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The effect of temperature on pressure tolerance of the shallow-water shrimp *Palaemon serratus*.

Tesi di laurea in

Adattamenti degli animali all'ambiente marino

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CONTENTS

1. ABSTRACT

Tolerance to low temperature and high pressure may allow shallow-water species to extend bathymetric range in response to changing climate, but adaptation to contrasting shallow-water environments may affect tolerance to these factors. The brackish shallow-water shrimp *Palaemon varians* demonstrates remarkable tolerance to elevated hydrostatic pressure and low temperature, but inhabits a highly variable environment: environmental adaptation may therefore make *P. varians*' tolerances unrepresentative of other shallow-water species. Critical thermal maximum (CT_{max}), critical hydrostatic pressure maximum (CP_{max}), and acute respiratory response to hydrostatic pressure were assessed in the shallow-water shrimp *Palaemon serratus*, which inhabits a more stable intertidal habitat. *P. serratus*' CT_{max} was 22.3°C when acclimated at 10°C, and CP_{max} was 5.9, 10.1, and 14.1 MPa when acclimated at 5, 10, and 15°C respectively: these critical tolerances were consistently lower than *P. varians*. Respiratory responses to acute hyperbaric exposures similarly indicated lower tolerance to hydrostatic pressure in *P. serratus* than in *P. varians*. Contrasting tolerances likely reflect physiological adaptation to differing environments and reveal that the capacity for depth-range extension may vary among species from different habitats.

2. INTRODUCTION

2.1 Colonisation of the deep sea

The deep sea, deeper than 200 m, is the largest environment on earth, covering >90 % of the surface area of the oceans (Brown and Thatje 2014). Deep-sea fauna are thought to result from the colonization of the deep sea by shallow-water species following regional mass extinction events (Kussakin 1973; Tyler et al. 2000). Regional deep-sea extinctions resulted from dysoxic events in the deep oceans that were driven by shifts in ocean circulation in response to climatic change during the Paleozoic and Mesozoic eras. These periods of deep-sea hypoxia and anoxia resulted in the extinction of most species, particularly benthic crustaceans and molluscs (Rogers 2000). Consequently, extant deep-sea fauna contain both ancient and relatively recent shallow-water lineages (Wilson 1999). Phylogenetic analyses have supported the relatedness of extant deep-sea and shallow-water species, predominantly consistent with diversification and invasion of deep-sea environments from shallow-water, albeit over differing timescales (see Brown and Thatje 2014 and references cited therein). Phylogenetic relationships, for the initial colonization of deep continental margins, have been determined between shallow-water and deep-sea lineages of the lithodid crab (Hall and Thatje 2009). Similarly, evidence to represent multiple deep-sea colonisations over differing timescales has been provided by Raupach et al. (2009) and for multiple colonization of the deep by at least four major lineages of isopods within the Asellota. Distel and colleagues (2000) showed Bathymodiolinae mussels from hydrothermal vents and cold seep were closely related to other deep-sea taxa. These mussels have been found on sunken wood and whalebones and all these appeared to have derived from shallow-water Mytilinae mussels. Furthermore, phylogenetic relationships representing hydrothermal vent colonisation by taxa more closely related to the present study species have been determined between shallow-

water palaemonid shrimp and alvinocarid hydrothermal vent shrimp (Tokuda et al. 2006; Li et al. 2011).

The cold temperatures prevailing in the deep sea are thought to limit colonisation by shallow-water species, which are adapted to the warmer conditions of the upper ocean (except at high latitudes) (Young et al. 1997). Colonisation of the deep ocean may therefore have occurred during the Mesozoic and early Cenozoic periods (roughly from 250 Ma to 65 Ma ago) when the oceanic water column was warm and isothermal. The formation of isothermal water columns permits access to the deep-sea without presenting a pronounced temperature gradient (Young et al. 1997, Brown and Thatje 2014). Similar conditions currently exist within the Mediterranean Sea, where the sill at Gibraltar prevents incursion of the cold dense isotherm water that keeps the abyssal temperature throughout most of the ocean below 4°C. Bottom temperature in the Mediterranean Sea at 2000 or 3000 m is between 12 and 14°C, that are the warm isotherm water, which at some times of the year is only a few degrees cooler than the surface temperature. For this reason, temperature is not expected to limit migrations to greater depth in the Mediterranean, and over evolutionary timescale (Young et al. 1997). Indeed, currently profiles of temperature and pressure are probably similar to those prevailing throughout Mesozoic and Cenozoic seas (Young et al. 1997). It has also been suggested that cold-adapted species at high latitudes may have colonised the deep-sea through regions of deep-water formation (Tyler and Dixon 2000; Thatje et al. 2005). High latitude shallow-water species are adapted to deep-sea temperature, due to their cold-stenothermal lifestyle, which is thought to make deep-sea colonisation possible in regions of deep-water formation (Tyler and Young 1998). Subsequent increases in biodiversity occurred through differentiation and adaptive radiation (Brown and Thatje 2014). Dispersal into the deep ocean may be possible for shallow-water invertebrates, facilitated by the great temperature and pressure tolerances of their embryonic and larval stages (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; but see Mestre et al. 2013).

Recently, has also been suggested that range extension of shallow-water species towards the deep-sea could be driven by climate change causing warming of surface waters (see Brown & Thatje 2015). Depending on the emission scenario, projections of oceanic warming predict an increase in global average sea-surface temperature, ranging from about 1°C to more than 3°C for the period 2081–2100, relative to the period 1986–2005 (Collins et al. 2013). Increasing temperature will affect taxa inhabiting the continental shelf and upper continental slope first. The entire ocean will eventually warm up reasonably uniformly by the amount of the surface increase, affecting even abyssal organisms (see Brown and Thatje 2015). Climate projections to 2300 predict that sea surface and subsurface temperatures will rise by between 6 and 7 °C at low latitudes and ~10 °C at high latitudes, with the deep-sea warming by 2–5 °C (Schmittner et al. 2008). Changing ocean temperatures may result in increases in depth range extensions by shallow-water species (Tyler and Young 1998), but these species must also tolerate hydrostatic pressure, and oxygen and carbon dioxide concentrations in the deep sea (see Brown & Thatje 2015 and citations therein). Species that are unable to respond to environmental changes may otherwise face extinction (Peck 2005).

2.2 Temperature and hydrostatic pressure tolerance

Temperature and hydrostatic pressure are critical factors determining the distribution of organisms in the oceans (Pradillon and Gaill 2007). Tolerance of low temperature and high hydrostatic pressure by shallow-water animals has previously been assessed to determine differences in physiological responses among species. Hydrostatic pressure is the only constant gradient with depth in the oceans, increasing by 0.1 MPa every 10 m. Temperature has a strong gradient with latitude, generally decreasing towards the poles, and typically also

has a strong gradient with depth, generally decreasing with increasing depth (Gage & Tyler 1991). The effects of both low temperature and high pressure must be overcome for survival in the deep sea: they are two key stressors that may act as physiological limit on the ability of shallow-water organisms to tolerate deep-sea conditions and it has been suggested that the synergistic effect of both may cause of a physiological bottleneck at 2,000–3,000 m (Brown and Thatje 2011; 2014). As thermodynamic variables, temperature and hydrostatic pressure affect biochemical equilibrium and rates of biological processes in similar ways (Brauer and Torok 1984). Low temperatures reduce the energy available in a system or reaction. High hydrostatic pressure increases the rate of reactions that require a volume decrease, but retards those that require a volume increase (Somero 1992; MacDonald 1997). Lipid bilayers of cell membranes are influenced by temperature and hydrostatic pressure (Farkas et al. 1988; Behan et al. 1992). Increased ordering of the lipids due to low temperature and high hydrostatic pressure reduces the fluidity and permeability of the membrane, limiting the movement of molecules across the bilayers and impeding membrane functions such as cell signalling (Hazel and Williams 1990). The effect of these physiological stress conditions may be loss of coordination defined 'Loss of Equilibrium' (Ravaux et al. 2003; Shillito et al. 2006; Oliphant et al. 2011). Reducing the ordering effects on the membrane is necessary to allow survival under high pressure and low temperature, as otherwise membrane functions cease (Hazel 1995; Somero 1992) and the effects of low temperature and high pressure are moderated by homeoviscous acclimatization that allows increasing the proportion of unsaturated to saturated fatty acids in lipid bilayer membranes (Hazel 1995; Somero 1992). This increases the fluidity of the membranes because unsaturated fatty acids decrease ordering of the lipids (Cossins and MacDonald 1989).

Examining temperature and hydrostatic pressure tolerance in shallow-water fauna can reveal constraints to range extension imposed by these factors and deliver insight into the

mechanism of deep-sea colonisation. The shallow-water shrimp *Palaemon varians* (previously *Palaemonetes varians,* because the genus *Palaemonetes* is demonstrated to be a junior synonym of the genus *Palaemon*; DeGrave & Ashelby 2013) has emerged as model taxon for hyperbaric and thermal stress physiology (e.g. Cottin et al. 2010, 2012; Oliphant et al. 2011, Ravaux et al. 2012; Smith et al. 2012; New et al. 2014; Morris et al. 2015a,b). These studies investigated *P. varians*' temperature and hydrostatic pressure tolerance to assess its thermal and pressure tolerance window, and have explored behavioural, respiratory, and molecular responses. Moreover, these studies assessed how temperature influenced pressure tolerance and demonstrated that decreasing temperature decreased pressure tolerance.

There is clear evidence that *Palaemon varians*, which lives in shallow-water (0-10 m), can tolerate a wide scale of temperature and pressure combinations for a sustained period (Cottin et al. 2012; New et al. 2014). It has been hypothesised that this tolerance could reflect the physiological capability of an ancestral species to colonise bathyal depths (Cottin et al. 2012). However *Palaemon varians* may not be representative of shallow-water marine shrimp species: *P. varians* inhabits brackish waters with variable temperature and salinity and is both eurythermal and euryhaline. Shallow-water species that experience a more typical shallow-water marine environment such as *Palaemon serratus* may be more representative and may be less tolerant to variable conditions.

2.3 *Palaemon varians* and *Palaemon serratus*

Palaemon varians (Fig 2.1) is found in brackish waters from Northern Europe to the coasts of Morocco and in the Mediterranean Sea. This species is commonly found in saline, lagoons, saline ponds, and salt marsh ponds (e.g. Fig. 2.2) (Muus 1967; Barnes 1994; Hayward and Ryland 1995). These environments are typically of highly variable salinity and temperature (González-Ortegón et al. 2013): *Palaemon varians* in these environments is exposed to wide

range of temperatures, from 0°C to 33°C across seasons, with an increase or decrease of more than 5°C that can occur in only 12 h period (Jefferies 1964). These environments typically also have large and rapid shifts in oxygen concentration, and *P. varians* is tolerant of variability in oxygen concentration and even hypoxia (Gonzalez-Ortegon 2013).

Fig. 2.1 ***Palaemon varians***

Fig. 2.2 Lymington salt marsh: a typical habitat inhabited by ***Palaemon varians*****.**

Palaemon serratus (Fig. 2.3; 2.4) inhabits intertidal and subtidal waters up to 40 m (Hayward and Ryland 1995). This species can grow up to 11 cm and is exploited as a fishery resource, especially in Great Britain and Ireland (Vinagre et al. 2013). *P*. *serratus*' environment is typically less variable than *P*. *varians*, with temperature, from a minimum of 5°C during winter to a maximum of 22°C during the summer, and the salinity level is almost constant during the year at 35‰. Similarly, *P. serratus* experiences less oxygen variability and hypoxic events in its habitat. Since its habitat is a typically less variable environment than *P*. *varians*, *P*. *serratus* may be expected to have a narrower environmental tolerance window than *P. varians*.

Fig. 2.3 ***Palaemon serratus.***

Fig. 2.4 Size comparison between *Palaemon varians* (left) and *Palaemon serratus* (right).

2.4 Purpose of the study

P. varians may be representative of physiological responses to high hydrostatic pressures and low temperatures for shallow-water crustaceans. However *P. varians* may be preadapted to tolerate hydrostatic pressure through adaptations to the fluctuating temperature and salinity experienced within its habitat. Consequently, the aim of this study was to determine whether hyperbaric tolerance and its interaction with temperature differ in *P. serratus*, a congener inhabiting a less variable shallow-water marine environment.

The objectives of this study were to:

1) assess the Critical thermal maximum of *Palaemon serratus* in order to determine its temperature tolerance window;

2) assess the Critical pressure maximum of *Palaemon serratus* at different temperatures, 5, 10, 15°C, below the critical thermal maximum, to determine the maximum pressure tolerance of this species at each temperatures;

3) determine how different combinations of temperature and pressure influence the respiration rate of *Palaemon serratus*.

3. MATERIALS AND METHODS

3.1 Sampling methods

Sampling was conducted from March to May 2015 (20-24/04; 7-8-19-20/05) at Calshot, Fawley, New Forest, UK (50°48' N, 1°19' O) (Fig. 3.1). Sampling was performed using hand-held nets in shallow-water (<1 m water depth) during low tide. *Palaemon serratus* were placed inside 10 l buckets containing seawater from the point of collection. Shrimp were transported to the National Oceanography Centre Southampton, UK (NOCS) and were maintained in a recirculating seawater aquarium system set at the water temperature of the sampling location (Fig. 3.2) to reduce potential acclimation stresses and allow specimens to recover from sampling. Prior to experimental treatments shrimp were transferred to 10 l tanks filled with filtered seawater (1-µm filtered; salinity 32.7) and submerged in water baths controlled by HAAKE EK20 chiller and a HAAKE DC10 heater to maintain constant temperature (Fig. 3.3; 3.4). Temperature was initially set at maintenance temperature, and acclimated stepwise to the desired experimental temperatures (5, 10, 15°C) for a period of 3 days; photoperiod was set to 12h:12h light:dark. Aeration was provided using bubble stones attached to air pumps. Shrimp were not fed during this period to reduce potential variability in responses during experimental treatments due to differences in digestive state.

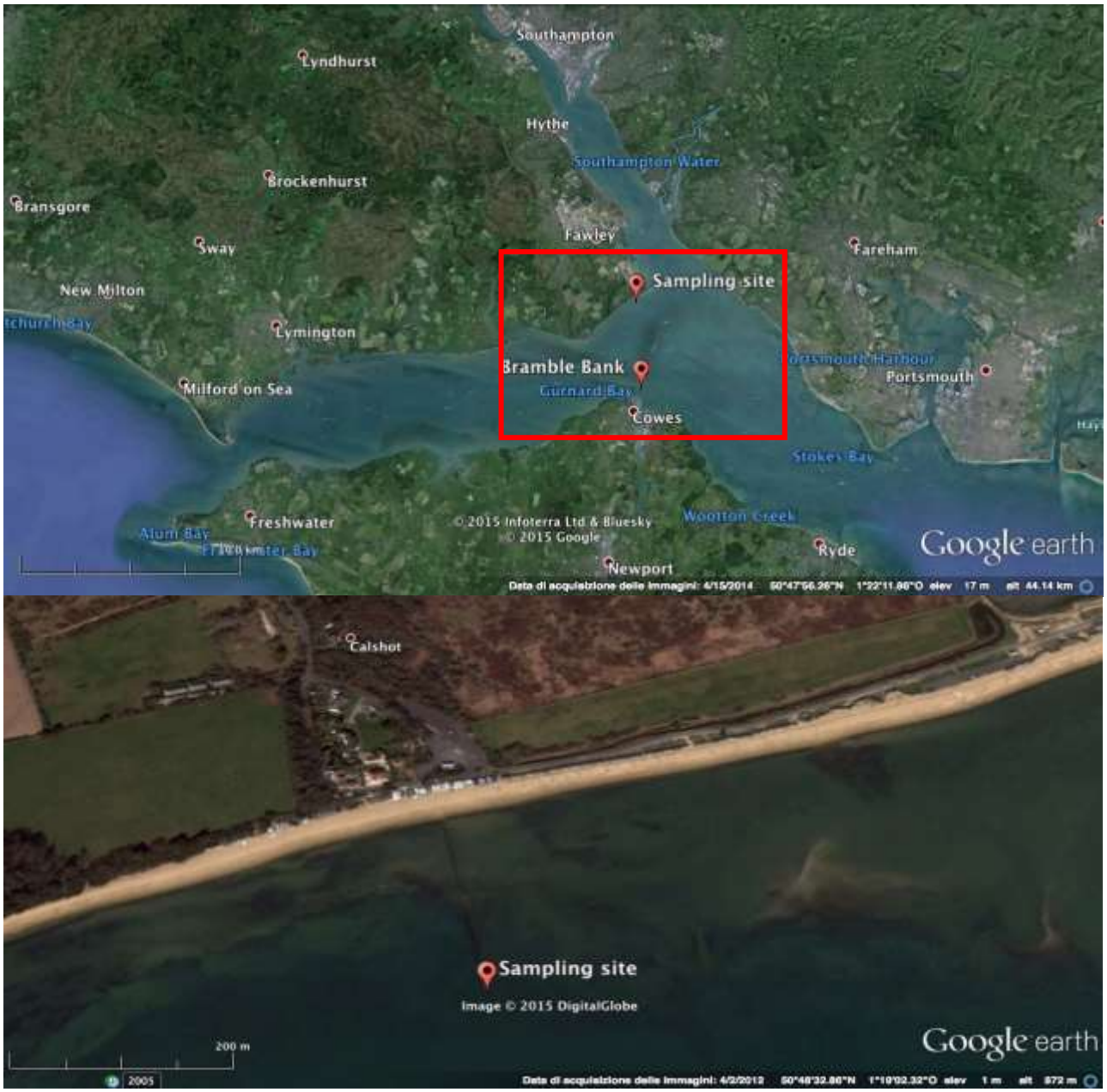

Fig. 3.1 Sampling location at Calshot, UK.

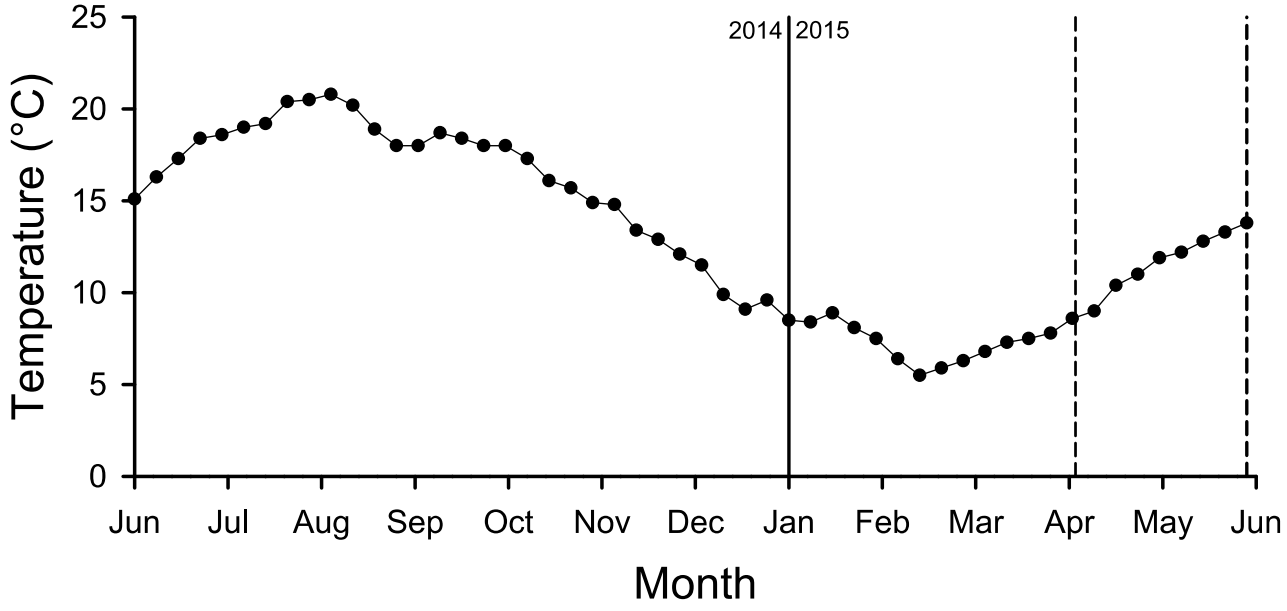

Fig. 3.2 Mean water temperature on Bramble Bank (50°46'21''N; 1°17'56''O) each Monday from June 2014 to May 2015, used as a proxy for water temperature at Calshot (data obtained from www.bramblemet.co.uk). Vertical dashed lines show the beginning and the end of the sampling of *Palaemon serratus*.

Fig. 3.3 Aquaria where specimens of *Palaemon serratus* were maintained prior to acclimation to experimental temperature.

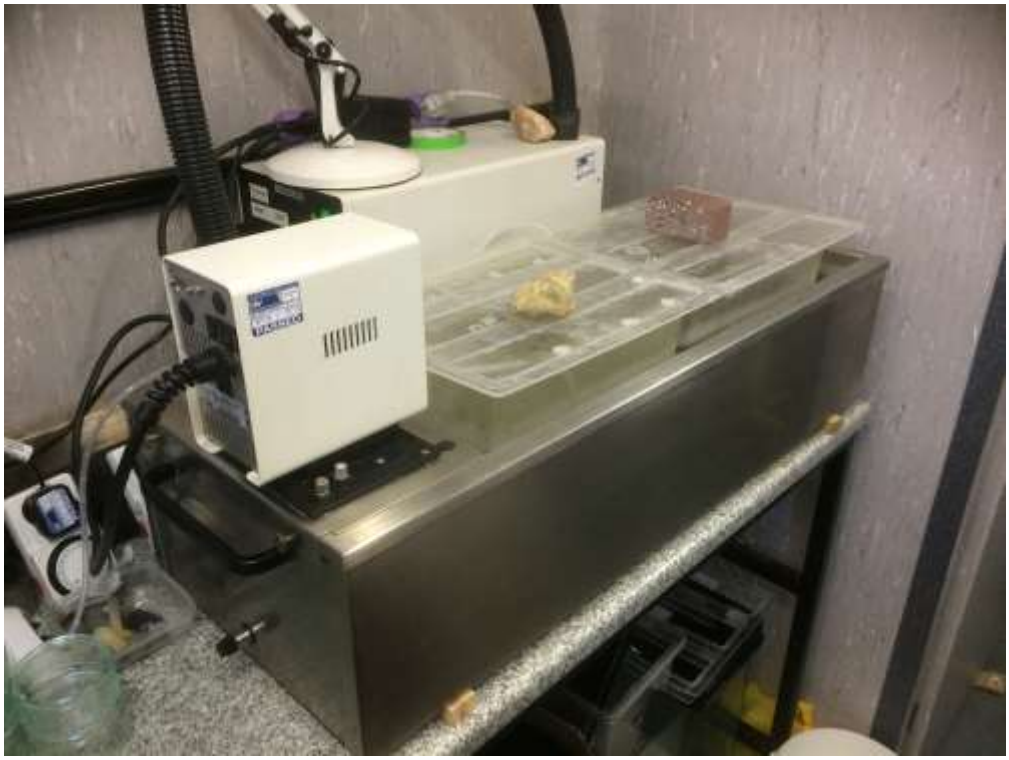

Fig. 3.4 Example of water bath where shrimps were acclimated to experimental temperature prior to treatment. Weighted covers were used to avoid shrimps escaping from the tanks.

3.2 Pressurized incubator IPOCAMP

All treatments to determine critical thermal maximum (CT_{max}) and critical pressure maximum (CP_{max}) were performed within the high-pressure flow-through IPOCAMP (Incubateur Pressurisé pour l'Observation et la Culture d'Animaux Marins Profonds) pressure system. The stainless steel IPOCAMP pressure chamber has a volume of ca. 19 l, with an aperture of 20 cm, capable of simulating pressure conditions up to 3000 m depth (30 MPa) (Shillito et al. 2014) (Fig. 3.5, 3.6). IPOCAMP is a flow-through system which circulates seawater at flow rates that may reach 20 l/hour. It is designed to allow *in vivo* experimentation at elevated pressure (Shillito et al. 2014). A tank that provides seawater to the main system is connected to the high-pressure pump. The pump pressurizes the water, which is further circulated through a heat-exchange device prior to its entry in the vessel to ensure water is acclimated to

experimental temperature (Shillito et al. 2014). 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.

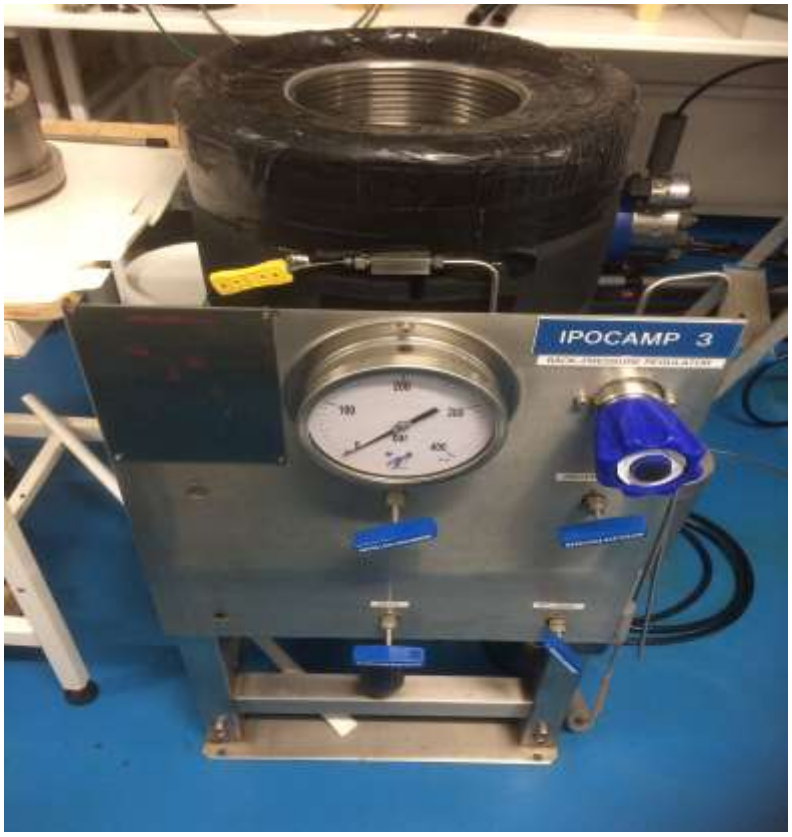

Fig. 3.5 IPOCAMP pressure chamber.

Pressure oscillations due to pump strokes (100 r.p.m.) are <0.1 MPa at working pressure. The temperature of the flowing seawater (filtered at 0.1 μm) is measured constantly, under pressure, in the inlet and outlet lines (±1°C). Temperature regulation is powered by a regulation unit (Huber CC 240, Offenburg, Germany), which circulates ethylene glycol through steel jackets that surround the main chamber, and around the seawater inlet line. Finally, IPOCAMP allows video observations of the organisms through three separate view-ports, each offering a vertically descending view inside the pressure vessel (Shillito et al. 2006). Inside IPOCAMP specimens are placed within a PVC cylinder of diameter 9 cm, which is mounted on a tripod platform, topped by an inclined translucent lid, resulting in a height of 15 cm. An endoscope (Fort, Dourdan, France) connected to a CCD camera (JVC, TK-C1380) is inserted in

a given vertical view-port. The platform elevates the cage, allowing a clear view via an endoscope inserted into a central viewing port in the lid of the IPOCAMP (Ravaux et al. 2003; Shillito et al. 2006) (Fig. 3.6). The resulting view of the inside of the pressure vessel is then displayed on a TV monitor (JVC), and recorded with a SONY digital HDV recorder (HVR-M25AE). Prior to each treatment IPOCAMP was set to run for a 2 h period to ensure the whole system was acclimated to the desired experimental temperature. Both temperature and pressure within the system were recorded using a temperature/pressure data logger (SP2T4000, NKE instrumentation) during critical thermal and critical pressure maximum treatments (Fig. 3.7). Water temperature within the chamber was also measured using a thermometer prior to experimental treatments.

Fig. 3.6 The lid of the IPOCAMP comprises two circular parts. Windows 1, 2, 3 are used to provide lighting; window 4 is used to view the inside of the pressure vessel using an endoscope.

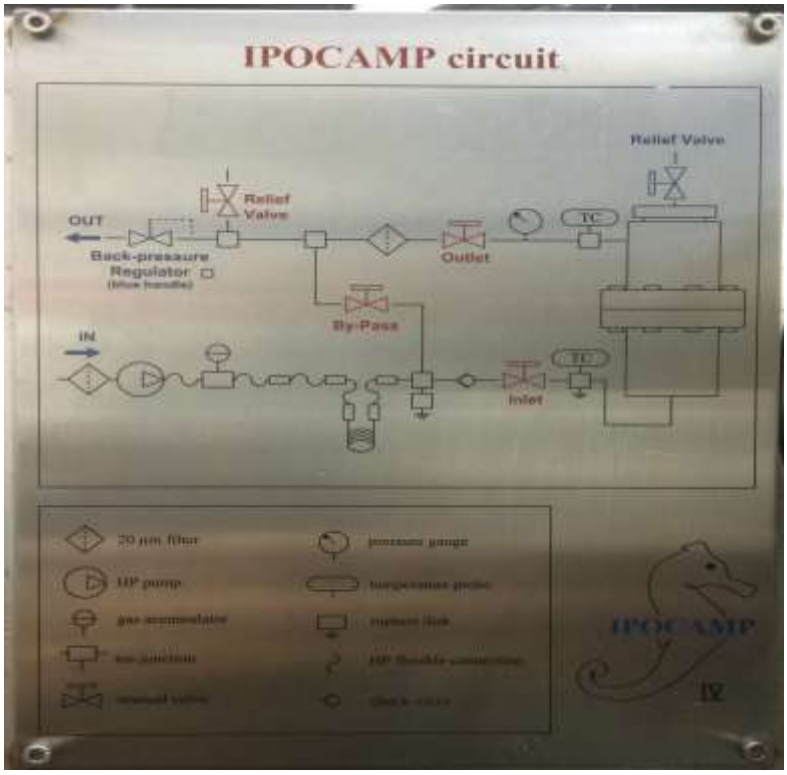

Fig. 3.7 IPOCAMP system water circuit diagram.

3.3 Assessment of CT_{max}

During CT_{max} treatments, the IPOCAMP system was run at atmospheric pressure (0.1 MPa) at all times. In each of three replicates, ten shrimp were placed inside the PVC cage with an inclined lid within the IPOCAMP and allowed a 1 h acclimation and recovery period at atmospheric pressure. The temperature within the system was then increased at a constant rate by 0.29°C min^{-1} from 10°C up to 35°C (after New et al. 2014). Upon reaching 35°C the system was switched off: shrimp were removed from the IPOCAMP immediately and preserved at -80°C for subsequent measurement and sex determination.

3.4 Assessment of CP_{max}

Results from the CT_{max} treatments identified the window of thermal tolerance of *Palaemon serratus* and consequently determined the exposure temperatures for CP_{max} treatments. CP_{max} treatments were performed at three different temperatures: 5, 10, 15°C. In each of 3

replicates at each experimental temperature, ten shrimp were placed inside the PVC cage with an inclined lid within the IPOCAMP and allowed a 1 h acclimation and recovery period at atmospheric pressure. The pressure within the system was then increased stepwise by 1 MPa 5 min^{-1} up to 30 MPa and then subsequently decreased to 0.1 MPa in the same stepwise manner (0.1 MPa = 10 m water depth). To identify the period of pressure increase during video analysis, lighting was briefly dimmed during every pressure change. Once back at atmospheric pressure, the system was left to run overnight before shrimps were removed from the IPOCAMP and preserved at -80°C for subsequent measurement and sex determination (Fig. 3.8).

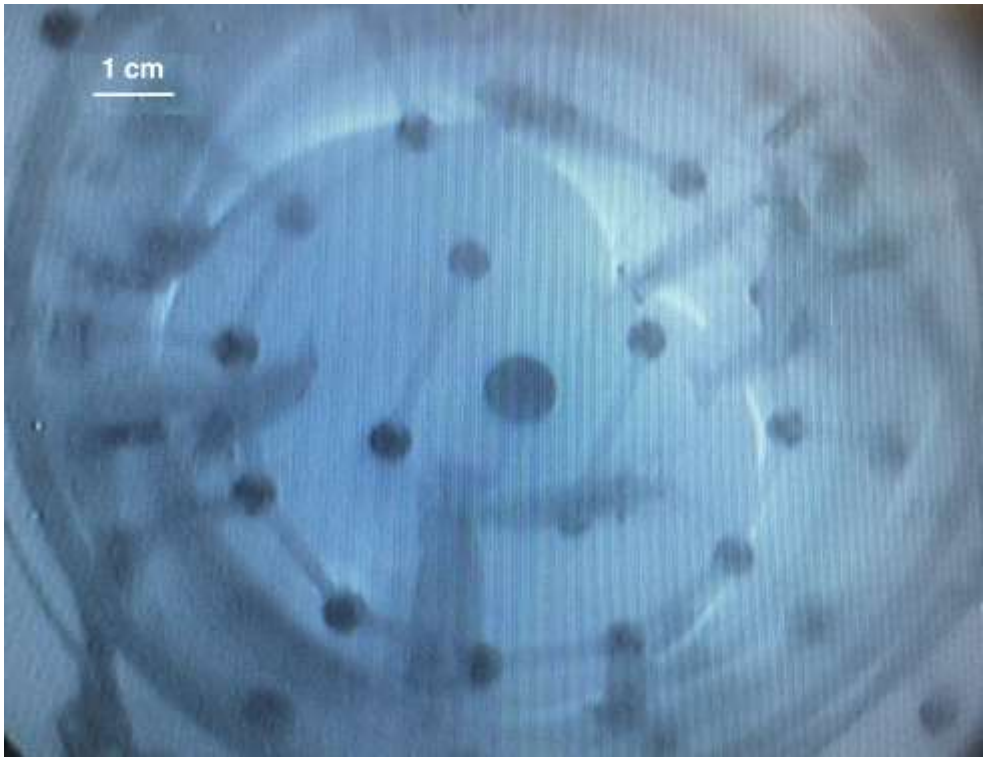

Fig. 3.8 Vision of specimens of *Palaemon serratus* at 15°C at 11 MPa.

3.5 Behavioural responses to increasing temperature and pressure

The behaviour of each individual shrimp was assessed post-treatment through video analysis. For CT_{max}, behaviour was analyzed for the 15 s before and after the temperature increasing of 1°C within the system (New et al. 2014). For CP_{max}, behaviour in response to pressure was

determined for the final 30 s at each pressure (Oliphant et al. 2011); this allowed animals an opportunity to recover from acute shock initiated by each pressure increase. Each individual was identified and its behaviour classified into four categories according to previous studies (following Ravaux et al. 2003; Shillito et al. 2006; Oliphant et al. 2011; Ravaux et al. 2012; New et al. 2014; Morris et al. 2015), as follows:

- 'Motionless'; when no movement was detected at normal tape-reading speed; in this category there were also individuals that, even if neighbouring shrimps were 'pushing', they were not reacting in any apparent way.
- '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' (AM); when the shrimp moved (walked or swam) a distance exceeding their body length in less than 30 s.
- 'Loss of equilibrium' (LoE); when a given shrimp layed on the bottom in either an 'upside-down' or a 'sideways' position for more than 2 s (Fig. 3.9).

LoE could co-occur with any of the other three categories; Movement, Active Movement and Motionless were all mutually exclusive. AM and LoE were interpreted as indicator behaviours of stress and loss of function, respectively (following Ravaux et al. 2003; Shillito et al. 2006; Oliphant et al. 2011; Ravaux et al. 2012; New et al. 2014; Morris et al. 2015). CT_{max} and CP_{max} were assessed as the temperature or pressure at which 50 % of shrimp experienced LoE. Values for CT_{max} and CP_{max} were calculated from the equations of survival curves fitted to LoE data (see statistical analysis below).

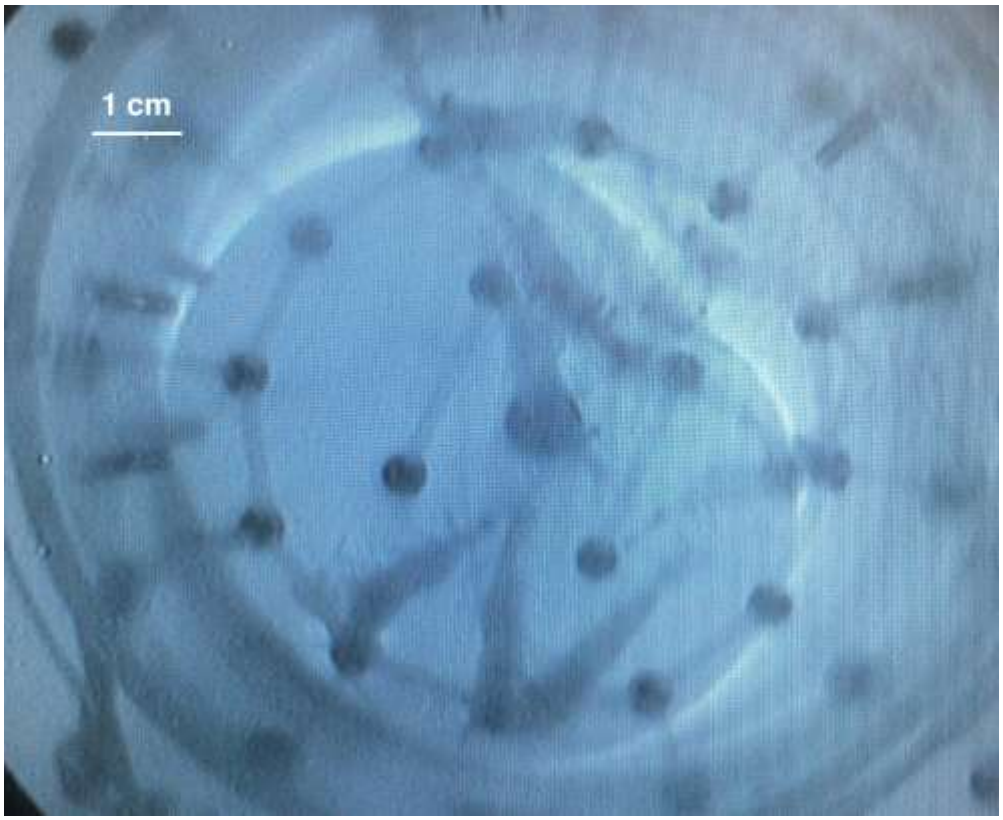

Fig. 3.9 Specimens of *Palaemon serratus* at 15°C at 21 MPa. Shrimps were laying laterally, indicating Loss of Equilibrium.

3.6 Oxygen consumption at different temperatures and pressures combinations

Individual animals were transferred to 55 ml plastic vials filled with filtered seawater (1µm filtered; salinity 32.7) 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. A single vial was placed inside a pressure vessel (Fig. 3.10) (Mestre et al. 2009) filled with freshwater; both the pressure vessel and the freshwater were pre-incubated at the experimental temperature (5, 10, 15°C). Pressurisation to experimental pressure (0.1, 5, 10, 15, 20, 25, 30 MPa) was continuous and was achieved using a MAXIMATOR anlage, model manual hydraulic pump, model 3310-0923 (Fig. 3.11) (see Mestre et al. 2009). Pressurisation was acute, taking approximately 10 s or less. Pressure vessels were placed in a temperature-controlled incubator during the experimental period. 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 exposure duration to ensure that the final oxygen concentration within the vial did not fall below 50% of initial concentrations to avoid possible effects of hypoxia on the respiratory capacity of the shrimps, determined through preliminary treatments (Hagerman & Uglow 1984; Nielsen & Hagerman 1998; Gonzalez-Ortegon et al. 2013). Animals were isolated for 40min at 5°C, 30 min at 10°C, 20 min at 15°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 micro-optode (Fig. 3.12) (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). The micro-optode was calibrated with fully aerated seawater that had been left to settle for 15 min (100% O_2 saturation) and seawater deoxygenated by over-saturation with sodium sulphite (Na_2SO_3) (0% O_2 saturation) (Thatje et al. 2010). Calibration solutions were incubated at experimental temperature. Calibrations were performed every day prior to treatments.

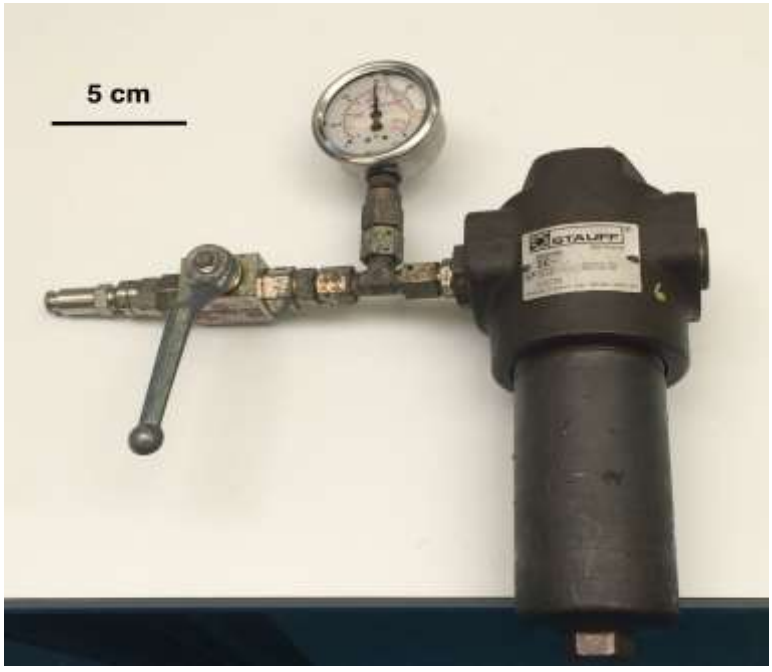

Fig. 3.10 Pressure vessel pressurized at 30 MPa. Inside the vessel the vial, containing a shrimp in seawater, was submerged in freshwater to avoid corrosion.

Fig. 3.11 MAXIMATOR hydraulic pump used to pressurize the vessels.

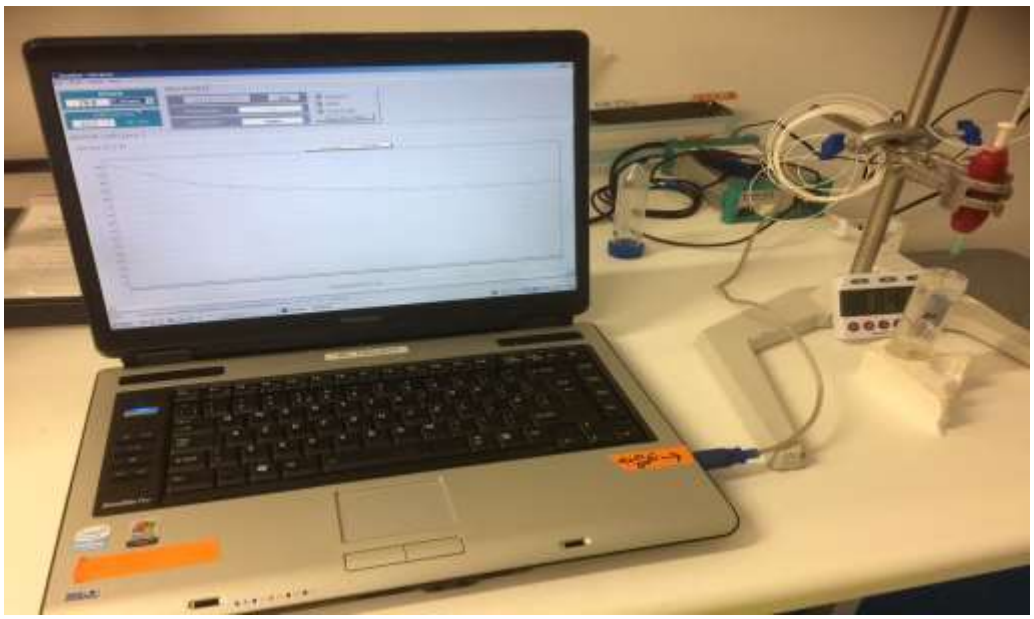

Fig. 3.12 Microx TX3 system used to measure the oxygen concentration. The syringe contains the micro-optode.

The oxygen concentration of 100% oxygen-saturated seawater was calculated according to Benson and Krause (Benson and Krause 1984). Oxygen consumption was calculated from the difference in final oxygen concentration between blank vials, containing no animals and

subjected to experimental treatments, and experimental vials as described by Thatje et al. (2010): this method discounts oxygen consumption by microbial activity. Five replicates and three blanks were run at each temperature/pressure combination. After oxygen measurements were performed, animals were removed from their vials and preserved at -80°C for subsequent weighing, measurement and sex determination. Animals were defrosted, blotted to remove excess water, and weighed to obtain their fresh mass in mg. They were also measured using a Vernier callipers and an optic microscope (LEICA MZ 16) to determine the length, in centimetres from the tip of the rostrum to the end of the tail, and the sex determined. Males can be distinguished from the females by the shape of the appendix internal of the first pleopod, and by the presence of an appendix masculine on the second pleopod, these being the normal secondary sex characters (see Foster 1951).

3.7 Statistical analysis

Statistical analysis was performed using Minitab Statistical software (version 17) and "Sigmaplot" software (version 12.5). Probit analysis of pooled LoE data from replicate treatments was used to model the LoE response to increasing temperature and pressure and to predict CT_{max} and CP_{max}, assuming a logistic distribution based on the sigmoidal data pattern: $\ln[y/1-Y] = a+bX$, where X is the exposure temperature or pressure and Y is the proportion of individuals demonstrating LoE. Multiple pairwise comparisons of pressure treatments using probit analysis were employed to identify which treatments yielded significantly different LoE responses; the Holm–Bonferroni correction was used to maintain the family-wise error rate during multiple comparisons within the experiment.

The effect of temperature on Active Movement was analysed using a general linear model (GLM) analysis of variance (ANOVA) comparing AM among temperatures. Data were proportional and therefore were arcsine-square-root transformed prior to statistical analysis to achieve homogeneity of variance. The effect of pressure on AM was also analysed using a GLM ANOVA comparing arcsine-square-root transformed AM data among pressure and temperatures. The post hoc multiple pairwise comparisons Šidák Simultaneous test was used to determine which treatments were significantly different in both GLM ANOVA analyses.

Oxygen consumption data were analysed using two-way ANOVA with temperature and pressure as factors, and significant differences were explored using the post-hoc Holm-Šidák multiple comparisons test.

4. RESULTS

The specimens of *Palaemon serratus* used for the experiments were all male, with a total length (from the tip of the rostrum to the posterior margin of the tail) between 4.5 and 6 cm. Both LoE and AM depended on temperature (Fig. 4.1, Table 1) (Pearson= 49.0339, respectively p= 0.002 and p=0.442). LoE was first observed at 21°C and rose to 100% at 25°C where it remained approximately constant to 35°C C. AM decreased gradually from 100% at 10°C to 0% at 30°C. CT_{max} was 22.3°C.

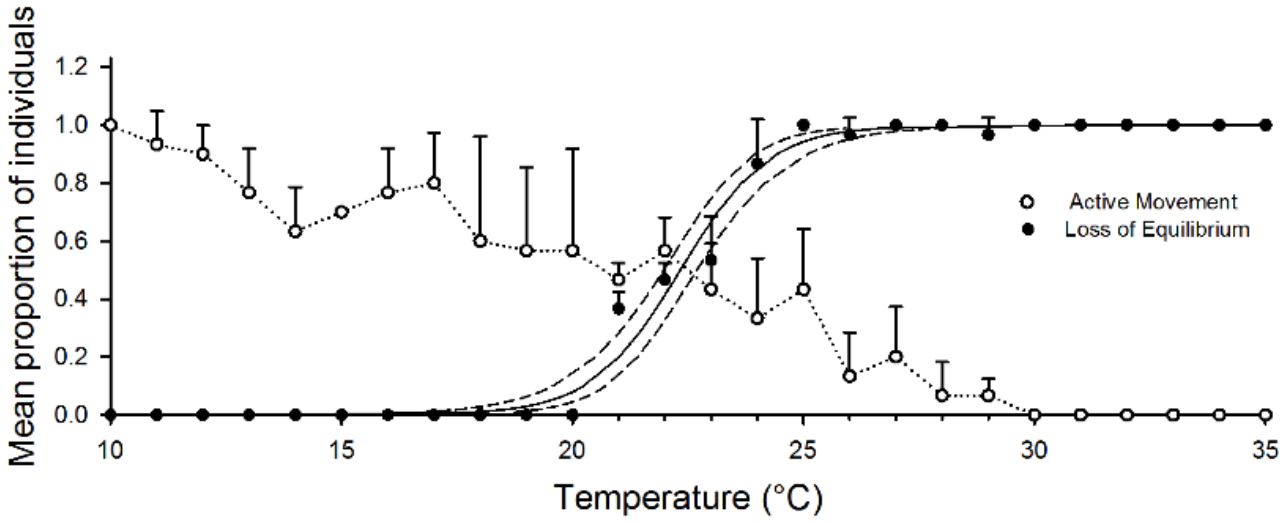

Fig. 4.1 Temperature tolerance of *Palaemon serratus* acclimated to 10°C. The solid line and dashed lines represent Loss of Equilibrium and 95% confidence intervals modelled using probit analysis of Loss of Equilibrium data (closed circles), assuming a logistic distribution. The dotted line represents Active Movement data (open circles). Data are presented as mean +1 standard deviation for clarity (n = 3 replicates with 10 individuals per replicate). See Materials and Methods for explanation of behavioural categories.

LoE and AM also depended on pressure, with increasing sensitivity to pressure with decreasing temperature (Fig. 4.2, Table 1) (respectively p=0.240 and p=0.455). At 15°C LoE remained constant at 0% from 0.1 MPa to 10 MPa. From 11 MPa to 19 MPa there was an increase in LoE from 10% to 90%. From 20 MPa, occurring concurrently with 0% AM, LoE was 100% until the end of the treatment. CP_{max} at 15°C was 14.1 MPa. LoE at 10°C from 0.1

MPa to 7 MPa remained at 0%. From 8 MPa to 12 MPa there was a sharp increase in LoE from 10% to 90% and from 13 MPa the LoE remained 100% until the end of the treatment. At 10°C CP_{max} was 10.1 MPa, lower than at 15°C ($p < 0.001$) and LoE also increased more acutely ($p < 0.001$). The pattern of LoE at 5°C was similar to 10°C, but at lower pressures. There were only two pressures, 0.1 MPa and 1 MPa, with 0% LoE. From 2 MPa to 7 MPa there was a sharp increase from 10% to 90% in LoE. From 8 MPa until the end of the treatment LoE remained constant at 100%. CP_{max} at 5°C was 5.9 MPa, lower than CP_{max} at 10°C which was 10.1 MPa ($p < 0.001$), but the rate of increase in LoE did not differ from 10°C ($p > 0.05$). Consequently, CP_{max} decreased with decreasing temperature.

The effect of pressure on AM also depended on temperature ($F_{60,186}= 6.91$, $p= 0.00$): AM decreased with decreasing temperature, and ceased at lower pressure at lower temperature (Fig. 4.2). At 15°C the AM was generally high (80-100%) until 7 MPa, then decreased sharply from 80% to 10% at 12 MPa. From 13 MPa to 20 MPa AM remained between 20% and 10%. At 21 MPa AM was 0% and remained constant up to the end of the treatment. At 10°C AM decreased less acutely than at 15°C because mean AM at 0.1 MPa was lower (65% at 10°C instead of 85% at 15°C). Between 7 MPa and 11 MPa AM decreased from 40% to 10%. At 12 MPa AM was 0% and remained constant up to the end of the treatment. At 5°C and 0.1 MPa AM was 10% and remained constant up to 7 MPa. From 8 MPa AM was 0% and remained constant to the end of the treatment.

Table 1 Loss of Equilibrium model parameters from critical temperature maximum (CT_{max}) and critical pressure maximum (CP_{max}) experiments, with Deviance goodness-of-fit tests indicating that no models differed significantly from the data it represented (P > 0.05), and values for critical thresholds (CT_{max} or CP_{max}).

Treatment	Acclimation Temperature	a	b	Goodness-of-fit	CT_{max} (°C) CP_{max} (MPa)
CT_{max}	10°C	-23.4306	1.04193	0.422	22.3
CP_{max}	5°C	-6.86596	0.11637	1	5.9
	10°C	-14.7685	0.14574	1	10.1
	15°C	-8.20356	0.08505	0.228	14.1

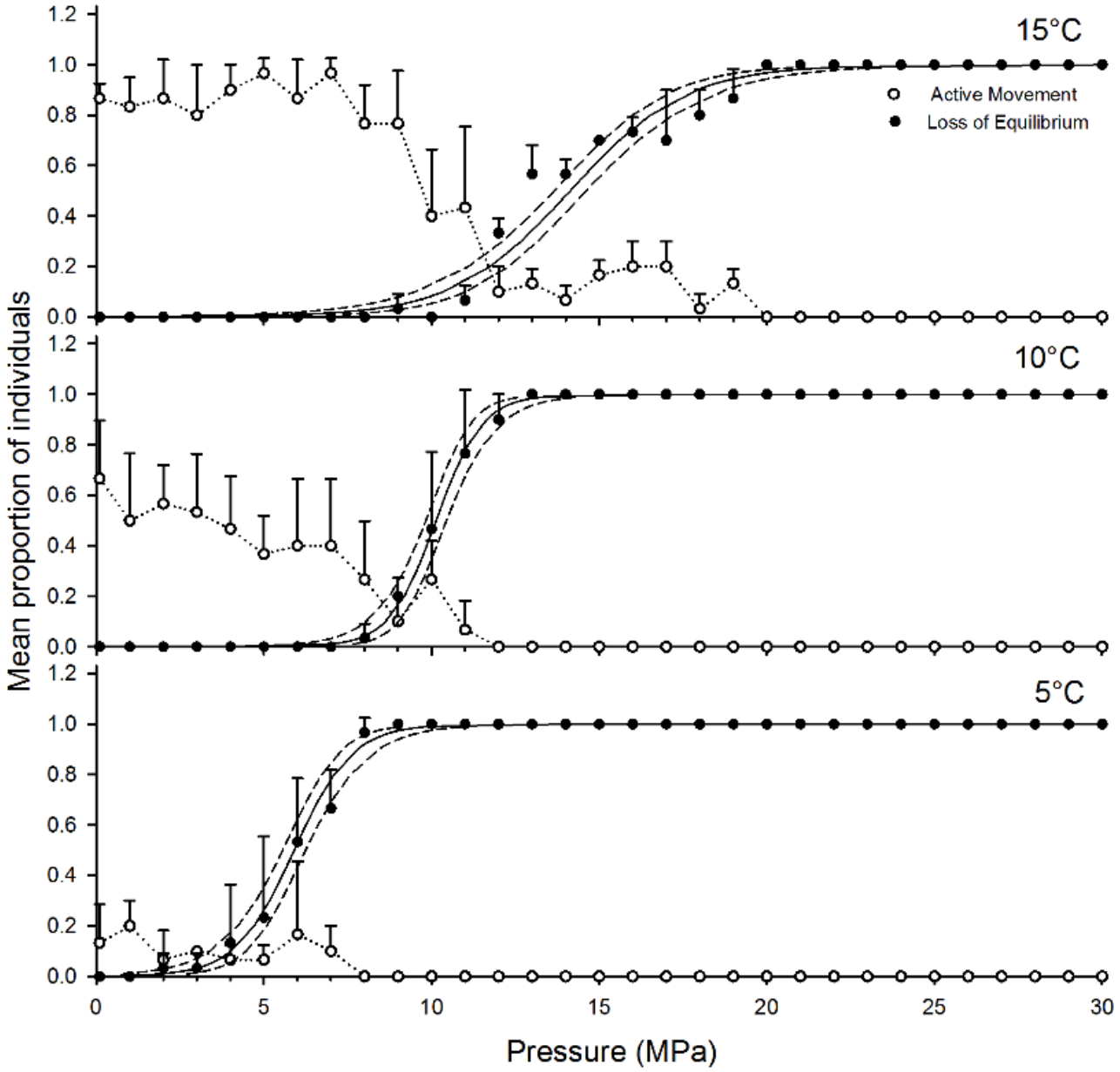

Fig. 4.2 Pressure tolerance of *Palaemon serratus* acclimated to 5, 10, 15°C. The solid line and dashed lines represent Loss of Equilibrium and 95% confidence intervals modelled using probit analysis of Loss of Equilibrium data (closed circles), assuming a logistic distribution. The dotted line represents Active Movement data (open circles). Data are presented as mean +1 standard deviation for clarity (n = 3 replicates for each temperature with 10 individuals per replicate). See Materials and Methods for explanation of behavioural categories.

The effect of pressure on respiration rate depended on temperature ($F_{12, 84}$=5.693, $p < 0.001$) (Fig. 4.3; Table 2). At 15°C, oxygen consumption rates at 0.1, 10, 15 MPa were not significantly different, but were higher than oxygen consumption rates at other pressures. The rate of oxygen consumption at 5 MPa was significantly different from any other pressure. Oxygen consumption rates at 20, 25, 30 MPa did not differ significantly, but they were lower than oxygen consumption rates other pressures. At 10°C, oxygen consumption rates at 0.1 and 5 MPa did not differ significantly, while at 10 MPa the oxygen consumption rate was significantly higher than at any other pressures. At 5 and 15 MPa, oxygen consumption rates did not differ significantly. At 15 MPa the oxygen consumption rate was not significantly different from 20 and 25 MPa. Finally oxygen consumption at 20, 25 and 30 MPa were not significantly different between each other.

At 5°C, oxygen consumption rates at 0.1 and 5 MPa were not significantly different, but they were lower than at 10 and 15 MPa and higher than at 20, 25, 30 MPa. Rates of oxygen consumption at 10 and 15 MPa were not significantly different than at other pressures. Oxygen consumption rates at 20, 25 and 30 MPa were not significantly different.

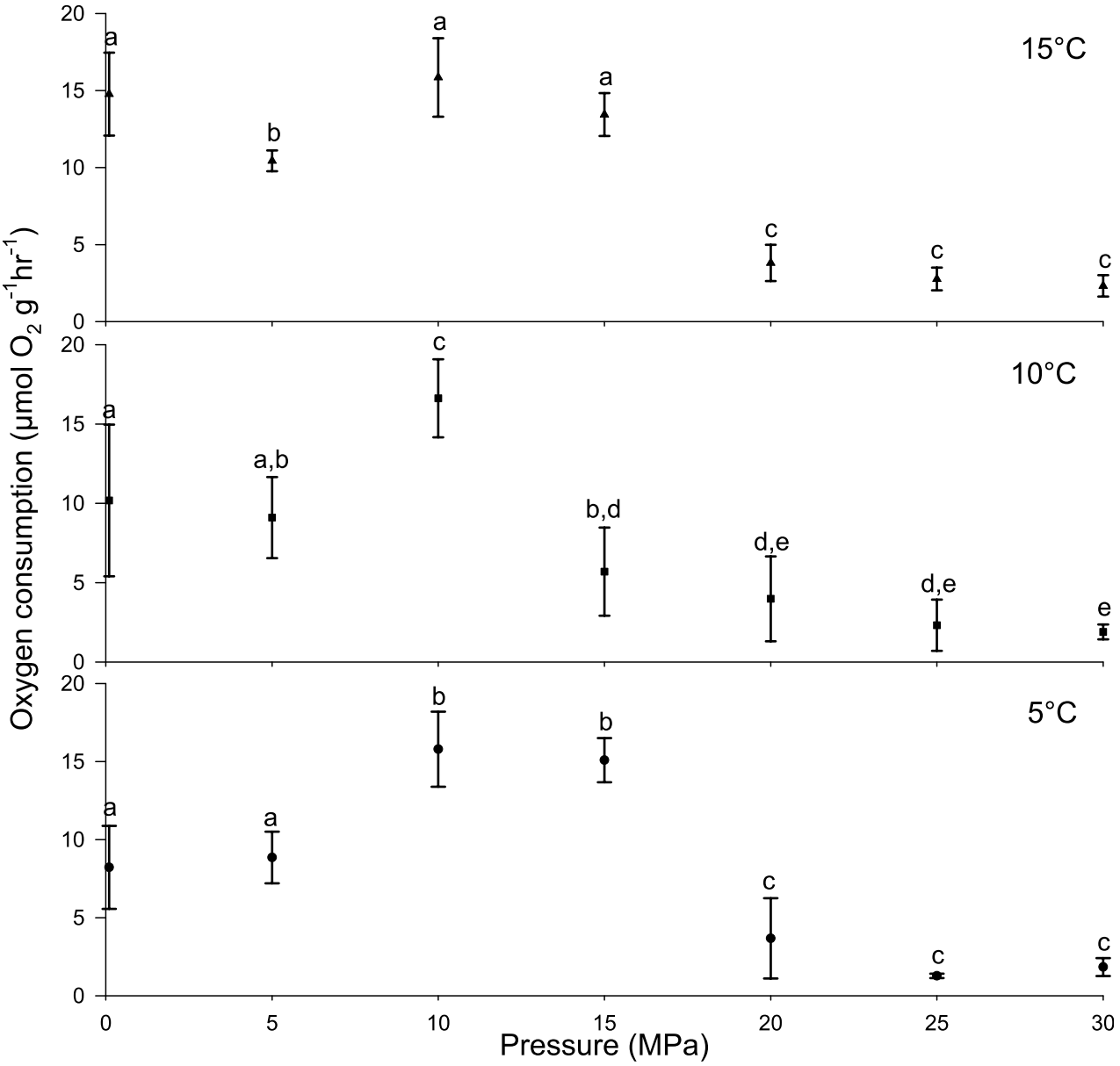

Fig. 4.3 Respiratory response to hydrostatic pressure of *Palaemon serratus* acclimated to different temperatures (mean ± 1 standard deviation, n = 5). Values that do not share a common letter are significantly different.

Table 2 Two-way analysis of variance, ANOVA on respiration rates of *Palaemon serratus* incubated at three temperatures (5, 10, 15°C) and seven pressures (0.1, 5, 10, 15, 20, 25, 30 MPa).

Source	D.F.	SS	MS	f-value	p-value
Temperature	2	67.2683	33.891	7.396	0.001
Pressure	6	2646.139	441.023	96.240	<0.001
Interaction	12	313.036	26.086	5.693	<0.001
Error	84	384.934	4.583		

5. DISCUSSION

Critical thermal maximum, critical pressure maximum and respiration rates of *Palaemon serratus* were assessed to clarify the thermal and pressure scope of this species. Comparison of *P. serratus*' and *Palaemon varians*' responses to temperature and pressure were made to determine whether thermal and pressure scope differs among these congeners, which live in contrasting habitats.

5.1 Critical thermal maximum

CT_{max} experiments are a commonly used index of thermal tolerance, which are linked to the acclimation temperature (see Ravaux et al. 2012). Acclimation period influences the capacity to tolerate high temperatures in animals (e.g. Ravaux et al. 2012). *Palaemon serratus*' CT_{max}, was assessed as 22.3°C following three days acclimation to 10°C. Specimens of *P. serratus* were caught between April and May 2015 when the average environmental temperature was approximately 11°C. The highest and lowest temperature experienced by *P. serratus*, from June 2014 to May 2015, were 20.9±1°C in August and 5.8±0.5°C in February. The 10°C acclimation temperature was similar to the environmental temperature of April and May and is likely to accurately represent the *in situ* CT_{max} at this time.

The highest temperature experienced by *P. serratus* in its habitat (21.4°C) was lower than *P. serratus*' CT_{max}, indicating that *P. serratus* is capable of tolerating the maximum summer temperature *in situ*, even when acclimated to spring temperatures. However, CT_{max} can vary over time: e.g. CT_{max} is generally higher in warm-acclimated and summer-captured specimens of *P. varians* (Ravaux et al. 2012) and decreases during acclimation to low temperature (New et al. 2014), demonstrating the capacity for thermal acclimation in palaemonid shrimp. Indeed, the CT_{max} of *P. serratus* collected in Portugal and acclimated at 20°C for 7 days was 33°C (Vinagre et al. 2013). Whilst contrasting thermal adaptation in different populations of *P.*

serratus may contribute to differences in CT_{max} among studies, it is likely that acclimation temperature and duration has a significant role in establishing differences in *P. serratus*' and *P. varians*' 10°C-acclimated CT_{max}: the 22.3°C CT_{max} of *P. serratus* was lower than the approximately 31°C CT_{max} of *P. varians* acclimated to 10°C for at least 1 month (Fig. 5.1) (Oliphant et al. 2011), suggesting that *P. serratus* is less tolerant of acute temperature rises than *P. serratus*. However, absolute thermal tolerance may be similar: the CT_{max} of *P. serratus* and *P. varians* differ little following sustained 20°C acclimation (respectively 33°C and 36°C).

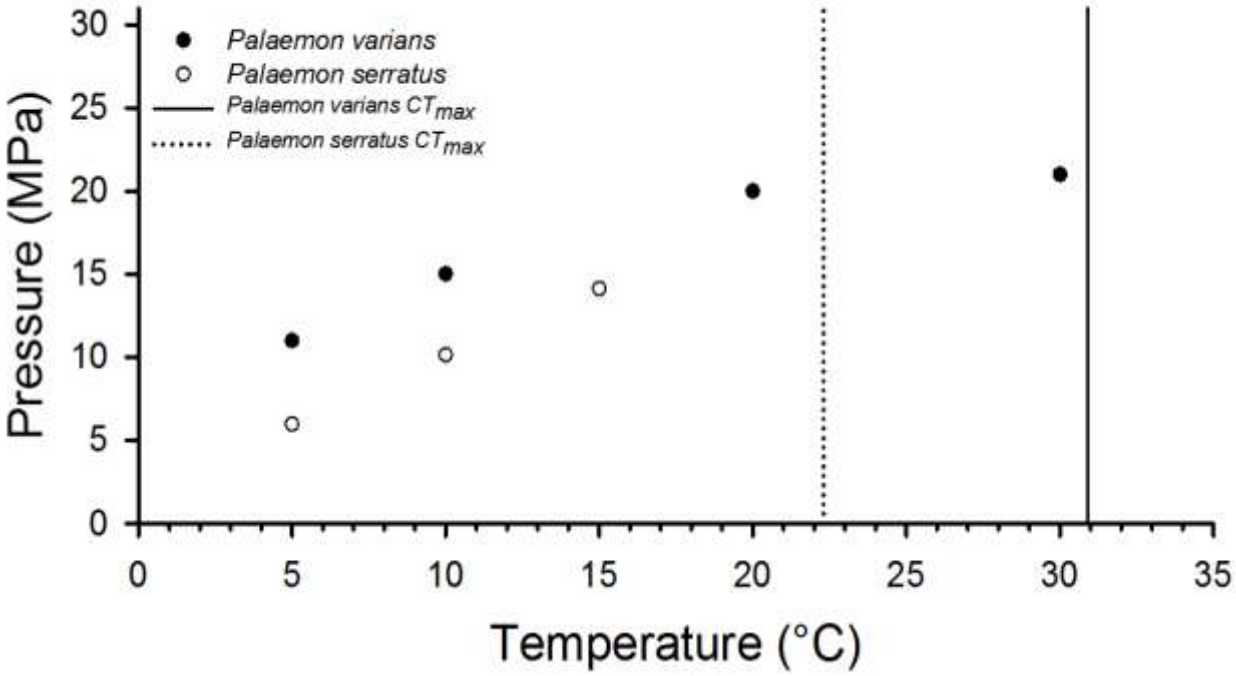

Fig. 5.1 CP_{max} of *Palaemon varians* and *Palaemon serratus* at different temperatures. CT_{max} of 10°C acclimated animals are expressed with vertical lines. Data for *Palaemon varians* are from Oliphant et al. (2011).

Differences in tolerance to acute temperature change between these species may result from adaptation to their contrasting habitats. *P. varians* inhabit salt marshes, brackish pond and lagoon where the temperature varies from 0°C in winter to 33°C in summer, and by >5°C of variation in only 12 h period (Jefferies 1964). In contrast, *P. serratus* inhabits the less variable intertidal zone, where the annual temperature range is between 5°C in winter and 21°C in

summer and the maximum variation is only 2°C in 24 h period (Foster 1951). Similarly, there are differences in environmental salinity among these species' habitats. Tolerance to salinity variations allows cell membranes to better tolerate higher temperatures (see Ravaux et al. 2012), which may preadapt species to tolerate thermal variation.

5.2 Critical pressure maximum

The effects of pressure and temperature on biological systems can be antagonistic, an increase in pressure has similar effects to a decrease in temperature, reducing kinetic energy and causing ordering of molecules (Pradillon and Gaill 2007), and this arrangement can restrict molecular movement causing neuronal impairment (Bartlett 1995). For this reason low temperature amplifies the effects of high hydrostatic pressure in shallow-water marine invertebrates, e.g. *Palaemon varians* (Oliphant et al. 2011; Cottin et al. 2012; New et al. 2014; Morris et al. 2015a,b). The effect of temperature on the critical pressure maximum in *Palaemon serratus* is consistent with this pattern: CP_{max} decreased progressively with decreasing acclimation temperature, indicating reduced hyperbaric tolerance at low temperature. Further, the onset of critical effects was more acute at low temperature. At 0.1 MPa temperature affected metabolism, indicated by lower AM at 5 and 10°C AM than at 15°C. Therefore the effect of high hydrostatic pressure was more acute at lower temperature.
The synergistic effects of high hydrostatic pressure and low temperature may act together to limit the bathymetric distribution of species (e.g. Brown and Thatje 2011). Lipid bilayers of biological membranes appear one of the most pressure-sensitive molecular assemblages (Somero 1992). High-pressure increase orders structures and reduces flexibility in lipids, nucleic acids and carbohydrates (Behan et al. 1992; Balny, Masson & Heremans 2002). An increase in pressure of 100 MPa is equivalent to a decrease in temperature of approximately 13–21°C depending on membrane composition (Somero 1992). Instead a temperature

increase of 2.8°C has been reported to reverse the reduction in membrane fluidity imposed by a hydrostatic pressure of 10 MPa (De Smedt et al. 1979). However high hydrostatic pressure and low temperature affect membrane fluidity in the same way. Both variables decrease the fluidity of the membranes, causing a packing of the lipid bilayer of the cells that reduces molecular motions and cellular signalling, which may interfere with neurotransmission (Siebenaller & Garrett 2002) causing motor coordination impairment, spasm and even paralysis (Treude et al. 2002; Oliphant et al. 2011). Deep-sea species exhibit homeoviscous adaptations to maintain membrane fluidity under high pressure (Somero 1992). For examples accumulation of higher levels of lipid and an increased proportion of unsaturated fatty acids have been observed, counter-acting pressure and temperature-induced decrease in membrane fluidity (Hazel 1995). Membrane fluidity may also be preserved by the adjustment of sterol or protein concentrations (Winter & Dzwolak 2005). *P. serratus*' lower capacity to tolerate acute pressure rises than *P. varians* (Fig. 5.1) may also result from adaptive influences on these species' physiology imposed by their contrasting habitats: selection pressure may have favoured faster physiological responses to environmental change in the more variable habitat of *P. varians*, conferring greater resilience to acute environmental stress. Sustained pressure acclimations will be required to determine whether absolute hyperbaric tolerances differ among these species.

5.3 Respiratory response to pressure and temperature

Oxygen consumption rates increased with temperature at 0.1 and 5 MPa and trends in the effect of hydrostatic pressure were similar across temperatures. Increasing oxygen consumption with increasing temperature at 0.1 and 5 MPa is unsurprising given that metabolic rate increases exponentially with temperature as a result of elevated kinetic energy in biological systems (Clarke and Fraser 2004). Subsequently hydrostatic pressure was the

dominant factor influencing respiration rates: oxygen consumption was similar at all three different temperatures, suggesting that metabolism was limited: oxygen consumption at 10 MPa was higher than every other pressures and almost independent of temperature. At greater pressure, oxygen consumption decreased, with the decrease occurring earlier at 10°C than at 5 or 15°C. The decreases in oxygen consumption likely reflect reduced membrane function by high hydrostatic pressures (Hazel 1995). Amplified homeostatic effort is required to reduce this loss of function, however mitochondrial oxygen demand is not directly compensated by increased respiratory capacity given through ventilation and circulation (see Frederich and Pörtner 2000). Where the demand of mitochondrial oxygen exceeds the respiratory capacity of the animal the mitochondrial respiration change from aerobic to anaerobic and this occurs at the critical threshold (Somero 2005). For example, ventilation and circulation may be limited by the aerobic capacity of mitochondria at low temperatures (Sommer & Pörtner 2002). Vital metabolic processes can be maintained before the critical threshold, but non-essential processes such as growth, breeding, feeding, and active movement are reduced (Peck et al. 2008). Since complex animals use ventilation and circulatory system to supply their cells with oxygen, under stressful conditions (e.g. high hydrostatic pressure or low temperatures) larger animals typically have greater difficulty maintaining oxygen supply than smaller ones (see Brown and Thatje 2014 and references cited therein). *P. serratus* is bigger than *P. varians* - the former can reach 11 cm of total length while the latter can only reach 6 cm - and this difference in size may therefore influence their ability to tolerate stressful conditions. The delayed onset of critical hyperbaric effects on *P. serratus*' metabolism at 5 and 15°C may be due to cross-tolerance provided by stress responses to shifts away from the environmental temperature during sampling (see Oliphant et al. 2011 and Morris et al. 2015a and citation therein).

Oliphant et al., 2011 demonstrated that temperature had considerable effects on the pressure tolerance of *P. varians*. The patterns were similar, but the decrease in oxygen consumption occurred at higher pressures in *P. varians* than in *P. serratus*. The respiratory responses of *P. serratus* and *P. varians* (Oliphant et al. 2011) indicate that respiration rates increase with temperature, as in other species (Clarke and Fraser 2004), thus revealing temperature as the dominant factor for oxygen consumption. At 5°C and 10°C, oxygen consumption was slightly higher in *P. serratus* than in *P. varians*. Consequently *P. serratus* could have been more stressed than *P. varians*. This may be explained by their different habitats, where *P. varians* is more exposed to lower temperatures than *P. serratus*.

As in *P. varians*, at higher pressures (20, 25, 30 MPa) it was evident that specimens were experiencing LoE (Oliphant et al. 2011). As evidenced in the CP_{max} treatments, their motion capacities were clearly compromised at higher pressures. Hydrostatic pressure intolerance is commonly expressed through perturbations of neural and muscular functioning (Morris et al. 2015a,b). Possible hyperbaric effects on neurotransmission could be the reduction of the signalling between neurons due to the high packing of the cells membrane caused by high pressure or the drop in metabolism because of a compromised enzyme functionality (Brown and Thatje 2011). Neurological interference resulting from hyperbaric effects on biomembranes can also affect cardiac function with clear implications for aerobic scope (Mickel & Childress 1982; Airriess & Childress 1994). Consequently, the respiratory capacity of *P. serratus* at higher pressures could have been compromised and this may explain why oxygen consumption rates were similar at pressures beyond 10 MPa regardless of the temperature.

6. CONCLUSIONS

This study provides the first data allowing comparison of critical thermal maxima, critical hydrostatic pressure maxima, and respiratory responses to hydrostatic pressure at different temperatures in congeneric shallow-water species. Acute thermal and hyperbaric scope in the intertidal shrimp *Palaemon serratus* is narrower than in the salt marsh and brackish-water shrimp *Palaemon varians*, indicating that adaptation to differing habitats has resulted in differing physiological tolerance to stress conditions. However, the patterns of temperature effects on hyperbaric tolerance were similar, suggesting that the mechanism of oxygen- and capacity-limited hyperbaric tolerance is consistent among species.

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