained constant up to 23 MPa. Total movement then decreased steadily from 10±0 individuals at 23 MPa to 1.3±1.2 individuals at 30 MPa. At 5°C, active movement was low relative to that at 10, 20 and 30°C (Fig. 2B–D); the mean level of active movement at 5°C was significantly lower than that at 10, 20 and 30°C (P<0.001). Active movement decreased from 6.3±2.5 individuals at 0.1 MPa to 0.0±0.0 individuals at all pressures ≥14 MPa; this decrease was statistically significant (P=0.001).

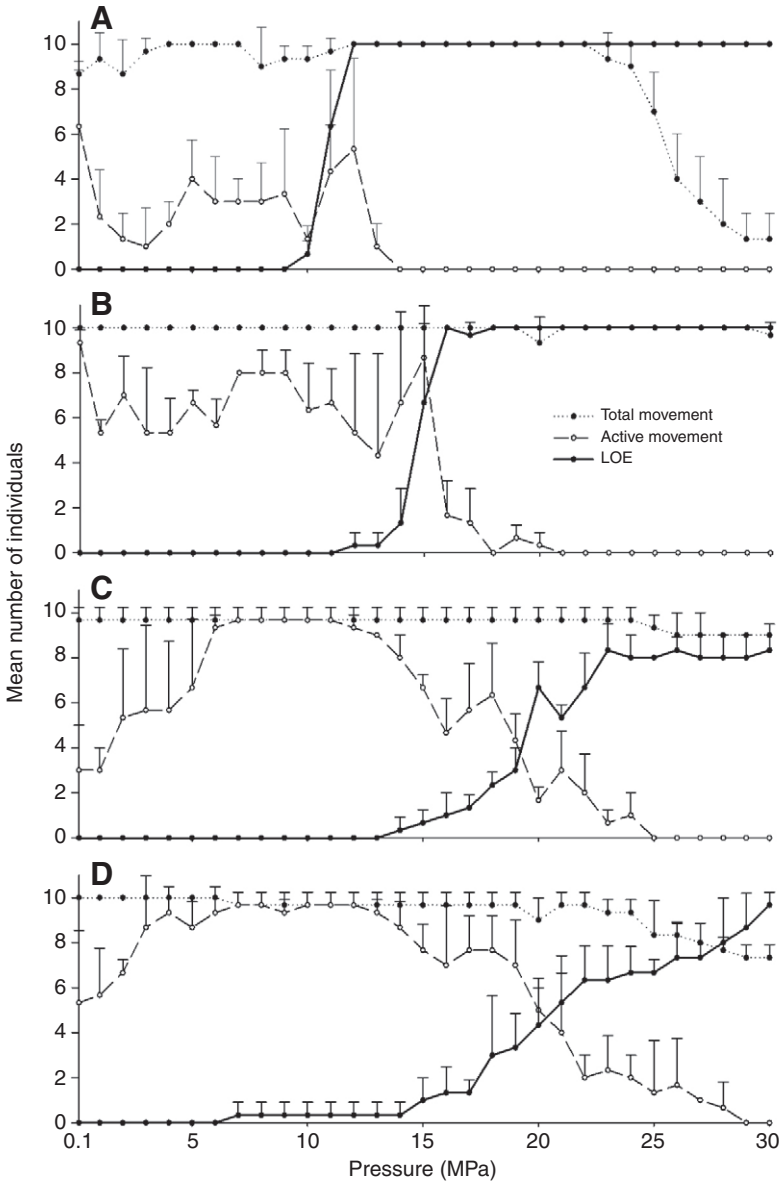

Fig. 2. Distribution of behavioural categories during pressurisation applied to *P. varians* shrimp (sampled and acclimatised in the UK) at four different temperatures: (A) 5°C, (B) 10°C, (C) 20°C, (D) 30°C. Data are presented as means (and s.d.) for three replicates of *N*=10 specimens (i.e. a total of 30 individuals at each temperature). Behavioural categories: total movement (i.e. movement + active movement = 10 – motionless), active movement and LOE. For a full explanation of the behavioural categories, see Materials and methods. NB for 20°C a total of 29 individuals were averaged; a mean of 9.7 individuals corresponds to all shrimp analysed.

At 5°C, LOE was acute, increasing from 0±0 individuals at 9 MPa to 10±0 individuals at 12 MPa. LOE in ≥50% of individuals occurred at 11 MPa. Increases in LOE observed between 10 and 11 MPa and between 11 and 12 MPa were statistically significant (P<0.001 and P<0.001, respectively).

At 10°C, total movement was 10.0±0.0 individuals at all pressures except 20 and 30 MPa (9.3±1.2 and 9.7±0.6 individuals, respectively; Fig. 2B). Relative to 5°C, active movement was also higher at 10°C; mean active movement was significantly higher than that at 5°C, and significantly lower than that at 20 and 30°C (P<0.001, P<0.001 and P<0.001, respectively). Most apparent was the shift in the pressure at which LOE occurred. At 10°C, LOE in ≥50% of individuals occurred at 15 MPa. Increased LOE observed between 14 and 15 MPa and between 15 and 16 MPa was statistically significant (P<0.001 and P=0.005, respectively). The decrease in active movement at 10°C from 8.7±2.3 individuals at 15 MPa to 1.7±1.5 individuals at 16 MPa was statistically significant (P<0.001). Significant decreases in active movement (15–16 MPa) coincided with significant increases in LOE (14–16 MPa).

During the second replicate at 20°C, a single shrimp appeared moribund prior to pressurisation. Although the shrimp was active on entry into the IPOCAMP™, it was neither upright nor active prior to the initiation of pressurisation. This individual was consequently omitted from behavioural analysis. Importantly, as only 29 individuals were considered for analysis, a mean of 9.7 individuals corresponds to all shrimp analysed. At 20°C, as pressure was increased, total movement was 9.7±0.0 individuals up to 24 MPa. At 25 MPa, total movement decreased to 9.3±0.6 individuals and reached 9.0±0.0 individuals at 26 MPa, where it remained up to 30 MPa. At 20°C, levels of active movement were initially low, increasing to their highest level at 7 MPa (9.7±0.6 individuals). Relative to levels of active movement at 0.1 MPa, the higher levels of active movement at 7–11 MPa were statistically significant (P=0.003). Active movement declined from 9.7±0.6 individuals at 11 MPa to 1.0±0.6 individuals at 24 MPa; this was statistically significant (P<0.001). No active movement was observed at pressures ≥25 MPa. At 20°C, LOE was gradual, beginning at 14 MPa (0.3±0.6 individuals) and only reaching 8.3±1.15 individuals

at 30 MPa; this was statistically significant (P<0.001). LOE in ≥50% of individuals occurred at 20 MPa.

At 30°C, levels of active movement were initially relatively low (5.3±3.2 individuals at 0.1 MPa) and increased to their highest value at 7 MPa (9.7±0.6 individuals). Unlike at 20°C, this increase was not statistically significant (P=0.954). Levels of active movement were greater than those at 20, 10 and 5°C and were statistically significant (P<0.001, P<0.001 and P<0.001, respectively). Active movement decreased gradually from 9.7±0.6 individuals at 12 MPa to 0.7±1.2 individuals at 28 MPa; this was statistically significant (P<0.001). No active movement was recorded at pressures greater than 28 MPa. LOE generally occurred between 15 and 30 MPa (reaching 9.7±0.6 individuals); this increase was statistically significant (P<0.001). LOE in ≥50% of individuals occurred at 21 MPa. A gradual decline in total movement was observed at 23 MPa, decreasing from 9.3±0.6 individuals to 7.3±0.6 individuals at 30 MPa. Combined, the effect of the interaction between pressure and temperature on behaviour was significant (P<0.001 for both active movement and LOE).

Post-experiment mortality was assessed over 3 days, at the end of which the following survival rates were observed: 5°C, 0.0% individuals; 10°C, 0.0% individuals; 20°C, 26.7% individuals (this value excludes the individual that was moribund prior to the initiation of pressurisation); 30°C, 43.3% individuals.

Effects of temperature and pressure on oxygen consumption rates

Respiration rate experiments used shrimp sampled and acclimated in the UK.

Effect of temperature

When oxygen consumption rates for a single temperature were averaged across all pressures, greater rates of oxygen consumption were observed at higher temperatures (Fig. 3A). The standard deviations also increased with temperature. The lowest mean rate of oxygen consumption occurred at 5°C [2.929(±1.973)×10^{-3} µmol O_2 mg^{-1} h^{-1}]; the highest was at 30°C [0.039±0.018 µmol O_2 mg^{-1} h^{-1}]. The differences in rate of oxygen consumption between temperatures were statistically significantly (P<0.001) except for that between 5 and 10°C (P=0.639). From 10 to 30°C, the rate of oxygen consumption increased in an approximately linear pattern. The effects of temperature on the oxygen consumption rates of adult *P. varians* were significant (P<0.001; Table 1).

Effect of pressure

The effect of pressure on the rate of oxygen consumption was more complex than that of temperature (Fig. 3B). The highest rate of oxygen consumption occurred at 15 MPa (0.024±0.020 µmol O_2 mg^{-1} h^{-1}); the lowest rate was at 30 MPa [1.881(±4.779)× 10^{-3} µmol O_2 mg^{-1} h^{-1}]. At 5 MPa (0.015±0.013 µmol O_2 mg^{-1} h^{-1}), the oxygen consumption rate was lower than that at 0.1 and 10 MPa (0.021±0.021 and 0.021±0.017 µmol O_2 mg^{-1} h^{-1}, respectively); this was statistically significant (P=0.029 and P=0.016, respectively). The rate of oxygen consumption at 5 MPa was also significantly lower than that at 15 MPa and higher than that at 30 MPa (P<0.001 and P<0.001, respectively). The difference in the rate of oxygen consumption between 0.1 MPa and 10, 15 and 20 MPa was not statistically significant (P=1.000, P=0.807 and P=1.000, respectively). At 25 MPa, the rate of oxygen consumption was significantly lower than that at 0.1, 10 and 15 MPa (P=0.014, P=0.007 and P<0.001, respectively) but was not different from that at 5 and 20 MPa (P=1.000 and P=0.065, respectively). At 30 MPa, the rate of oxygen consumption was significantly lower than that at all other pressures (P<0.001). The standard deviations were similar at all pressures except 30 MPa, where it was small (±4.779×10^{-3} µmol O_2 mg^{-1} h^{-1}). The effect of pressure on the oxygen consumption rate of adult *P. varians* was significant (P<0.001; Table 1).

Combined effect of temperature and pressure

Temperature influenced the effect of pressure on the rate of oxygen consumption in *P. varians*. At 5 and 10°C, changes in the rate of oxygen consumption with pressure were relatively small (Fig. 3C). At 20 and 30°C, these changes were much greater. The greatest variation in the rate of oxygen consumption between 0.1 and 30 MPa was observed at 30°C (s.d. of ±0.018 µmol O_2 mg^{-1} fresh mass h^{-1}); the smallest was at 5°C (s.d. of ±1.973×10^{-3} µmol O_2 mg^{-1} h^{-1}; Fig. 3C). At 20 and 30°C, an initial decrease in the rate of oxygen

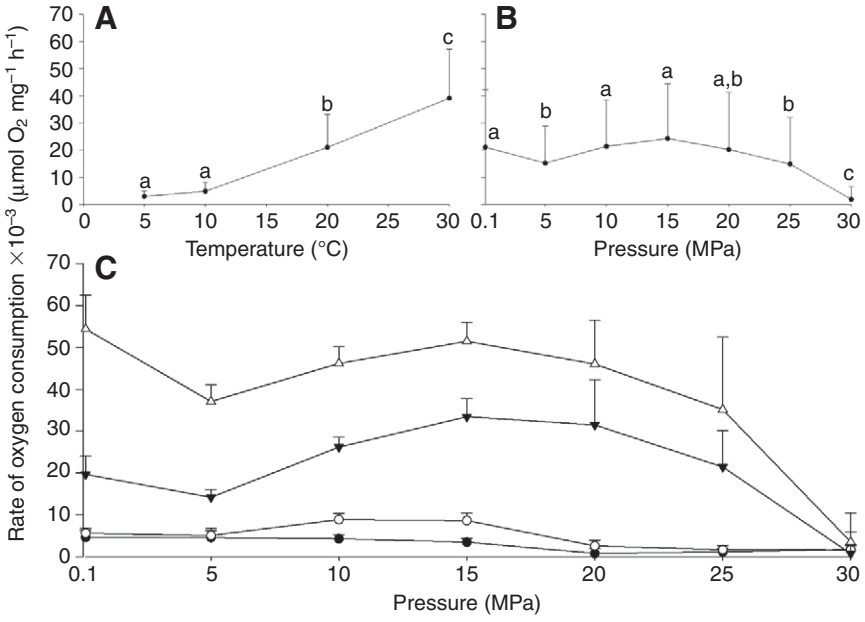

Fig. 3. (A) Rate of oxygen consumption (µmol O_2 mg^{-1} h^{-1}) of *P. varians* shrimp (sampled and acclimated in the UK) at four different temperatures (data are presented as means and s.d.). Values with different letters are significantly different. NB each value is a mean over seven pressures (0.1, 5, 10, 15, 20, 25 and 30 MPa) at a single temperature. (B) Rate of oxygen consumption (µmol O_2 mg^{-1} h^{-1}) at seven different pressures (data are presented as means and s.d.). Values with different letters are significantly different. NB each value is a mean over four temperatures (5, 10, 20 and 30°C) tested at a single pressure. (C) Rate of oxygen consumption (µmol O_2 mg^{-1} h^{-1}) under pressure at four temperatures: open triangles, 30°C; filled triangles, 20°C; open circles, 10°C; filled circles, 5°C.

Table 1. General linear model ANOVA on rates of oxygen consumption of *Palaemonetes varians* shrimp incubated at four temperatures and seven pressures

Source	d.f.	ss	ms	*F*-value	*P*-value
Temperature	3	0.0297711	0.0099237	312.34	<0.001
Pressure	6	0.0066986	0.0011164	35.15	<0.001
Interaction	18	0.0062876	0.0003493	10.99	<0.001
Error	112	0.0035585	0.0000318		

The general linear model ANOVA was carried out with pairwise comparisons using the Holm–Šidák method for 95% simultaneous confidence interval. d.f., degrees of freedom; ss, sum of squares; ms, means squares.
Shrimp were sampled and acclimated in the UK. See Fig. 3 for data.

consumption at 5 MPa was followed by an increase in rate up to 15 MPa. A subsequent decrease was then observed up to 30 MPa. This pattern was more pronounced at 30°C than at 20°C.

Pressure also influenced the effect of temperature on the rate of oxygen consumption. The greatest difference in oxygen consumption rate observed at 5 and at 30°C occurred at a pressure of 0.1 MPa ($0.049\,\mu mol\,O_2\,mg^{-1}\,h^{-1}$); the smallest occurred at 30 MPa ($0.002\,\mu mol\,O_2\,mg^{-1}\,h^{-1}$; Fig. 3C). Remarkably, at 5 MPa the difference between the oxygen consumption rates observed at 5 and at 30°C was smaller than that at 0.1, 10, 15 and 20 MPa.

The effect of the interaction between pressure and temperature on the rate of oxygen consumption was significant ($P<0.001$; Table 1).

DISCUSSION

During experiments assessing behavioural responses to pressure, LOE indicated that the normal functioning of shrimp was compromised. We are unaware of any study using LOE as an indicator of pressure-induced loss of function. However, LOE is well established as an indicator of temperature-induced loss of function (e.g. Herrera et al., 1998; Díaz et al., 2002).

Temperature and pressure effects on rates of oxygen consumption and behaviour

Palaemonetes varians lives in very shallow water habitats, where the seasonal fluctuations of environmental temperature can range from 0°C in December to 33°C in July (Lofts, 1956; Jefferies, 1964; Healy, 1997). The assessment of the lower thermal limit of specimens caught in autumn showed that *P. varians* tolerates sustained exposure to very low temperatures close to 0°C, which is consistent with the temperature encountered in its natural environment. This is considerably lower than the minimal thermal limits of other adult palaemonids reported to date, i.e. 20.5–23.0°C for *Macrobrachium tenellum* (Rodríguez and Ramirez, 1997), from 11.0±0.15 to 16.2±0.52°C for *M. acanthurus* (Díaz et al., 2002) and 14.9–16.9°C for *M. rosenbergii* (Manush et al., 2004). However, these other palaemonids are tropical species and the lowest acclimation temperature tested in the above studies was 20°C. In contrast, the lower thermal limit of *P. varians* is very similar to that of the deep-sea vent Alvinocarididae shrimp *R. exoculata*, which is assumed to be below 2°C, as neither signs of stress nor mortality were observed when decreasing the temperature to 2°C (Ravaux et al., 2009).

CT_{max} is a commonly used index for thermal tolerance, and depends on the acclimation temperature and experimental procedures (Rodríguez and Ramirez, 1997; Herrera et al., 1998; Díaz et al., 2002; Chown et al., 2009). Here we obtained a CT_{max} of approximately 31°C (based on LOE and spasm responses) for *P. varians* specimens acclimated at 10°C and subsequently subjected to a temperature ramp of $1°C\,min^{-1}$. This is lower than the CT_{max} obtained for the other adult palaemonid shrimps: *M. tenellum*, 37–43°C (Hernandez et al., 1996); *M. acanthurus*, 34.2±0.48 to 39.8±0.28°C (Díaz et al., 2002); *M. rosenbergii*, 40.7–41.96°C (Manush et al., 2004); and *P. kadiakensis*, 37–40°C (Nelson and Hooper, 1982). These tropical freshwater shrimps all live in water where the temperature is rarely cooler than 20°C (usually 28–30°C) and acclimation was therefore conducted at temperatures above 20°C. The critical thermal maximum determined for *P. varians* acclimated at 10°C is also lower than that of the closely related deep-sea Alvinocarididae shrimp *R. exoculata* (38.5°C) and *M. fortunata* (36°C) (Shillito et al., 2006), which are supposed to thrive in water with a mean temperature of 10°C and encounter temperature changes of similar amplitudes to *P. varians*. The CT_{max} of *P. varians* is more comparable to that of the temperate climate palaemonid shrimp *Palaemon serratus*, which naturally encounters temperatures in the 14–25°C range along the Mediterranean coast (Richard, 1978), and for which extreme temperature limits (the temperature at which immediate death occurs upon exposure) were reported to be in the 31–37°C range, when acclimated at temperatures within the natural range.

During experiments investigating the effects of temperature and pressure on the rate of oxygen consumption and behaviour, the maximum temperature condition studied was 30°C, which is very close to the CT_{max} of 31°C. The shrimps subjected to pressurisation experiments at 30°C did not, however, appear stressed by heat. *Palaemonetes varians* specimens subjected to the CT_{max} determination experiments were acclimated at 10°C and brought to 31°C in less than 1 h; those subjected to pressurisation experiments were progressively acclimated to 30°C for 3 days. As both acclimation temperature and thermal ramping rate are known to affect CT_{max} (Rodríguez and Ramirez, 1997; Herrera et al., 1998; Díaz et al., 2002; Chown et al., 2009), this could explain the apparent difference in thermal tolerance between these two batches of shrimp.

An attempt was made to assess pressure tolerance at 35°C; however, at this temperature 82% mortality was observed after a 3 day acclimation period. A second trial was made by repeating the acclimation process and the shrimps then tolerated temperatures of approximately 33°C for a 24 h period; however, at 35°C mortality reached 84% within 14 h. The highest temperature at which the pressure tolerance of *P. varians* was assessed during this study was therefore 30°C.

The oxygen consumption rate obtained for *P. varians* increased with temperature. This has previously been demonstrated for caridean shrimp (Achituv and Cook, 1984; Allan et al., 2006) and is not surprising considering that metabolic rate is known to increase exponentially with temperature as a result of elevated kinetic energy in biological systems (Clarke and Fraser, 2004). The marginal effect of pressure on the rate of oxygen consumption at 5 and 10°C indicates that temperature is the dominant factor affecting metabolic rate. This is in agreement with the results of Thatje and colleagues, who found that the standard metabolic rate of the shallow-water hermit crab *Pagurus cuanensis* was more greatly influenced by temperature than by pressure (Thatje et al., 2010). The capacity for oxygen supply is often limited within organisms at upper and lower temperature extremes; consequently, a loss of functional performance results from the inability to maintain aerobic respiration (Pörtner, 2001). Oxygen supply is therefore thought to be a critical determinant of CT_{max} (and critical minimum

temperature, CT_{min}); the performance of respiration and circulatory systems being uncoupled from the oxygen demands of the tissue (Pörtner, 2001). The relationship between respiration and circulatory system performance and oxygen demand in the tissue of an organism with respect to hydrostatic pressure is poorly studied and remains unclear (Thiel et al., 1996).

During behavioural experiments, levels of activity were found to increase with temperature. Consequently, elevated rates of oxygen consumption observed at higher temperatures might be attributed to increased rates of metabolism [see Clarke and Fraser (Clarke and Fraser, 2004) and references therein]. At 20 and 30°C, levels of active movement initially increased with increasing pressure and high levels of active movement generally occurred between ~7 and 10 MPa. Increased levels of activity may indicate a pressure-initiated stress response. Elevated swimming activity in response to increased hydrostatic pressure has been observed in crustacean larvae as an example of behavioural homeostasis (Hardy and Bainbridge, 1951; Knight-Jones and Qasim, 1955; Gherardi, 1995). Elevated levels of active movement exhibited by *P. varians* may represent an escape response to maintain its optimal bathymetric distribution. Such behaviour indicates that pressure was detrimental to individuals and suggests that the pressure tolerance demonstrated here might be short term. Pressure appears to initiate a stress response whereas temperature, limiting metabolic potential, constrains the magnitude of this response.

Temperature had considerable influence on the tolerance to hydrostatic pressure exhibited by *P. varians*. At 5 and 10°C, LOE in ≥50% of individuals occurred at 11 and 15 MPa, respectively. At 20 and 30°C, LOE in ≥50% of individuals was delayed until pressure reached 20 and 21 MPa, respectively. The effects of pressure and temperature on biological systems are usually antagonistic; i.e. an increase in pressure has similar effects to a decrease in temperature, reducing kinetic energy and causing ordering of molecules (Pradillon and Gaill, 2007). Indeed, low temperatures are known to exacerbate the effects of pressure on shallow-water invertebrate fauna (e.g. Young et al., 1997; Villalobos et al., 2006). Similarly, in a study investigating the effects of pressure/temperature combinations on the hydrothermal vent crab *Bythograea thermydron*, Airriess and Childress concluded that higher temperatures precluded disruption of membrane systems by elevated hydrostatic pressure (Airriess and Childress, 1994).

Lipid bilayers are highly sensitive to the effects of pressure and as such have been extensively studied (e.g. MacDonald, 1984; Somero, 1992; Pradillon and Gaill, 2007). In shallow-water organisms, pressure increases tend to reduce membrane fluidity; their deep-sea counterparts exhibit homeoviscous adaptations to maintain membrane fluidity under high pressure (Somero, 1992). Antagonistic to pressure, increasing temperatures enhance membrane fluidity (Hazel, 1995). In shallow-water organisms (excluding fauna of high latitudes), high temperatures may alleviate some pressure effects through enhanced membrane fluidity. This may explain the greater tolerance of pressure exhibited by *P. varians* under warmer conditions. At 30 MPa, rates of oxygen consumption across all temperatures were very low and analogous. This aggregation of data points may indicate a threshold pressure above which the antagonistic effects of temperature become insignificant.

Post-experimental mortality was temperature dependent, occurring after pressurisation at 20 and 30°C only. Mortalities of 26.7 and 43.3% were observed at 20 and 30°C, respectively. Whilst the effects of pressure and temperature on most biological systems are usually antagonistic, their effects on proteins are known to be synergistic; promoting the denaturing of proteins and, consequently, loss of function (Balny et al., 1997; Pradillon and Gaill, 2007). At 20 and 30°C, the combined effects of temperature and pressure may have resulted in the denaturation of proteins, leading to the observed mortalities. The zero mortality following depressurisation at 5 and 10°C suggests that the effects of pressure at these temperatures were reversible. The reversible effects of pressure observed at low temperatures can probably be attributed to a reduced kinetic energy in biological systems.

In summary, we have demonstrated *P. varians*' physiological capacity to tolerate hydrostatic pressures found outside its normal bathymetric distribution under varied thermal conditions. It is probable that this physiological capability was inherited from an ancestral species. As such, physiological capability coupled with the bathymetric distribution of closely related extant bresiliid shrimp species may reflect the baro-physiological plasticity of a common ancestral species.

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