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Elder, L. E., & Seibel, B. A. (2015). The thermal stress response to diel vertical migration in the hyperiid amphipod *Phronima sedentaria*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 187*, 20-26. doi: 10.1016/j.cbpa.2015.04.008
Available at: https://doi.org/10.1016/j.cbpa.2015.04.008

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The thermal stress response to diel vertical migration in the hyperiid amphipod *Phronima sedentaria*

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Running title: Thermal Stress response in *Phronima sedentaria*

ms. has 29 Pages, 6 figures, 1 table

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Abstract

The hyperiid amphipod *Phronima sedentaria* experiences a temperature change of 15°C during diel migration in the Eastern Tropical North Pacific (ETNP) from 8-10°C at depth to 25-27°C at night in the surface waters. The aim of this study was to determine if the natural temperature gradient experienced by *Phronima sedentaria* results in a thermal stress response. Individuals were initially exposed to their night time temperatures (23°C) and subsequently subjected to temperatures within and above the range they typically experience. In the Eastern Tropical North Pacific *P. sedentaria* tolerates its normal night-time temperature (~23°C), but only for the duration of its stay there (~9 hours). Longer exposures (24 hours) result in elevated heat shock

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protein (hsp) expression. 29°C results in hsp expression, increased lactate production and 50% mortality at all exposure durations. This represents an upper critical temperature. Understanding the adaptations of pelagic amphipods to their current environment will help predict the physiological impacts of global warming for amphipods and their predators.

Keywords: Phronima, hsp70, thermal stress, hyperiid amphipod, diel vertical migration, critical temperature, oxygen limited thermal tolerance

1. Introduction

Environmental changes that cause a reduction in performance or fitness are known as stress factors (sometimes referred to as stressors) (Schulte, 2014). Abiotic stress factors include hypoxia, acidification, and thermal extremes to name a few. Stress factors vary in temporal scale and level of intensity; from gradual seasonal changes, to drastic tidal or migratory variation. When one of these stress factors is impacting biological function of an organism (fitness) it is referred to as stressful or a stress (Schulte, 2014; Sørensen et al., 2003). The stress response of an organism is the behavioral and physiological adjustments to attempt to maintain fitness (Schulte, 2014). During thermal stress enzymatic and structural proteins denature (unfold) which impacts their stability and kinetic properties (Somero, 1995). The stress response to thermal stress includes expressing heat shock proteins (hsps) (DuBeau et al., 1998) as well as antioxidases, proteases and DNA repairs systems (Sørensen et al., 2003). Hsps act as molecular chaperones that are able to prevent/reduce denaturing of proteins and target those that are irreversibly denatured for removal from the cell via the ubiquitin-proteosome pathway. Hsp 70 is one of the

most highly conserved heat shock proteins expressed in response to hypoxia and osmotic stress and is especially noted for its role in recovery from thermal stress (Feder and Hofmann, 1999). During stress the amount of denatured proteins increases, which requires hsp 70 to chaperone these proteins to prevent damage to the cell. In response to thermal stress there is an upregulation of hsp 70 concentrations proportional to the amount of denatured proteins. Therefore Hsp 70 is a biochemical indicator for the degree of protein unfolding in a cell and an indirect gauge of protein damage (Hofmann, 2005). At a certain upper temperature beyond optimal conditions, referred to as the critical temperature, organisms are not able to perform normally. At this critical temperature there is an increase in hsp expression and a failure of ventilatory or circulatory systems which results in reduced aerobic scope. This reduction in aerobic scope occurs even in oxygenated conditions and results in the transition to anaerobic metabolism in an attempt to continue ATP production (Pörtner, 2010).

The thermal stress response is especially important for aquatic ectotherms since their body temperature fluctuates over the full range of their habitat temperatures (Sokolova and Portner, 2003). Diel migrators experience large temperature changes in their natural environment, spending the day in deeper colder waters and nighttime foraging near the surface. *Phronima sedentaria* (Forskål, 1775) is an abundant species of diel migrating hyperiid amphipod found throughout the world oceans (Shih, 1969; Shulenberger, 1977; Vinogradov et al., 1996; Voznesensky et al., 2004). *P. sedentaria* exhibits no apparent differences among age classes in its patterns of diel migration (Diebel, 1988).

While most pelagic species of amphipods "hitch-hike" on gelatinous zooplankton that serve as physical and metabolic substrate (Gasca and Haddock, 2004; Harbison et al., 1977; Madin and Harbison, 1977), the relationship between *P. sedentaria* and its parasitized host is unique in that

the host is transformed by the parasite (Land, 1992). Phronimids eat the internal tissue of their siphonphore or tunicate host leaving the remaining gelatinous matrix in a barrel shape brood chamber (Diebel, 1988; Hirose et al., 2005; Laval, 1978) that is propelled through the water with the urosoma (tail) (Land, 1992).

P. sedentaria may encounter a temperature change of 15°C during its diel vertical migrations, experiencing surface temperatures approaching 30°C in some regions. Such wide temperature variation within the natural range of a species can induce a stress response (Hofmann and Somero, 1995). Furthermore, the maximum habitat temperatures of many warm-adapted organisms (such as those found in the tropics) are near their upper critical temperature. Additional increases in temperature due to climate change may not be tolerated by such organisms (Somero, 2010). Oceanic temperatures have increased over the past century as a likely result of anthropogenic carbon dioxide emissions (Trenberth et al., 2007). Increasing environmental temperatures are predicted to affect the physiological performance, and consequently the vertical distribution and ecology of marine organisms (Doney et al., 2012; Saltzman and Wishner, 1997; Seibel, 2011; Somero, 2002). If existing night time temperatures are stressful for diel migrators and they are not able to adapt, their depth range will not be sustainable at current latitudes in the future. Determining how close to thermal limits zooplankton are currently living is an important step to project ecosystem response to climate change.

We determined the critical temperature for a tropical population of *Phronima sedentaria* from the Eastern Tropical North Pacific. The expression of hsp 70 and the production of the anaerobic end product, lactate, were quantified at temperatures spanning the range experienced by *Phronima sedentaria* across their vertical distribution to determine what temperatures induce a

stress response. Individuals were exposed to these temperatures for durations equivalent to the approximate time they are at the surface (9 hours) as well as shorter and longer time frames. We tested the hypothesis that the highest temperatures experienced within the natural range can induce a stress response that would result in an increase in synthesis in heat shock protein 70, and a shift to anaerobic metabolism.

2. Materials and methods

2.1.*Collection*

Phronima sedentaria (Figure 1) were collected in the Eastern Tropical North Pacific (ETNP) at the Costa Rica Dome (8.5°N; 90°W; Figure 1). The research cruise on the R/V Knorr (Woods Hole Oceanographic Institute) was from 8 December 2008 - 6 January 2009. Collection was done using a tucker trawl with a thermally insulated cod end (Childress et al., 1978). Individuals were identified according to published taxonomic keys (Shih, 1991; Vinogradov et al., 1996). Physical vouchers to confirm the identification were preserved in formaldehyde and housed in the Seibel lab at the University of Rhode Island.

Individuals were collected from two separate trawls on January 1st and 2nd 2009 in discrete tows between the depths of 250 and 300m with a speed of 1.5- 2 knots. The first trawl was opened at depth at 1509 local time (2109 GMT) at 09 º 10.4370 N, 89 º 56.5019 W and closed at 1539 (2139 GMT). The second tow was opened at depth at 1525 local time (2125 GMT) at 09 º 01.6328 N, 89 º 59.1241 W and closed at 1614 local time (2214 GMT). The shipboard CTD was SBE9+ (Sea-Bird electronics, USA) and included sensors for oxygen (SBE 43), temperature (SBE 3T), conductivity (SBE 4C), pressure (Digiquartz) and a SBE 5 pump. CTD data from the

same day show that the ambient temperature where these individuals were collected was approximately 12° Celsius (Figure 2). Sightings from blue water SCUBA diving, and other trawls have shown that this species can be found near the surface at night in water at temperatures of 23-25° Celsius (personal observations). Collection at depth provided control of the duration individuals were exposed to surface temperatures.

2.2. *Thermal stress*

We employed a unique experimental protocol designed to test both the time-relative tolerance to, and recovery from, the natural night-time temperatures experienced by *Phronima sedentaria*. Individuals were sorted immediately after retrieval and identified quickly under a microscope to reduce stress. Individuals in good condition (intact with no injuries) were separated into chilled filtered seawater until experimentation and held for a maximum of a half hour before initial treatments. For the initial exposure treatment (I-exposure) individuals were placed in plastic containers with 0.2 micron filtered sea water at their approximate nighttime temperature (23°C) for 3, 9 or 24 hours. The 9 hour exposure is similar to the duration diel migrators spend in surface waters.

For the subsequent exposure (S-exposure) individuals were then transferred to open scintillation vials (25ml volume) containing 0.2 micron filtered seawater at 23°C and placed in separate wells of an aluminum thermal gradient block (Figure 3, (Henkel and Hofmann, 2008). The vials took ~15 minutes to get to the desired temperature. S-exposures lasted five hours at temperatures: 10, 15, 20, 23, 25 and 29 ± 1 °C.

The thermal block consisted of a piece of aluminum with holes drilled through each end fitted with brass inlet and outlet ports to accommodate heating and chilling lines. The heating and chilling lines were connected to temperature controlled water baths (Lauda, Germany). Water

flowed directly against the aluminum for optimal thermal transfer. Evenly spaced wells were drilled in the top of the block in rows of four to allow up to four replicated experiments at each temperature. Prior to experiments the wells were filled with fresh water and allowed to come to temperature. Once the wells were at the appropriate temperature the scintillation vials with filtered seawater and the individual *P. sedentaria* were floated in the well.

Table 1 outlines the number of individuals for each treatment. During the experiment the thermal block was loosely covered by black plastic bags to block light. Oxygen concentrations of the water in experimental vials was checked using a Clark-type oxygen electrode (1302 Strathkelvin Instruments, United Kingdom; (Clark, 1956)) to make sure water remained well above the published critical oxygen partial pressure of 2.11 kPa (28 µM at 10°C (Childress, 1975). For this study 24 hour I-exposure specimens were frozen at 0100, 9 hour I-exposure specimens were frozen at 1300 and 3 hour I-exposure specimens were frozen at 0700. No significant differences were found between individuals run at different times of day and results are combined for all analyses. Following S-exposure, individuals were then taken out of the vial with feather forceps and blotted dry before being immersed in liquid nitrogen and stored at -80 degrees Celsius.

2.3. *Lactate*

Individual whole frozen individuals were ground on ice in a prechilled glass tissue homogenizer (Kimble Chase, USA) using a 1/3 dilution with grinding buffer, 465mm NaCl, 19mm KCl, 20 mm Tris, 1mM EDTA, containing a 1 x protease inhibitor cocktail (Sigma p2714) and 0.1% detergent (IGEPAL Sigma 18896). The homogenate was centrifuged at 2000 rpm for five minutes at 4°C and the supernatant removed. L-lactate concentrations were measured on the Accutrend lactate meter using a 25 µl sample of supernatant. All samples were assayed in

duplicate and compared to a lactate standard curve (sodium lactate, L7022, Sigma- Aldrich, MO, USA) which was run daily. Remaining supernatant was flash frozen in liquid nitrogen and stored at -80 until needed for western blots.

2.4. *Western blots for hsp70 concentration*

Lysate was thawed on ice and centrifuged at 13400 rpm for 2 minutes. Protein concentration was determined using the Pierce BCA protein assay (Bio-Rad, USA). Thirty µg total protein of each sample lysate was mixed with 1/3 lysate volume of 4x NuPAGE LDS buffer containing 10% β-mercaptoethanol and then boiled for 10 minutes at 95ºC. Lysate was loaded on to 4-12% bis tris gels (Invitrogen). Heat shocked HeLa cells (Enzo, USA, ADI-LYC-HL101) were added as a control between gels by using their protein band for comparison of relative intensities between samples. Proteins were electrophoresed at 120V for 15 minutes, and 150V for approximately 2 hours in 1X MOPS running buffer. Gels were soaked in transfer buffer (5.82g Tris, 2.93g Glycine, 2x 940 µl 20% SDS, 100mL Methanol, q.s. to 1000ml with deionized water) for 20 minutes and electroblotted (Bio Rad, Trans-blot 170-3940) for 30 minutes at 25 volts onto a polyvinylidene difluoride (PVDF) membranes (Fisher IPVH00010). The membrane was washed twice in 10X TBST (Tris Buffered Saline: 400g NaCl, 10g KCl, 150g Tris, 5mL tween into 4.5L of DI water, pH of 7.4. quantum satis DI water for 5L total). The membrane was then blocked in 5% milk powder (diluted in TBST) for one hour at room temperature. This was followed by 3 five minute TBST washes. The membrane was then incubated in a 1:1,000 dilution of hsp 70 antiserum (Stressgen SPA-822) overnight at 4 ºC. After washing, the secondary antibody (anti-mouse Igc:HRP-Linked, GE Healthcare Biosciences NA931) was added for one hour at room temperature.

Immunoreactive proteins were then visualized with Chemiluminecent substrate Western lightening (Perkin Elmer, NEL102001EA) for 2 minutes. Following a one minute exposure, on kodak biomax XAR film (Sigma, F5388-50EA) the film was developed and HSP 70 expression was determined semi-quantitatively using Image J software.

2.5. *Statistical analysis*

Statistics were performed using the software SAS version 9.3 (SAS institute inc. USA). One-way Analysis of Variance (ANOVA), with between subjects design were conducted to compare differences in lactate accumulation or hsp 70 concentration between treatments.

3. Results

3.1 Collection

At the time of collection surface temperatures of the ETNP were between 23 and 25°C. The maximum surface temperature recorded in the ETNP during this cruise was 27°C. Based on published distribution for *Phronima sedentaria*, temperatures at the deepest range of daily migrations are between 8 and 10°C. This indicates *Phronima sedentaria* may experience a temperature change of 13-17°C in the ETNP during diel migration in the ETNP (Figure 2).

3.2 Thermal stress, and Lactate concentrations

There was no significant difference in mortality or lactate accumulation (ANOVA: $p>0.1$) for 3, 9 or 24 h I-exposure to nighttime temperature (23°C). Data for those exposure times are averaged within each temperature for subsequent analyses of lactate concentrations. There was no

significant difference in any parameter between experiments conducted at different times of the day. No further evaluation of diel rhythms was conducted.

There was no mortality of individuals between 10 and 20°C. At 23°C, 1 of 7 individuals died (13%) and at 25°C, 2 of 8 individuals died (30%); Table 1). The most significant mortality occurred at 29°C, at which temperature 50% of the experimental individuals died (4 out of 8 total individuals; Table 1). Dead individuals had a significantly higher accumulation of lactate, and so are not included further.

Exposure to 29°C resulted in a significant increase in lactate accumulation relative to all other temperatures (Figure 4; one way ANOVA, $F(5,15)=8.26$; $p=0.0025$). At 29°C the average L-lactate production in live individuals was 20.5 ±4.52 μmol g^{-1}. For all other S-exposure temperatures (10-25°C) there was no significant difference in lactate accumulation. The average lactate accumulation after S-exposure to 10, 15, 20, 23 or 25 °C was 2.89±0.797 μmol g^{-1}. A previous study on *P. sedentaria* found that individuals frozen immediately after collection had very high levels of lactate (≥20 μmol g^{-1}) indicating use of anaerobic metabolism in oxygenated conditions, which is thought to be a result of capture stress (Elder and Seibel, In Revision). The low values measured here at temperatures below 29°C indicated that acclimation time was sufficient to recover from collection stress.

3.3 Western blots

Western blot analysis using an antibody for hsp 70 revealed one band occurring at approximately 70kDa (Figure 5). Due to low sample size, no significant differences were found between individuals S-exposed to 23 or 25°C following I-exposure at 23°C. These individuals were combined and are designated 24°C S-exposure in Figure 6. Individuals I-exposed to 23°C for

only 3 hours had significantly lower hsp70 levels than either 9 or 24 hour I-exposed individuals at all S- exposure temperatures (ANOVA: $F(2,47)=7.82$; $p<0.0012$; Figure 6). There was no difference in hsp70 expression between the 9 and 24 h I-exposures at 10-20°C S-exposure. For individuals in the 24 h I-exposure, hsp70 levels were elevated at 29°C compared to lower temperatures for that I-exposure duration (Figure 6, ANOVA: $F(5,24)=2.57$, $P<0.0535$). Elevated temperature (29°C) did not induce hsp70 expression in individuals I-exposed for 3 hours at 23°C (Figure 6). There were no significant differences in hsp expression within a single temperature for the S-exposures other than 29C. At 10°C (one-way ANOVA $f(2,8)=1.63$, $P>0.2548$) and 15°C (one-way ANOVA $f(2,8)=1.85$, $p>0.1675$) there were nearly significant differences.

4. Discussion

During daily migrations *Phronima sedentaria* experiences a temperature change of ~15°C (Figure 2) with sustained upper temperatures near 23°C at night. *P. sedentaria* migrates between the surface and 200-350m during diel migration (Shih, 1991; Shulenberger, 1977). This temperature change when migrating through the thermocline would be rapid, with a change of up 10°C degrees across 50m (Figure 2). For this study we assessed mortality, lactate and hsp 70 accumulations in individuals initially exposed to nighttime temperature (23°C) for varying durations to assess both the time-sensitive stress response and required recovery temperature. The stress response consists of physiological adjustments to attempt to maintain fitness. We predicted that the temperatures routinely experienced by *P. sedentaria* within its natural range would induce a stress response. If this stress response occurred, it would result in a shift to anaerobic metabolism due to oxygen-limitation (discussed below; Pörtner, 2002) that can be

measured as an increase in lactate production under oxygenated conditions. In addition, a stress response would result in an increase in hsp 70 concentrations. Three hour initial exposure individuals had consistently lower hsp 70 levels than individuals with 9 and 24 hour initial exposures (Figure 6). This indicates that longer durations did induce some stress response. There was an increase in both lactate and hsp70 at 29°C. Although this temperature was not experience during our expedition (January), surface temperatures in the Eastern North Tropical Pacific can reach 29°C (Pennington et al., 2006). It is possible that *P. sedentaria* adjusts their physiological temperature tolerance seasonally, a follow up study in this region in the summer would determine that. At the S-exposure temperatures other than 29°C, there was no significant difference in hsp expression when comparing the I-exposure duration. This is in part because of large variation in hsp expression for the 9 and 24 hour individuals. At 10 and 15°C at least one individual at 9 and 24 hours had a low hsp expression similar to the 3 hour individuals.

The 13% mortality at 23°C and 30% mortality at 25°C (Table 1) may indicate some amount of stress at night time temperatures (although sample sizes are too low to place much confidence in the mortality analysis). The modest heat-shock response observed may be necessary for these amphipods to survive the 8 hours typically spent in near-surface waters. In all initial exposure treatments, including 3-hour individuals, subsequent exposure to 24°C for five hours did not result in additional significant hsp70 expression. The less than 30% mortality and lack of an increase in lactate or hsp 70 suggests that *P. sedentaria* is tolerant of nighttime temperatures for at least 8 hours, equivalent to its nightly exposure duration before returning to cooler depths. Pörtner (2002) has suggested that upper critical temperatures are related to a mismatch between oxygen supply and demand. This is supported by an elevation in lactate at 29°C. However, lactate levels did not increase at temperatures below 29°C at any initial exposure duration. This

suggests that the heat-shock response in the 9 and 24-hour initial exposures is independent of oxygen stress. In addition temperatures below 23°C did not result in a reduced amount of lactate production or hsp70 concentrations (Figures 5 and 7), indicating that the low lactate levels measured were a true "basal" level. There was no significant mortality at temperatures below 23°C (Table 1). This suggests that the modest heat-shock response at temperatures below 29°C was successful at protecting the individual from detrimental effects of thermal stress.

At 29°C *P. sedentaria* had a significant increase in lactate production (Figure 4), hsp 70 concentrations (Figure 6), and mortality (Table 1). This indicates that the critical temperature range for *Phronima sedentaria* in the ETNP is between 26 and 29°C, which is slightly higher than the ambient surface temperatures during our winter expedition. Summer temperatures can surpass 29°C in the ETNP (Pennington et al., 2006).

The increase in lactate production at 29°C represents the onset of anaerobic metabolism. At their critical temperature, individuals may experience a failure of ventilatory or circulatory systems to meet elevated oxygen demand, which results in reduced aerobic scope and a transition to anaerobic metabolism under oxygenated conditions. This loss of system function is thought to reflect the earliest level of thermal stress and is known as oxygen and capacity limited thermal tolerance (Pörtner, 2010). Our measurements suggest that thermal stress begins earlier than this critical or "pejus" (Latin for 'turning worse') temperature but protective mechanisms are effective, at least for finite periods of time. Although we did not test heart or ventilatory function directly, the onset of anaerobic metabolism in aerobic conditions is consistent with this mismatch in oxygen supply and an inability to deliver enough oxygen to the body (Pörtner, 2010). Survival beyond the critical temperature leads to a decline in performance and is time limited due to low ATP yield from anaerobic glycolysis (Pörtner, 2002; Pörtner, 2010).

The pejus range is the range when individuals are past optimum conditions but can still survive with reduced aerobic activity (Jost et al., 2012). During the pejus range, there is an increase in ventilation rate with temperature to compensate for increasing oxygen demand with temperature. At the upper pejus temperature ventilation rate reaches a maximum level and haemolymph Po_2 begins to decrease (Frederich and Pörtner, 2000). Oxygen supply to tissues and overall aerobic scope, is obviously linked to fitness and functioning at the ecosystem level (Clark et al., 2013; Pörtner, 2010).

Lactate accumulation at 29° C in this study (20.5 ±4.52 µmol g^{-1}, Figure 4) is similar to the lactate level of 17.15±4.75 µmol g^{-1} in the same species subjected to five hours of environmental hypoxia (1% oxygen) at the intermediate temperature of 20°C. Lactate concentrations at 25°C and below were comparable to levels in the previous study when exposed to normoxic conditions 2.85±0.40 µmol g^{-1} (Elder and Seibel, In Revision). This indicates that individuals were experiencing tissue level hypoxia at 29°C despite access to high seawater oxygen levels. This tissue level hypoxia could be due in part to failure of ventilatory or circulatory systems. However, factors other than oxygen transport can also be thermally limited and potentially cause decline in performance and temperature tolerance. These factors could include cell damage, membrane fluidity, enzyme function, and neural function (Clark et al., 2013).

A critical temperature of approximately 30°C is found in several other crustacean species. The spider crab *Maja squinado* from Roscoff France has a critical temperature close to 30°C, which was indicated by accumulation of anaerobic end products succinate and lactate. This coincided with very low arterial Po_2 values (Frederich and Pörtner, 2000). The critical temperature range at which anaerobic metabolism begins in the intertidal crabs *Carcinus maenas* and *Cancer irroratus* is 34°C and 30°C, respectively. Interestingly, hsp70 was not detected in either of these

crabs, but it may be due to the experimental design, which included a rapid rate of temperature increase (Jost et al., 2012). Our results suggest at least an 8 hour lag (3 h initial exposure and 5 hour subsequent exposure) in the onset of hsp70 expression following exposure to stressful temperatures.

The majority of studies on heat shock response in ectothermic invertebrates have focused on intertidal organisms, especially mussels. A theme from these studies is the plasticity of hsp expression, where past thermal history has an impact on induction temperature (Hofmann, 2005; Hofmann et al., 2002). In the intertidal, thermal history can vary with season and tide level. In temperate regions of the pelagic realm, seasonal changes can have an effect on surface temperatures. In the tropics however, temperature gradients are steep but relatively stable (Fernández-Álamo and Färber-Lorda, 2006). Vertical migators experience drastic temperature changes during their transit between surface and deeper waters. The lack of a full stress response in *Phronima sedentaria* at 23°C indicates that this species is adapted to the current, relatively constant, surface temperatures of the region.

Vertically migrating calanoid copepods (*Calanus finmarchicus*) from the temperate waters of the Gulf of Maine demonstrated a heat shock response when exposed to their maximum summer habitat temperature (20°C) (Voznesensky et al., 2004). After 30 minutes at 20°C individuals exhibited a ~ 4 fold increase in hsp 70 expression (Voznesensky et al., 2004). The heat shock response in these vertically migrating copepods may increase survival by allowing them to tolerate high temperatures while at the surface before migrating down to deep, cooler waters (Voznesensky et al., 2004). The individuals of *P. sedentaria* examined here were acclimated to their winter temperatures. Summer temperatures may reach 30°C (Pennington et al., 2006).

High constitutive levels of hsp 70 are thought to provide a general protective mechanism against heat shock, and possibly other stresses, in freshwater amphipods (Bedulina et al., 2013). There was a stronger hsp response in intertidal amphipods from a variable habitat (sub-littoral) versus a less variable habitat (supra-littoral) (Bedulina et al., 2010). This may indicate that the heat-shock response is critical for tolerating natural temperature fluctuations, even below critical extremes. Rhythmic pre-synthesis of hsps to prepare for potential heat stress, such as prior to low tide, has not been found in rocky intertidal organisms (Hofmann et al., 2002). The dependable timing of diel migration compared to the variability of low tide levels, suggest that vertical migrators would be more likely to have an anticipatory increase in hsp production than intertidal organisms. For this study 24 hour I-exposure specimens were frozen at 0100, 9 hour I-exposure specimens were frozen at 1300 and 3 hour I-exposure specimens were frozen at 0700. At 0100 diel migrators would have been at the surface for a few hours, while at 0700 they would have recently arrived at depth and at 1300 they would have arrived at depth several hours prior. If *P. sedentaria* were producing hsp in anticipation of vertical migration, one would expect lower levels of hsp in the group subjected to the same temperature frozen at 1300 compared to the group frozen at 0100. However, there was no significant difference in the hsp concentrations or level of mortality between the freezing times.

5. Conclusions

Upper thermal tolerance limits are correlated with the maximum habitat temperatures in intertidal organisms (Stillman and Somero, 2000). In the midwater environment *Phronima sedentaria's* critical temperature of 29°C may be reached in summer and, due to global warming (Deser et al., 2010), during future winters. The Eastern Tropical Pacific warms by approximately

0.8-1.0°C per century (Deser et al., 2010). If organisms are already close to their critical temperatures, global warming may cause some species to exceed their thermal limits and may affect their biogeographic range. Increasing temperature and decreasing oxygen supply (Keeling et al., 2010; Stramma et al., 2008) will compress the night time habitat of vertically migrating species (Elder and Seibel, In Revision; Seibel, 2011). This change will have important impacts on zooplankton physiology, ecology, and vertical distribution as well as carbon cycling (Vinogradov and Voronina 1962; Seibel 2011; Somero 2002).

Acknowledgments. Thanks to Gretchen Hofmann for numerous insightful discussions. The Hofmann Lab provided the thermal gradient block design which was fabricated by the Equipment Development Lab at the URI Graduate School of Oceanography. This work would not have been possible without the Captain and Crew of the R/V Knorr. Thank you to the members of the Seibel lab for assistance in net deployment and recovery, and to Kendra Daly for her organization of the ETP research expedition. A special thanks to Niall Howlett, and the members of the Howlett lab (especially Rebecca Boisvert and Meghan Rego), for use of equipment, assistance with western blot methods and troubleshooting. Thanks to Terry Bradley, Karen Wishner and three anonymous reviewers for helpful comments and suggestions on earlier versions of the manuscript that improved our work. Funded was provided by the National Science Foundation grants: OCE-0526545 to Kendra Daly and OCE-0526502 to Karen Wishner and Brad Seibel. Additional funding was provided by The Crustacean Society Graduate Student Fellowship to Leanne Elder and the University of Rhode Island Enhancement of Graduate Research grant, also to Leanne Elder.

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Legends

Figure 1. Eastern Tropical North Pacific sites. This map displays the station in the Eastern Tropical North Pacific (ETNP) during collection aboard the R/V Knorr in Dec 2008- Jan 2009. Individuals for these experiments were collected at the Costa Rica Dome (8.5°N, 90°W) using a tucker trawl.

Figure 2. CTD profile for the Costa Rica Dome. The Costa Rica Dome CTD profile of oxygen (black line) and temperature (grey line). Boxes represent approximate day and night time distributions of *Phronima sedentaria* based on published distributions (Shih, 1991; Shulenberger, 1977).

Figure 3. Thermal gradient block. The thermal block consisted of a piece of aluminum with holes drilled through each end fitted with brass inlet and outlet ports to accommodate heating

and chilling lines. The heating lines are to the left side of the block and separate chilling lines are on the right side. Water flows from the water bath through the tubing and the block and back to the water bath. By having the two water baths at opposing extreme temperatures there is a temperature gradient in the wells on the top.

Figure 4. *Phronima sedentaria* lactate accumulation from thermal stress experiments. Average accumulation of lactate in µmol g^{-1} for *Phronima sedentaria* at each subsequent exposure (S-exposure) temperature. All values are mean ±se. There was a significantly higher accumulation of lactate at 29°C.

Figure 5. Western blot analysis of hsp 70 levels in *Phronima sedentaria.* Representative Western blot analysis of levels of hsp 70 in *Phronima sedentaria* relative to control (HELA cells first lane on the left). The marker from the protein ladder at 75 Kda is indicated in the figure, to show that the band is at 70 Kda. This gel consists of the samples with a 24 hours Initial exposure to 23°C and subsequent exposure to the designated temperatures. The last three lanes (samples that have been kept at 29°C) had significantly higher relative intensity than the other samples, indicating significantly higher hsp 70 concentration.

Figure 6. *Phronima sedentaria* mean hsp70 concentrations following thermal stress experiments. Mean hsp 70 concentration ±se for individuals initially exposed to night time temperature of 23°C for 3(gray), 9 (black) or 24 hours (white) followed by subsequent exposure (S-exposure) to the designated temperatures. * indicates there was a significantly lower hsp 70 concentration at 3 hours compared to 9 and 24 hours (p value < 0.05). ** indicates there was a

nearly significant increase in hsp 70 concentration in individuals acclimated to 23C for 24 hours before a five hour incubation at 29°C

Figure 1

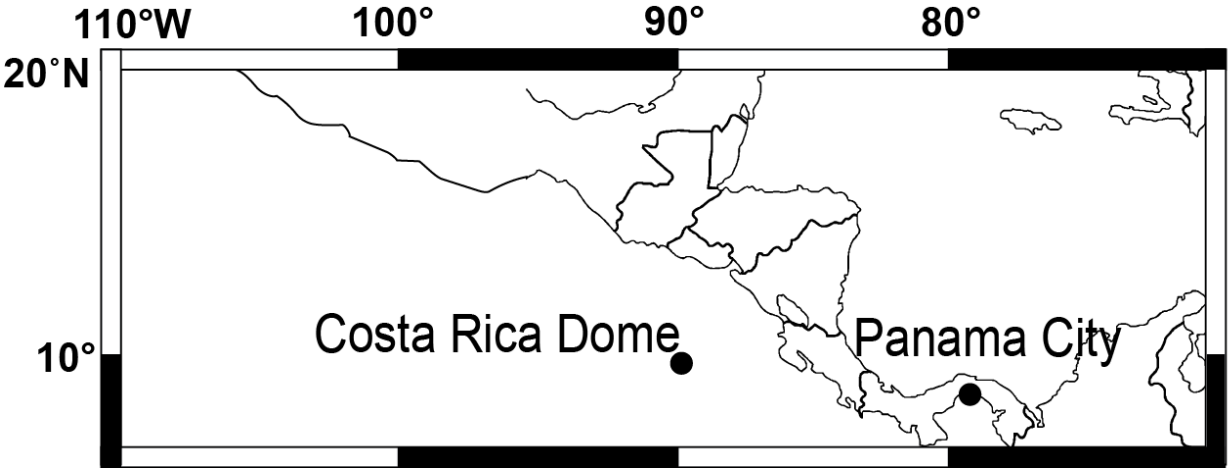

Figure 2

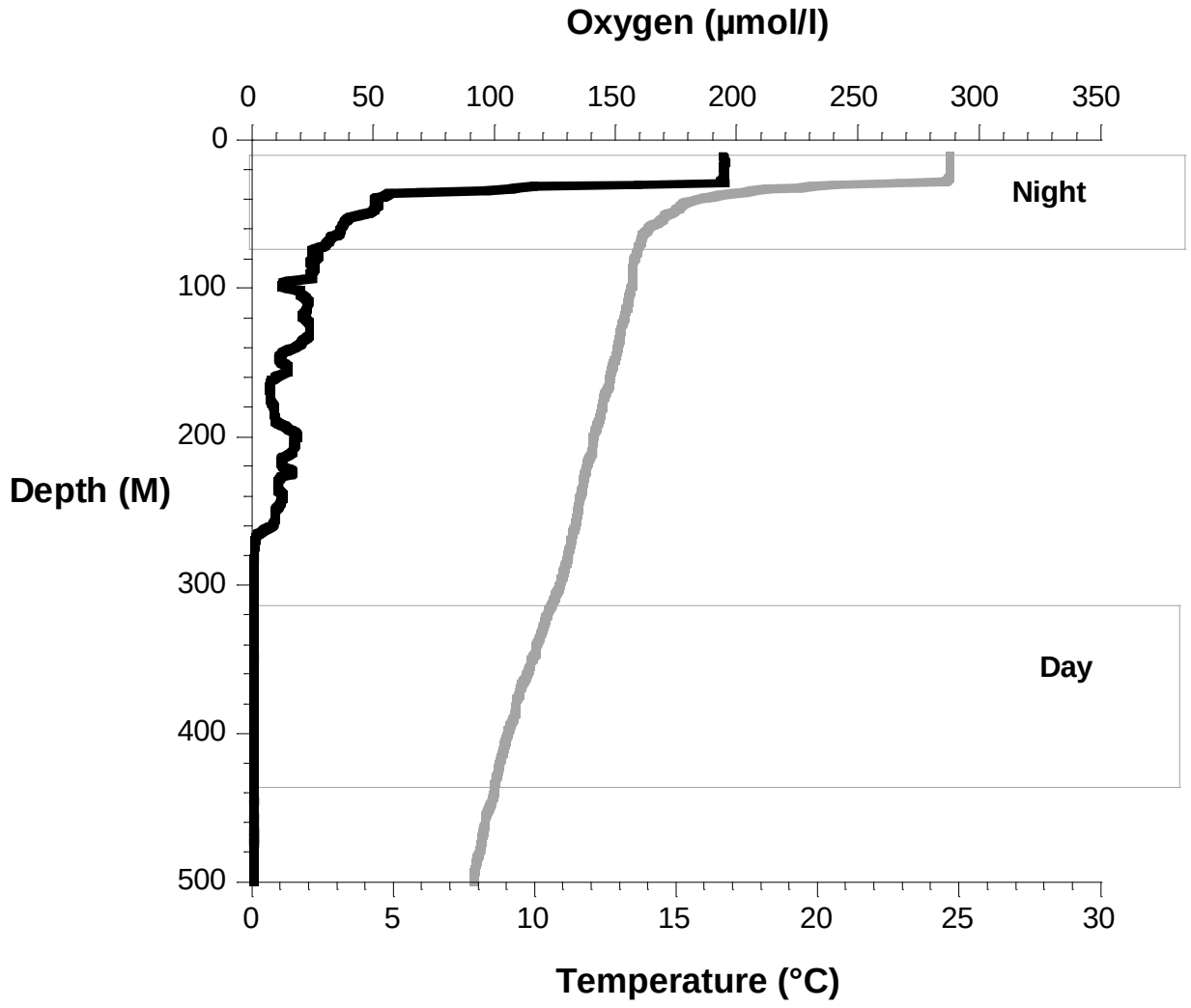
Oxygen (µmol/l)
0
50
100
150
200
250
300
350
0
100
200
300
400
500
Depth (M)
Night
Day
0
5
10
15
20
25
30
Temperature (°C)

Figure 3

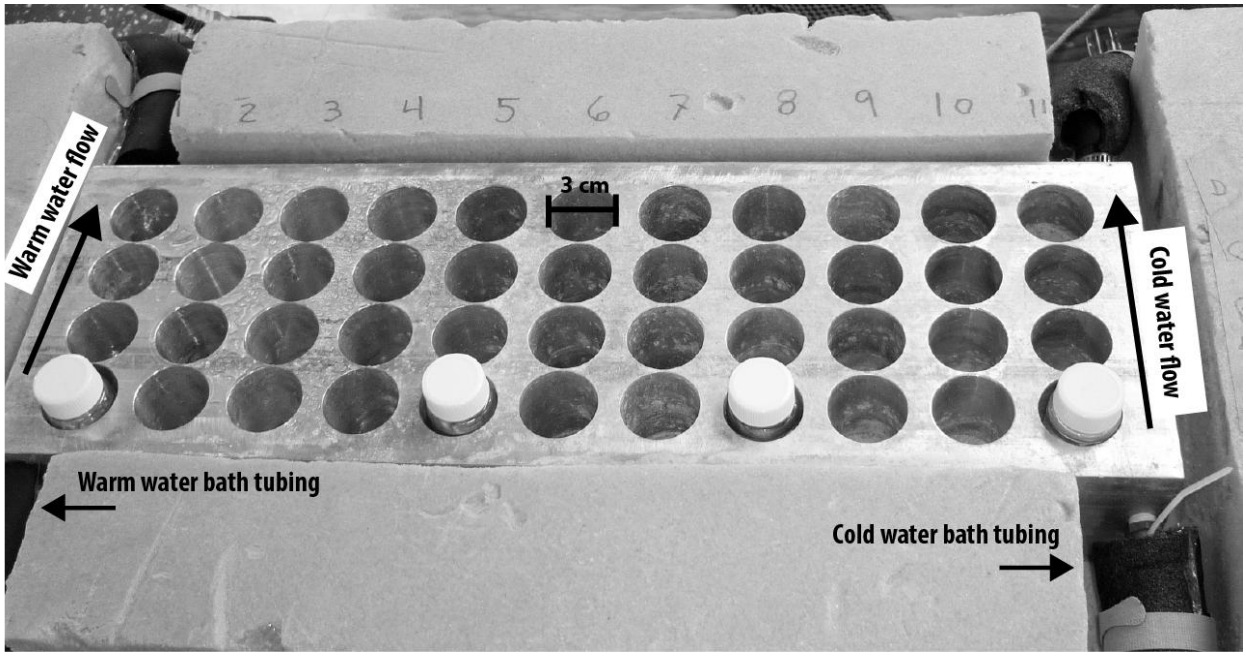
1 2 3 4 5 6 7 8 9 10 11
Warm water flow
3 cm
Cold water flow
Warm water bath tubing
Cold water bath tubing

Figure 4

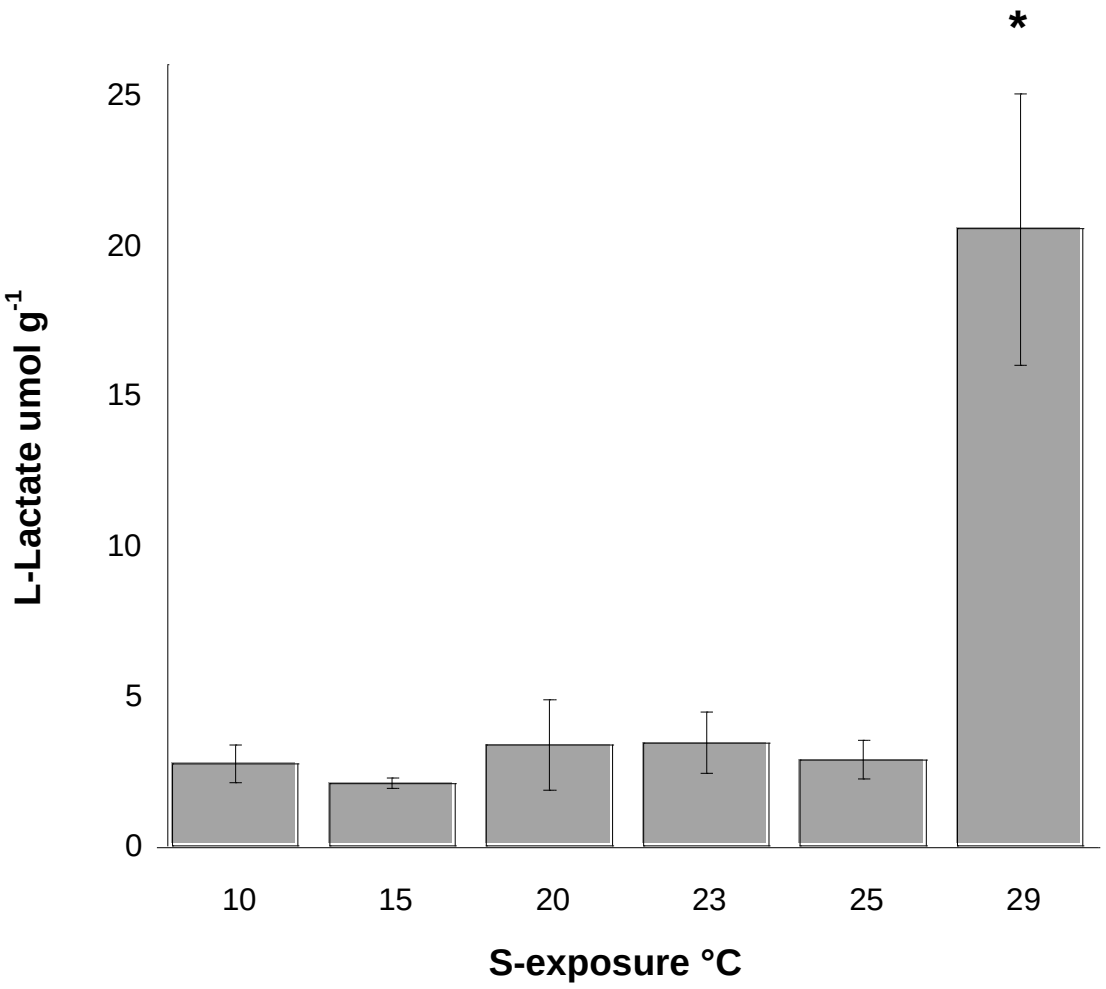

Figure 5

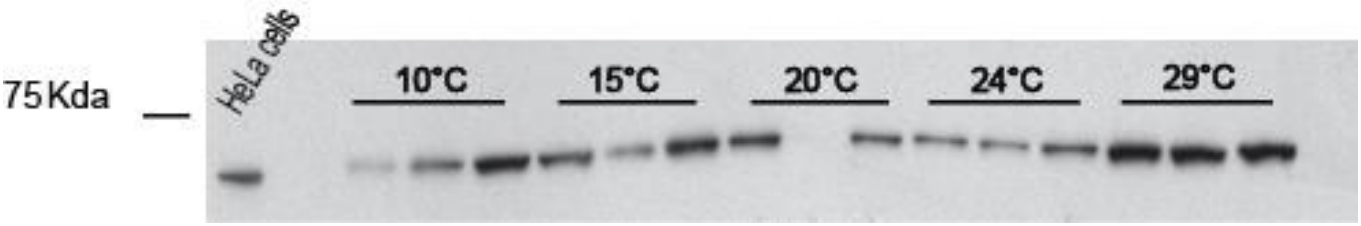

Figure 6

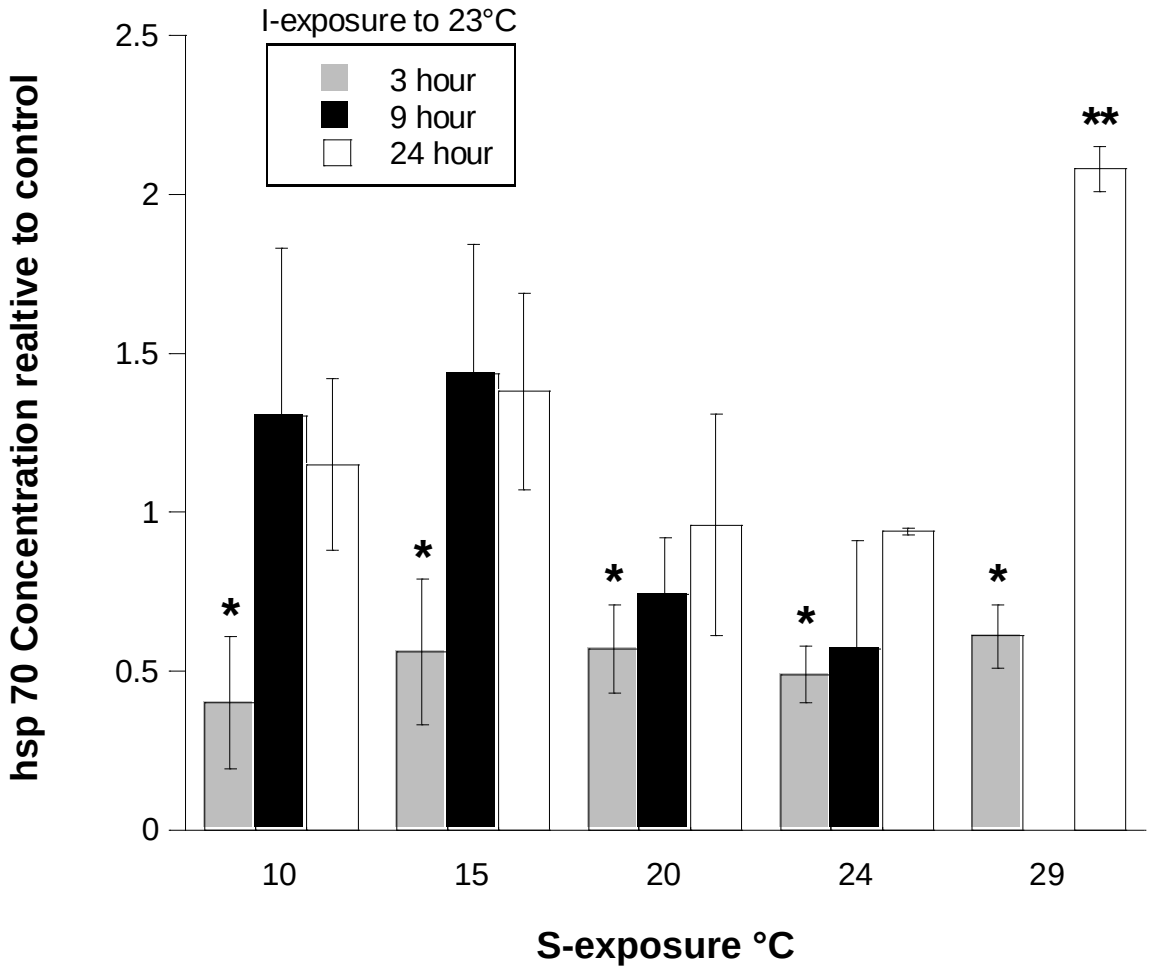
I-exposure to 23°C
3 hour
9 hour
24 hour
hsp 70 Concentration realtive to control
2.5
2
1.5
1
0.5
0
*
*
*
*
*
**
10
15
20
24
29
S-exposure °C

Table 1. Experimental design for thermal stress experiments on *Phronima sedentaria*.

I-exposure to 23°C	S-exposure temperature °C	n	n dead at end
3 hours	10	4	
	15	4	
	20	4	
	23	4	
	29	4	3
9 hour	10	4	
	15	4	
	20	4	
	23	3	1
	25	3	2
	29	1	
24 hour	10	3	
	15	3	
	20	3	
	25	3	
	29	3	1

Thermal stress experimental setup for initial exposure (I-exposure) to night time temperature of 23°C for 3, 9 or 24 hours and subsequent exposure (S-exposure) for five hours to the designated S-exposure temperature. n is number of individuals kept at those conditions. n deceased at end is the number deceased at the end of each experiment.