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### Permalink

<https://escholarship.org/uc/item/73p5x630>

### Journal

Marine Biology, 163(2)

### ISSN

0025-3162

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### Publication Date

2016-02-01

### DOI

10.1007/s00227-015-2779-5

Peer reviewed

# Role of sequential low-tide-period conditions on the thermal physiology of summer and winter laboratory-acclimated fingered limpets, *Lottia digitalis*

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Received: 23 March 2015 / Accepted: 26 October 2015 / Published online: 18 January 2016  
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**Abstract** The rocky intertidal zone is among one of the most highly variable environments on Earth, with rapid and unpredictable changes in temperature on a daily basis. Numerous studies have investigated the thermal physiology of intertidal animals; however, few have focused on an organism's capacity to withstand repeated heat stress during the emersion of low-tide periods and how previous exposures to sublethal increases in temperature may modulate the capacity to withstand severe heat stress. *Lottia digitalis*, a species of limpet ubiquitous along the Pacific coast of North America, were acclimated under ambient ocean conditions in the laboratory during the summer and winter months. Limpets were aerially exposed with or without preliminary heat exposures of varying magnitudes (15–35 °C for 2 h) the day before being challenged to a lethal temperature increase to investigate how sequential low-tide-period conditions affect upper thermal tolerance and temperature sensitivity. Limpets with a preliminary aerial

exposure of 25–35 °C (summer) and 20 °C (winter) had greater upper critical thermal limits of cardiac performance as determined by final breakpoint temperature (~1.5–6 °C increase) and flatline temperature (~1–2 °C increase) than limpets with no previous exposure. The magnitude of temperature increase that conferred significant increases in thermal tolerance differed in summer and winter, reflecting seasonal differences in the thermal environment in nature. Fingered limpets' upper thermal tolerance is plastic and likely modulated by the previous day's low-tide exposure, demonstrating the importance of incorporating the repeated nature of stress into thermal physiology research in intertidal organisms.

## Introduction

The rocky intertidal zone is a highly variable environment, with conditions ranging from fully aquatic to fully terrestrial over vertical distances of only a few meters and with conditions changing in a matter of hours (Wolcott 1973; McMahon 1990; Tomanek and Helmuth 2002). In nature, intertidal organisms do not experience single stressor events alone; rather, they experience multiple elevated temperature events in sequence, corresponding to sequential low-tide periods. To date, much of the work on the thermal physiology and thermal tolerance mechanisms of intertidal animals have focused on the response to one heat shock at a time, rarely the serial exposure to multiple high-temperature events of varying magnitude, more indicative of the natural environment (Somero 2002). The repeated nature of temperature fluctuations during low tides is likely an important factor modulating the thermal physiology of intertidal organisms such as upper temperature tolerance limits and cellular defense mechanisms. While daily

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Responsible Editor: H. Pörtner.

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Reviewed by undisclosed experts.

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**Electronic supplementary material** The online version of this article (doi:10.1007/s00227-015-2779-5) contains supplementary material, which is available to authorized users.

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exposure to low-tide conditions is predictable in nature, the degree of temperature change that an intertidal organism will experience is unpredictable, and it is unclear how elevated temperatures coupled with aerial exposure from previous low-tide periods modulates the capacity of intertidal organisms to tolerate a more severe increase in temperature (Denny et al. 2011). Acclimation to a fluctuating versus a constant thermal environment results in different physiological phenotypes (Widdows 1976; Podrabsky and Somero 2004; Todgham et al. 2006). There are many examples of both marine and terrestrial organisms inhabiting fluctuating environments that have higher stress tolerance under fluctuating conditions than when held under stable conditions in either the field or in the laboratory (e.g., Feldmeth et al. 1974; Krebs et al. 2001; Tomanek and Sanford 2003; Kingsolver et al. 2009; Fanguet et al. 2011; Oliver and Palumbi 2011).

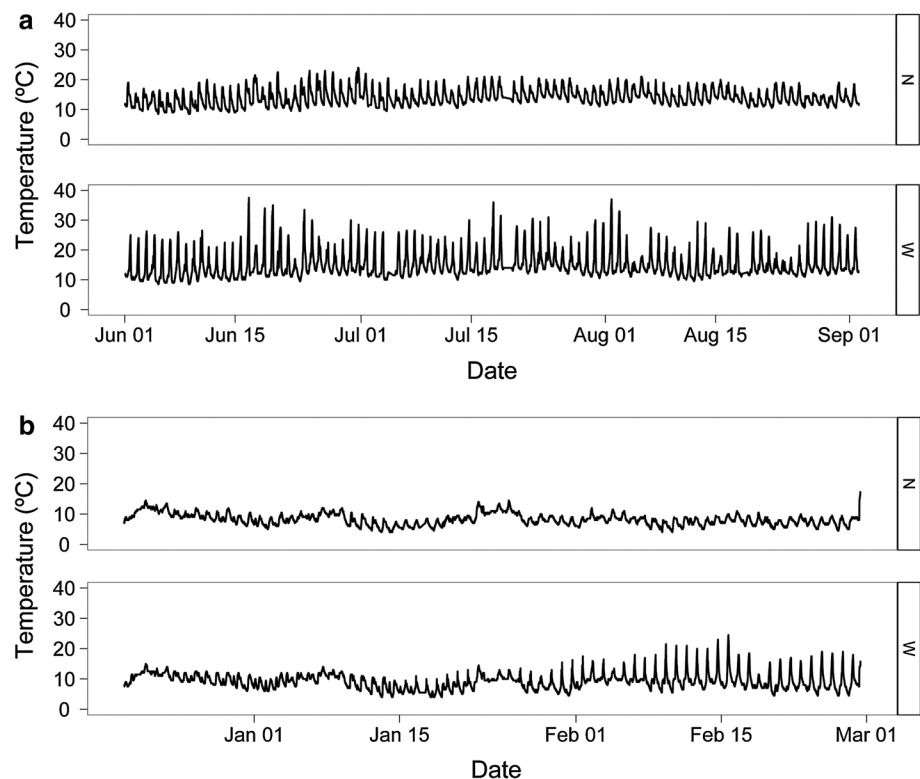
Laboratory studies provide strong evidence to suggest that preliminary mild increases in temperature can serve to prime an organism to tolerate a more severe second stressor (Maness and Hutchison 1980; DuBeau et al. 1998; Todgham et al. 2005; Middlebrook et al. 2008; Dong et al. 2010; Chen and Stillman 2012; Sinclair et al. 2013). Inducible stress tolerance (IST) occurs when an initial stressor has the ability to increase an organism's tolerance to a second stressor of homologous (e.g., temperature + temperature, heat hardening or inducible thermal tolerance) or heterologous (e.g., temperature + hypoxia, cross-tolerance) nature (Hahn and Li 1990) and is a phenomenon that is well described for *Drosophila* (for example, see Loeschcke and Hoffmann 2007; Marshall and Sinclair 2010; Sørensen et al. 2012). There is a physiological window within which inducible stress tolerance operates. The greater the magnitude or duration of the first (also known as priming) stressor, the greater the resistance gained by the organism to the second, subsequent stressor. However, if the priming stressor gets too severe or lasts too long, resulting in cellular damage, the organism will be more susceptible to or less tolerant of a second stressor (Krebs and Loeschcke 1994; Loeschcke et al. 1994; Todgham et al. 2005). IST is likely a fundamental but understudied strategy for intertidal organisms with limited mobility during low tide that have the potential to be exposed to sequential stressors with repeated low-tide periods.

Depending on an organism's vertical location within the intertidal zone and the tidal cycle, intertidal organisms experience aerial exposure daily for different magnitudes of time (McMahon 1990). Aerial exposure may act as an important factor modulating upper temperature tolerance, as this is a predictable aspect of environmental change in the intertidal zone (Gracey et al. 2008; Miller et al. 2009; Pöhlmann et al. 2011; Connor and Gracey 2012; Bjelde and Todgham 2013; Han et al. 2013; Huang et al. 2015).

Intertidal organisms are subject to highly variable conditions on a daily basis due to the tidal cycle and therefore are potentially adapted to take advantage of the predictable variability of their natural environment. There is strong evidence to suggest that short-term environmental variation and multivariate aspects of environmental stress in nature (i.e., fluctuating vs. constant temperature, emersion vs. immersion) are important in influencing how an organism responds to environmental change (Sinclair et al. 2007; Denny et al. 2011). The physiological adaptations of intertidal organisms to cope with the highly variable abiotic conditions of intertidal habitats have been an area of extensive investigation for many decades (Wolcott 1973; Garrity 1984; McMahon 1990; Branch 1981; Williams and Morritt 1995; Helmuth et al. 2002; Somero 2002; Williams et al. 2005); however, we still lack an understanding of how the repeated nature of aerial heat stress tailors the thermal tolerance of intertidal organisms over short timescales and whether this varies seasonally.

Our primary hypothesis is that exposure to repeated, sublethal increases in temperature during low-tide emersion are essential to the thermal tolerance of organisms inhabiting fluctuating environments and that sublethal increases in temperature of sufficient magnitudes will confer enhanced thermal tolerance to subsequent more severe thermal stress in intertidal limpets. To better understand the factors, such as chronic thermal history (season) and previous low-tide exposures, that modify upper temperature tolerance in limpets, we investigated the capacity of preliminary exposure to elevated aerial temperatures to increase the upper temperature tolerance of both summer and winter laboratory-acclimated fingered limpets, *Lottia digitalis* (Rathke 1833). *L. digitalis* is a species of limpet that is found at the highest edges of the intertidal zone, is ubiquitous along the Pacific coast of North America from the middle of California northward (Lindberg and Pearse 1990; Crummett and Eernisse 2007), and can experience daily fluctuations in temperature in California of +20–30 °C (Fig. 1). Previous studies have shown that cardiac performance of intertidal limpets is closely linked to their capacity to tolerate increased temperatures and their vertical zonation in the rocky intertidal environment (Chelazzi et al. 2001; Dong and Williams 2011; Bjelde and Todgham 2013), and therefore, we examined critical thermal limits of heart function in *L. digitalis* to examine inducible thermal tolerance. To better understand how a preliminary increased temperature exposure might confer protection against a more severe heat stress, we investigated the levels of damaged proteins tagged for degradation (through ubiquitin-conjugated proteins) and desiccation in limpets prior to the more severe heat stress. We predicted the indices of stress would be reduced in animals that were heat-hardened. Lastly, glycogen content of foot tissue was measured following

**Fig. 1** Temperature profiles at Fort Ross, CA, of upper intertidal rock temperatures recorded in **a** summer 2012 and **b** winter 2013 every 10 min taken from temperature loggers located on north (N)- and west (W)-facing sites. Limpets for this study were collected from both north and west sites



laboratory acclimation in summer- and winter-acclimated limpets to examine whether there were differences in energy storage across seasons and therefore differences in energy available for thermal tolerance mechanisms (Santini and Chelazzi 1991; Santini et al. 2002). Intertidal organisms are thought to have already “stretched” their physiological tolerance to the limit as a strategy to take advantage of living in such a productive, but otherwise environmentally stressful, marine ecosystem (Stillman 2003; Tomanek 2008; Wethey and Woodin 2008; Jones et al. 2009). With limited acclimatization potential, additional environmental change predicted by climate change scenarios is forecasted to push these organisms past their tolerance limits (Hofmann and Todgham 2010; Somero 2012). To best predict how intertidal organisms will respond to future environmental change, it is essential to incorporate repeated heat stress and capture the role of IST in modulating sensitivity to severe heat stress when investigating the capacity of intertidal organisms to tolerate future increased frequency of heat waves.

## Materials and methods

### Temperature profiling

Temperature loggers (ibuttons, Dallas Maxim, California) were secured to rocks using marine ZSPAR (Kop-Coat Inc.,

Rockaway, New Jersey) at two different sites (north and west) spanning the upper mid-intertidal zone at Fort Ross, California ( $38^{\circ}30'45.79''\text{N}$ ,  $123^{\circ}14'45.58''\text{W}$ ). Loggers recorded temperature every 10 minutes and were replaced on a monthly basis. *Lottia digitalis* were collected from rocks in close proximity to the loggers. Emersion times of limpets during daytime low-tide periods were approximated from the temperature logger data and ranged from 4.5 to 7 h across a tidal series at both sites. Heat budget models have demonstrated that substratum temperature is the primary determinant of limpet body temperature during low tides (Denny and Harley 2006), and therefore, logger data provided an estimated thermal history during emersion of *L. digitalis* over the course of the year (Fig. 1; Table 1). Preliminary laboratory trials confirmed that the temperature of the aluminum block on which limpets were placed closely matched the temperature of the limpet foot as block temperatures were increased ( $\pm 1^{\circ}\text{C}$ ), especially at elevated temperatures (Bjelde and Todgham 2013). Temperature logger data also provided information on the average rate of temperature increase during summer low-tide periods ( $6^{\circ}\text{C h}^{-1}$ ) to help design a ramping temperature profile in the laboratory that simulated natural conditions.

### Limpet collection and acclimation

For the summer laboratory acclimation trials, *L. digitalis* (length size range: 14.43–21.48 mm; mass range:

**Table 1** Temperature data from Fort Ross, CA, during the summer and winter of 2012–2013

	Average temperature (°C)				
	Site	Overall	Maximum	Minimum	$\Delta$ (daily range)
Summer 2012					
June	N	13.4 ± 1.5	19.1 ± 2.5	10.1 ± 0.9	8.3 ± 2.0
	W	14.4 ± 1.4	25.6 ± 4.7	10.5 ± 1.2	14.0 ± 4.2
July	N	14.6 ± 1.1	19.4 ± 1.5	11.9 ± 1.2	6.9 ± 1.6
	W	15.8 ± 1.1	26.5 ± 3.4	11.9 ± 1.2	13.1 ± 3.0
August	N	14.2 ± 1.0	18.2 ± 1.8	11.7 ± 0.9	5.9 ± 1.4
	W	14.9 ± 1.3	24.7 ± 5.2	11.6 ± 0.9	12.3 ± 5.1
Winter 2013					
December	N	9.7 ± 1.5	11.5 ± 1.5	8.2 ± 1.5	3.0 ± 0.8
	W	10.3 ± 1.4	12.3 ± 1.4	8.7 ± 1.5	3.4 ± 1.0
January	N	8.2 ± 1.7	10.2 ± 1.8	6.5 ± 1.7	3.2 ± 1.1
	W	8.5 ± 1.7	12.0 ± 1.7	6.2 ± 1.8	4.9 ± 1.7
February	N	7.6 ± 0.8	9.5 ± 1.0	5.9 ± 1.1	3.2 ± 0.7
	W	9.6 ± 0.9	18.1 ± 2.8	6.6 ± 1.4	10.1 ± 2.6

Data include monthly mean and SD of daily average temperature and average maximum, minimum, and the change in temperature during the daytime low tide at both the north and west site, where limpets were collected for experimentation

0.47–1.34 g) were collected in early June and early September 2012 from the intertidal zone during a low-tide period at Fort Ross, California. Limpets were carefully removed without damage from rocks using small flat-head spatulas. Only limpets that could be removed on the first try, before they could secure themselves tightly to the rock surface, were collected. Once removed, limpets were inspected for foot damage, immediately placed into water-filled coolers and transported back to the Romberg Tiburon Center of Environmental Studies (RTC), San Francisco State University, Tiburon, California, where they were transferred to indoor holding tanks. Limpets were held in large plastic containers with mesh sides to allow sufficient water flow and partially submerged in water tables with recirculating seawater under stable, ambient ocean conditions (12–13 °C, salinity 33–34) with no tidal cycle for 2–5 weeks before experimentation began. Algal-covered rocks were collected from Fort Ross, California, and placed in the acclimation tanks for limpet grazing and feeding. Limpets were allowed to self-regulate access to air by moving freely up and down the sides of the holding container. Approximately 25–35 % of limpets were out of water at a particular time. For the winter laboratory acclimation trials, limpets were collected in January 2013 (length size range: 16.13–23.48 mm; mass range: 0.63–1.93 g) and acclimated in the same manner as summer trials (2–5 weeks at

12–13 °C, salinity 33–34). Acclimation temperatures for both summer and winter trials were similar due to similar temperatures of incoming bay water from San Francisco Bay into our aquarium facility.

### Preliminary temperature exposure regimes

To test whether preliminary elevated temperature exposures of varying magnitudes had an effect on upper thermal tolerance and temperature sensitivity of *L. digitalis*, limpets were subjected to two increased temperature exposures in series with a recovery period in between. Specifically, limpets ( $n = 23$ ) were exposed to a sublethal increase in temperature on Day 1 in the morning, returned to their tanks at ambient ocean temperatures to recover overnight and then exposed to a lethal temperature increase the following morning on Day 2 to assess upper temperature tolerance and cardiac performance. The repeated heat stress exposure regime was selected to simulate serial daytime low-tide periods that are 24 h and 50 min apart. To simulate low-tide emersion, limpets ( $n = 15$ ) were placed in air on an aluminum temperature-controlled heat block and covered with an acrylic lid. On Day 1, limpets were held at ambient temperatures (~13 °C) for 15 min and then ramped to 20 °C, 25 °C, 30 °C, 32 °C, or 35 °C at a rate of 6 °C h<sup>-1</sup> (indicative of an average heating rate in nature during summer low tides) and held at that temperature for 2 h (Fig. 1). Limpets that were ramped to higher temperatures were emersed for longer periods of time due to the nature of the ramping protocol. One group of limpets was aerially exposed for 4 h and 15 min, the average amount of time limpets were aerially exposed during preliminary temperature exposures with heating, on the heat block but kept at ambient temperatures (15 °C) to test the effect of aerial exposure alone on upper temperature tolerance. Limpets were then immediately placed back in the original holding tanks at ambient ocean conditions overnight. A final group of limpets ( $n = 15$ ) received no preliminary elevated temperature or aerial exposure (NoPE) on Day 1 and was kept in tanks at ambient conditions up until the severe, lethal temperature increase on Day 2. Preliminary trials found that a preliminary temperature exposure of 35 °C was too high for limpets collected during the winter, as approximately one-quarter of the limpets tested were dead after the 2-h exposure. Thus, the 35 °C treatment group was taken out, and 32 °C was the highest preliminary temperature exposure on Day 1 of the winter laboratory-acclimation trials. We did not repeat preliminary elevated temperature exposure trials on immersed limpets as intertidal animals are unlikely to see changes in temperature of this magnitude when submerged under water.

## Upper critical limits of cardiac performance and inducible thermal tolerance

To test upper thermal limits of cardiac performance of *L. digitalis* on Day 2, limpet heart rates were recorded as limpets were exposed to increasing substratum temperatures until a lethal temperature increase was reached as described in Bjelde and Todgham (2013). Briefly, two days prior to a preliminary elevated temperature exposure, two small holes were drilled through the apex cavity of the limpet shells and animals were returned to the sea tables until experiments began. Preliminary trials demonstrated that drilling holes in a limpet shell did not affect survival for 3 weeks under laboratory conditions (data not shown). On the morning of Day 2, forty-gauge ceramic-coated copper wire (Belden, Illinois, USA) electrodes were implanted through the predrilled holes and glued into the air cavity between the shell and the limpet, directly above the heart. Limpets were placed in air on a temperature-controlled aluminum block and were allowed 30 min to recover from handling stress at an ambient temperature of 13 °C before ramping began. Temperature was then ramped at a rate of 6 °C h<sup>-1</sup> from 13 to 48 °C, a severe, lethal temperature increase. Cardiac performance of each limpet was recorded as changes in impedance, as in Bjelde and Todgham (2013), and converted to heart rate, in beats per minute (bpm) using PowerLab Chart 5 (ADInstruments, Colorado, USA).

### Cardiac performance analysis

Heart rate data were analyzed using R (R Development Core Team 2012) to determine four measures of cardiac performance. Final break point temperature (BPT), also known as Arrhenius break point temperature, and flatline temperature (FLT) are common indices of upper critical thermal maxima of intertidal species (Stillman and Somero 1996; Stenseng et al. 2005), including limpets (Bjelde and Todgham 2013; Huang et al. 2015). Final BPT was determined by plotting individual limpet heart rate (bpm) over temperature. Best-fit regression lines over the ascending portion of heart rate and over the rapid, descending portion of heart rate were fitted as described by numerous investigators (Stillman 2003; Bjelde and Todgham 2013; Huang et al. 2015). The intersection of both best-fit regression lines was determined to obtain final BPT for each individual limpet. A limpet's FLT was found by determining the temperature at which heart rate had completely ceased. Patterns of multiple breaks in heart rate were observed in limpets during increases in environmental temperature, as was seen by Bjelde and Todgham (2013) in *L. digitalis*. The number of breaks in heart rate (i.e., when heart rate would decrease, then increase again) per individual limpet and the temperature at which these breaks occurred were recorded.

Breaks were characterized as inflections in heart rate traces where heart rate decreased and then increased again until the final steep break in heart rate recorded at upper critical limits of cardiac performance (i.e., final BPT). If a limpet's heart rate never increased or decreased and the limpet maintained a steady slow heart rate for the entire thermal ramping trial, the limpet was considered to have no breaks in heart rate. Lastly, temperature sensitivity of heart rate was examined using thermal performance curves.

### Tissue sampling

Limpets ( $n = 8$ ) were sampled immediately before the preliminary aerial exposure and sublethal heat stress (Day 1) and immediately before exposure to the severe lethal stressor (Day 2) for each preliminary temperature exposure group. The limpets that received no preliminary elevated temperature or aerial exposure (NoPE) were sampled at the same time as the other treatment groups. Foot tissues were dissected from each limpet, frozen in liquid nitrogen, and then stored at -80 °C for subsequent analysis of ubiquitin-conjugated protein levels and glycogen content.

### Ubiquitin-conjugated protein

Frozen foot tissue samples were prepared for total protein and ubiquitin (Ub)-conjugated protein dot blot analyses following a slightly modified protocol in Bjelde and Todgham (2013). Total protein concentration of limpet foot tissue was determined using the bicinchoninic acid method (Smith et al. 1985) for  $n = 8$  limpets per temperature exposure for each season. For dot blot analysis of levels of Ub-conjugated proteins, equal amounts of total protein (10 µg) were blotted onto the pre-wetted nitrocellulose membrane (Whatman, 0.2-µm pore size) in triplicate by gravity filtration for 2 h using a BioDot dot blotter (Bio-Rad, Hercules, California, USA). Nitrocellulose membranes were blocked in 5 % nonfat milk powder in Tween-20 Tris-buffered saline (TTBS: 20 mM Tris-HCl, 140 mM NaCl, 0.1 % Tween-20, pH 7.6) and incubated in Ub-conjugated protein-specific primary antibody that detects polyubiquitinated proteins (1:5000 in 5 % blocking solution, rabbit polyclonal antibody produced by Cocalico Biologicals Inc., donated by G. Hofmann), followed by an alkaline phosphatase-conjugated secondary antibody (1:5000 in 5 % blocking solution, Sigma-Aldrich, St. Louis, Missouri, USA). Nitrocellulose membranes were developed in a nitro blue tetrazolium (NBT; 333 µg mL<sup>-1</sup>), 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 167 µg mL<sup>-1</sup>) solution in alkaline phosphatase buffer (0.1 M Tris-HCl, 0.1 M NaCl, and 10 mM MgCl<sub>2</sub>, pH 9.5) for approximately 15 min or until background color became visible and allowed to dry overnight. Using a Kodak Molecular Imager, colorimetric



intensity was detected and quantified. Values were standardized using dot blot intensity values from a standard reference foot sample of *L. digitalis* blotted in triplicates on each nitrocellulose membrane (referred to as “Internal Std” in figures).

### Percentage body water

To assess desiccation during mild and severe elevated temperature exposures, limpet in shell, wet weights were taken before ( $W_i$ ) and after ( $W_f$ ) both the preliminary and lethal heat exposures to calculate change in wet mass in response to heat stress. Care was taken to dry or blot the limpet during transfer to the aerial heat block to ensure mantle water storage was maintained but excess water removed. While we cannot be certain that all animals had the same water content at the start of the experiments, limpets in all treatments were handled similarly, and therefore, we hope any discrepancies would be consistent across treatments. To assess tissue water loss, limpets were dissected out of shells after exposure to a lethal temperature increase on Day 2 and kept in an oven at 65 °C overnight. Shell weights (SW) and dry weights (DW) were recorded to calculate percentage body water (%BW) to determine amount of tissue water before (%BW<sub>*i*</sub>) and after (%BW<sub>*f*</sub>) the preliminary temperature exposure and the severe heat stress, as:

$$\%BW_{i,f} = \left[ 1 - \left( \frac{DW}{W_{i,f} - SW} \right) \right] \times 100 \quad (1)$$

The percentage body water loss (%BW<sub>Loss</sub>) from exposure to both the preliminary and lethal heat exposure ramps was determined by subtracting %BW<sub>*f*</sub> from %BW<sub>*i*</sub> for each individual limpet for each specific exposure. Shell dimensions, including shell length, width, and apex height, were recorded to ensure limpets were in similar size ranges to eliminate size as a confounding factor.

### Glycogen content

Frozen foot samples from summer and winter laboratory-acclimated limpets (with no previous heat or aerial exposure) were ground into a fine powder under liquid nitrogen using an insulated mortar and pestle ( $n = 9$  for each season). Glycogen content was enzymatically digested and measured as in Bjelde and Todgham (2013), modified from Fanguie et al. (2008). Briefly, glycogen was extracted in ice-cold 8 % HClO<sub>4</sub> and homogenized. The homogenate was split into two microcentrifuge tubes, 200 μl was set aside for glycogen determination and held on ice, and the remaining 800 μl for free glucose determination was centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was transferred to a new microcentrifuge tube, neutralized with 3 M K<sub>2</sub>CO<sub>3</sub>, and centrifuged again at 10,000 g

for 10 min at 4 °C at frozen at −80 °C for later analyses. Glycogen samples were then digested enzymatically to glucose following Hassid and Abraham (1957), and all glucose samples were measured spectrophotometrically modified from Bergmeyer (1983). Glycogen content was then corrected for starting free glucose and calculated as μmoles glucosyl units g<sup>−1</sup> wet foot tissue.

### Statistical analyses

Statistical analyses were conducted in R (v. 2.15.0, R Development Core Team 2012) using Rstudio (version 0.98.1103) with  $\alpha$  values of 0.05. Models were visually inspected for normality and homogeneity of variances using Q–Q plots, fitted values vs. residuals and factor levels vs. residuals. If assumptions were not met, analysis was run on transformed data (log transformation: glycogen and temperature differences between BPT and FLT, cube transformation: winter BPT and FLT) or using nonparametric tests if normality assumptions were still not met (Kruskal–Wallis test: % body water loss and BPT and FLT by number of breaks). Due to unequal number of preliminary temperature exposure treatments between seasons (no 35 °C data in winter due to mortality during temperature exposure), data from each season were analyzed separately with treatment as a fixed factor (one-way analysis of variance (ANOVA) or Kruskal–Wallis test) followed by Tukey’s HSD or Dunn’s rank sum comparisons (*pgirmess* package, Giraudoux 2015) post hoc test on significant treatment effects. A two-way ANOVA was used to assess differences in ubiquitin-conjugated proteins with preliminary temperature treatment and day as fixed effects, while independent Kruskal–Wallis tests were used to compare the effect of treatment on preliminary and final % body water loss. Additionally, to compare seasonal differences in BPTs and FLT, data from 35 °C for summer were dropped, and a generalized least squares (GLS) model was used to incorporate significant heterogeneity within the fixed factors (treatment by season) into the model using “varIdent” variance structure (Zuur et al. 2009) with season and treatment as fixed factors (*nlme* package, Pinheiro et al. 2013). An *anova* function was then run on the GLS model, and a post hoc Tukey’s test was used to detect differences between treatments using the package *multcomp* (Hothorn et al. 2008). A *t* test was used to determine differences in glycogen reserves by summer and winter seasons.

To evaluate thermal performance curves of heart rate between preliminary temperature treatments within summer and winter seasons, we used generalized additive mixed modeling (GAMM) (Zuur et al. 2009; Angilletta et al. 2013) with the *mgcv* (Wood 2004) and *nlme* (Pinheiro et al. 2013) packages in R. The identity of individual limpets was included in the model as a random factor to account for repeated measures. An *anova* of the model

was used to test whether the fitted heart rate curve for each preliminary exposure treatment significantly deviated from the control treatment performance curve generated in both summer and winter.

## Results

### Fort Ross temperature profiles

Temperature data from approximately 3 months in the summer and winter months at Fort Ross (Fig. 1) were coarsely evaluated to better understand the temperature conditions experienced by limpets in their natural environment and how it might relate to the preliminary temperature exposures provided on Day 1 of the experiment. Specifically, for both north and west sites, we calculated 1) daily overall average temperature (high and low tides), 2) average maximum daily temperature (Max °C, during emersion), 3) average minimum daily temperature (Min °C, during immersion), and 4) daily change in temperature ( $\Delta$  °C = Max °C – Min °C) from ocean temperature during high tide to peak temperature during the daytime low tide (Table 1). The daily average temperature and the maximum, minimum, and change in temperature during the daytime low tide were all greater during the summer months than the winter months at both sites (Table 1). In addition, we calculated the number of days in the summer (94 days) and winter (72 days) months when maximum temperatures during daytime low-tide periods reached or exceeded each of the preliminary exposure temperatures (Table 2).

### Upper critical limits of cardiac performance and inducible thermal tolerance

#### Summer laboratory acclimation

A linear model of final BPTs as a function of preliminary temperature exposures during the summer season showed no overall effect of temperature exposure on upper thermal tolerance in cardiac performance (ANOVA,  $F(6, 93) = 1.818$ ,  $P = 0.104$ ); however, the model showed that preliminary aerial exposures alone did increase BPTs compared to limpets that experienced no preliminary aerial exposure, referred to as NoPE (Fig. 2, panel 1). BPTs of limpets exposed to 25, 30, 32, and 35 °C were significantly greater ( $P < 0.05$ ) than limpets with no preliminary exposure. The average BPT of limpets with no preliminary exposure was  $38.95 \pm 0.54$  ( $n = 8$ ), while mean final BPTs from limpets with preliminary exposures ranged from  $40.51 \pm 0.31$  to  $41.72 \pm 0.33$  °C, a 1.5–3 °C increase in upper critical limits of cardiac performance. Specifically, after a preliminary

**Table 2** Total number of days in the summer (94 days) and winter months (72 days) when maximum temperatures during daytime low-tide periods reached or exceeded each of the preliminary exposure temperatures used in experimentation

Temperature (°C)	Days at or exceeding temperature		
	Field site	Summer	Winter
15	North	90	1
	West	90	29
20	North	34	0
	West	82	7
25	North	0	0
	West	50	0
30	North	0	0
	West	15	0
32	North	0	0
	West	7	0
35	North	0	0
	West	4	0

The numbers of days at or exceeding the temperatures are described for each Fort Ross field site, north and west

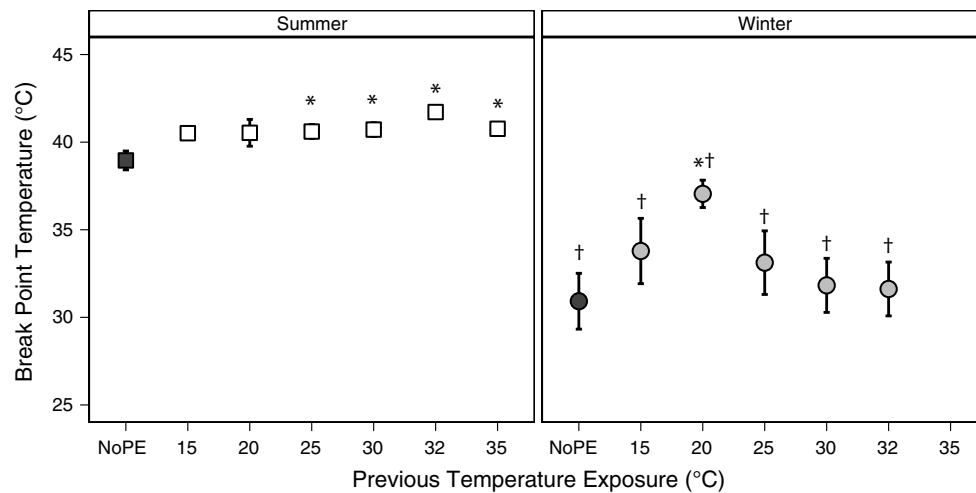
exposure to 15 °C, the average BPT increased by 1.55 °C ( $P = 0.053$ ,  $n = 12$ ) compared to that of the NoPE limpets, after exposure to 20 °C, BPTs increased 1.57 °C ( $P = 0.06$ ,  $n = 10$ ), 25 °C increased 1.64 °C ( $P = 0.03$ ,  $n = 14$ ), 30 °C increased 1.76 °C ( $P = 0.01$ ,  $n = 31$ ), 32 °C increased 2.76 °C ( $P = 0.001$ ,  $n = 10$ ), and after 35 °C, BPT increased by 1.80 °C ( $P = 0.03$ ,  $n = 10$ ).

A similar effect of preliminary temperature exposure on the FLT of limpets was demonstrated. Overall, there was no significant effect of preliminary temperature exposure on FLT (ANOVA,  $F(6,88) = 1.23$ ,  $P = 0.29$ ); however, the model summary showed preliminary aerial exposure to warming temperatures increased FLT compared to limpets with no preliminary exposure ( $P < 0.05$ , Fig. 3, panel 1). Limpets with no preliminary exposure had mean FLT of  $41.99 \pm 0.33$  ( $n = 8$ ), while mean FLT from limpets in treatment groups with preliminary exposures ranged from  $43.15 \pm 0.26$  to  $43.74 \pm 0.21$  °C ( $n = 10$ –31), a 1–2 °C increase in upper thermal limits of cardiac performance.

#### Winter laboratory acclimation

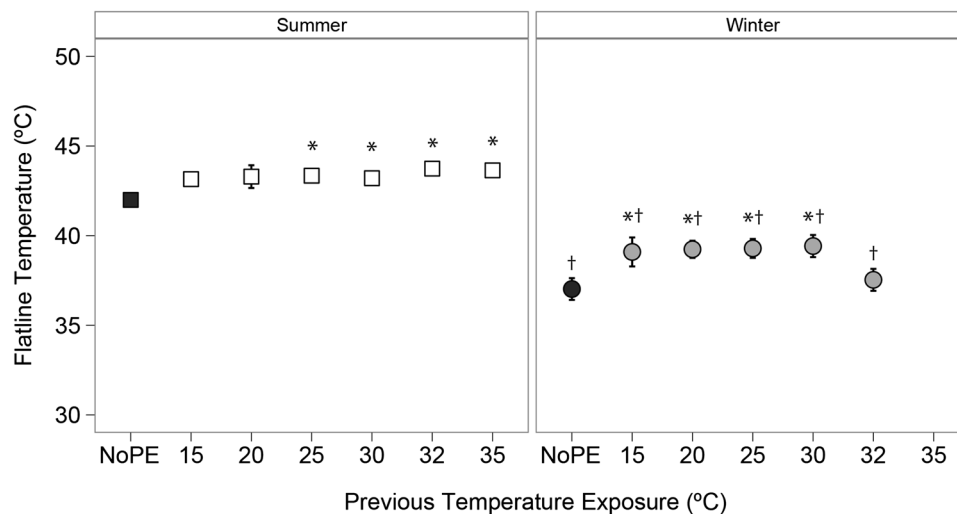
Preliminary temperature exposures did have a significant effect on final BPTs (ANOVA,  $F(5,96) = 2.72$ ,  $P = 0.024$ ) and FLT (ANOVA,  $F(5,128) = 3.057$ ,  $P = 0.012$ ) in winter laboratory-acclimated limpets (Figs. 2, 3, panel 2). Limpets subjected to a preliminary heat exposure of 20 °C on Day 1 had a significantly higher final BPT ( $P = 0.003$ ,





**Fig. 2** Final break point temperatures (BPT) in heart rate in summer (*open squares*, panel 1) and winter (*gray circles*, panel 2) laboratory-acclimated limpets during the lethal temperature ramp from 13 to 48 °C at a rate of 6 °C h<sup>-1</sup>. Data are presented as mean ± SEM. Asterisks indicate significant differences (ANOVA,  $P < 0.05$ ) between

final BPTs of the indicated preliminary temperature exposure to the group of limpets with no preliminary aerial or temperature exposure (NoPE, *black shapes*). Daggers indicate a significant difference (ANOVA,  $P < 0.05$ ) between BPTs of summer- and winter-acclimated limpets within each temperature exposure and NoPE



**Fig. 3** Flatline temperatures (FLT) in heart rate in summer (*open squares*, panel 1) and winter (*gray circles*, panel 2) laboratory-acclimated limpets during the lethal temperature ramp from 13 to 48 °C at a rate of 6 °C h<sup>-1</sup>. Data are presented as mean ± SEM. Asterisks indicate statistical differences (ANOVA,  $P < 0.05$ ) between FLTs of

limpets from indicated preliminary heat exposure treatments during summer or winter to the NoPE group (*black shapes*) of limpets with no preliminary aerial or heat exposure. Daggers indicate a significant difference (ANOVA,  $P < 0.05$ ) in FLTs by summer and winter season within each preliminary temperature exposure group

37.05 °C ± 0.78,  $n = 22$ ) than the limpets with no preliminary exposure (30.92 °C ± 1.54,  $n = 14$ ), a difference in final BPT of approximately 6 °C (Fig. 2). Limpets that were subjected to a preliminary exposure at 15, 20, 25, and 30 °C also had significantly greater FLTs ( $P < 0.05$ ) than the limpets in the NoPE group (37.02 °C ± 0.61,  $n = 20$ ). A preliminary exposure at 32 °C did not significantly increase FLT over the NoPE group (37.54 ± 0.61,  $P = 0.48$ ).

### Seasonal differences in cardiac performance

To compare seasonal differences in BPTs and FLTs, an ANOVA of a GLS model, including heterogeneity of seasons, was conducted and showed summer laboratory-acclimated limpets had significantly higher upper critical limits of cardiac performance as defined by final BPTs compared to winter laboratory-acclimated limpets ( $F(1,180) = 118.22$ ,  $P < 0.0001$ ) (Fig. 2). In addition,

there was an effect of preliminary temperature exposure ( $F(5,180) = 6.84$ ,  $P < 0.0001$ ) and a significant interaction between preliminary exposure and season ( $F(5,180) = 3.20$ ,  $P = 0.008$ ). Comparing summer and winter seasons within each preliminary temperature exposure treatment, BPTs were greater in summer-acclimated limpets. Specifically, BPTs were 6.7 °C greater at 15 °C ( $P = 0.013$ ), 3.5 °C greater at 20 °C ( $P = 0.047$ ), 7.5 °C greater at 25 °C ( $P < 0.01$ ), 8.9 °C greater at 30 °C ( $P < 0.01$ ), and lastly 10 °C greater at 32 °C ( $P < 0.01$ ) in summer compared to BPTs in winter. The cardiac performance of limpets with no preliminary aerial and temperature exposure (NoPE) in summer and winter also differed, such that BPTs in summer were 8 °C higher ( $P < 0.01$ ) than limpets in winter. *L. digitalis* collected in the summer were also able to reach significantly higher temperatures (~4–5 °C) before heart rate ceased (i.e., FLT) (ANOVA,  $F(1, 207) = 251.0$ ,  $P < 0.0001$ ) (Fig. 3). Summer laboratory-acclimated limpets at each preliminary temperature exposure experienced a flatline in heart activity at approximately 43 °C, all significantly greater temperatures ( $P < 0.01$ ) than winter laboratory limpets that flatlined at 39 °C or under.

Along with greater upper critical thermal limits in cardiac performance, summer laboratory-acclimated limpets also required an exposure to a higher preliminary temperature to confer increased thermal tolerance than winter laboratory-acclimated limpets (Fig. 2). Preliminary temperature exposures of 25, 30, 32, and 35 °C significantly increased the final BPT of summer limpets, whereas only a preliminary temperature exposure of 20 °C significantly increased the final BPT of winter limpets (Fig. 2). Preliminary temperature exposures of 25, 30, 32, and 35 °C significantly increased the FLT of summer limpets, and preliminary temperature exposures of 15, 20, 25, and 30 °C significantly increased the FLT of winter limpets (Fig. 3). A preliminary heat exposure of 32 °C caused two limpet mortalities in the winter and although not significant, decreased final BPT by approximately 2–3 °C and FLT by close to 2 °C compared to the other treatment groups exposed to lower preliminary temperatures during winter trials. Limpets with a preliminary temperature exposure of 35 °C in the summer survived, whereas in the winter, 25 % of the limpets could not tolerate a preliminary heat exposure of 35 °C, and therefore, this treatment group was removed from the winter experiment.

## Patterns of cardiac performance

### Multiple breaks in heart rate

Both summer and winter laboratory-acclimated limpets exhibited variable patterns in heart function as temperatures increased, demonstrated by multiple breaks in heart rate (Online Resource Fig. S1). Summer laboratory-acclimated

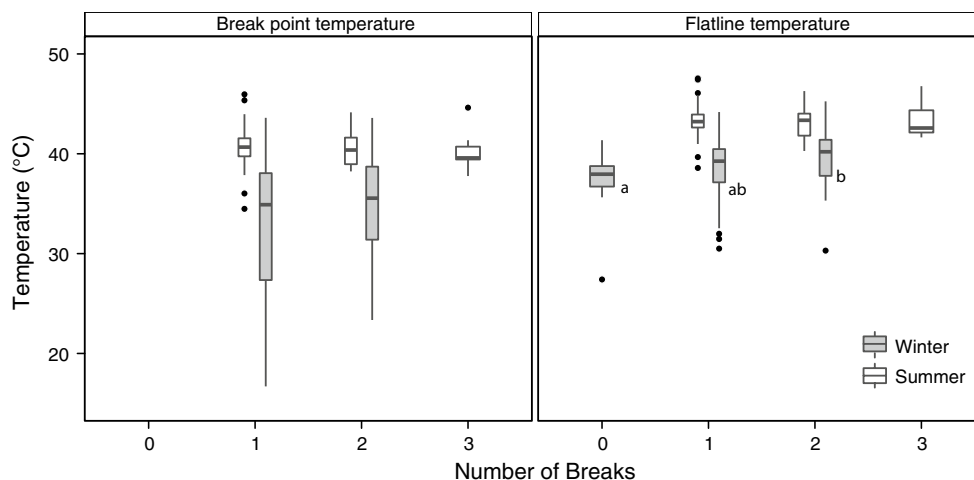
limpets had 1, 2, or 3 breaks in heart rate during the aerial temperature ramps, never exhibiting 0 breaks in heart rate (i.e., constant low heart rate). In contrast, winter laboratory-acclimated limpets had 0, 1, or 2 breaks in heart rate during the thermal ramp but never had 3 breaks in heart rate. Preliminary heat exposure temperature had no effect on number of breaks in either the summer or winter laboratory acclimations (Kruskal–Wallis test, summer:  $H_6 = 3.91$ ,  $P = 0.69$ ; winter:  $H_5 = 3.11$ ,  $P = 0.68$ ). The number of breaks did not affect upper thermal limits of cardiac performance as determined by final BPT or by FLT in summer laboratory-acclimated limpets (Kruskal–Wallis test, BPT:  $H_2 = 1.42$ ,  $P = 0.49$ ; FLT:  $H_2 = 0.06$ ,  $P = 0.97$ ) (Fig. 4). The number of breaks also did not affect final BPTs in winter laboratory-acclimated limpets (Kruskal–Wallis test,  $H_1 = 0.8$ ,  $P = 0.37$ ); however, the number of breaks did have a significant effect on FLTs in winter laboratory-acclimated limpets (Kruskal–Wallis test,  $H_2 = 8.05$ ,  $P = 0.018$ ), in which limpets with no breaks had significantly lower FLTs ( $P < 0.05$ ) than limpets with two breaks in heart rate (Fig. 4).

### Temperature difference between BPT and FLT

Winter laboratory-acclimated limpets had greater temperature differences between final BPT and FLT (ANOVA,  $F(5,96) = 3.55$ ,  $P = 0.005$ ), along with much greater levels of variability in the difference in temperature between final BPT and FLT (Table 3). There was no significant difference in the temperature difference between final BPTs and FLTs between treatment groups in summer laboratory-acclimated limpets (ANOVA,  $F(6,88) = 1.51$ ,  $P = 0.184$ ). There was, however, a significant decrease in the temperature difference between final BPTs and FLTs for limpets subjected to a preliminary heat exposure of 20 °C compared to 30 °C ( $P < 0.01$ ) and 32 °C ( $P = 0.03$ ) in the winter experiment. Limpets subjected to a preliminary heat exposure of 20 °C had the greatest upper thermal tolerance (BPT) of the winter laboratory-acclimated limpets, which could explain why their heart rate patterns more closely matched those seen in the summer laboratory-acclimated limpets.

### Cardiac performance curves

Summer laboratory-acclimated limpets pre-exposed to 20, 32, and 35 °C the day before a lethal temperature increase had significantly different heart rate curves compared to NoPE limpets, with faster heart rates, especially at elevated temperatures (Fig. 5; Table 4). Winter laboratory-acclimated limpets in all pre-exposure treatment groups, except for 32 °C, had significantly different heart rate curves compared to NoPE limpets, showing a similar trend to summer laboratory limpets, with faster heart rates intensified at higher temperatures (Fig. 5; Table 4). Cardiac performance



**Fig. 4** Final break point temperatures (panel 1) and flatline temperatures (panel 2) separated by the total number of breaks in heart rate exhibited by limpets during the lethal temperature ramp (Day 2) for summer (*white*) and winter (*gray*) laboratory-acclimated limpets. The line of the *boxplots* represents the median of the data. The box

represents the inter-quartile range (IQR) and the *whiskers* extend  $1.5 \times$  IQR. Points beyond the *whiskers* are outliers and were included in the dataset. *Different letters* indicate significant differences between flatline temperatures and number of breaks in heart rate in winter laboratory-acclimated limpets (Kruskal–Wallis,  $P < 0.05$ )

**Table 3** Temperature difference between final BPT and FLT (mean and SEM) of winter and summer laboratory-acclimated limpets during a lethal temperature ramp (Day 2)

Treatment	Season	Final BPT	FLT	FLT-BPT	<i>n</i>
NoPE	Summer	38.96 ± 0.54	41.99 ± 0.33	3.04 ± 0.43 <sup>a</sup>	8
	Winter	30.92 ± 1.6	37.02 ± 0.61	6.01 ± 1.29 <sup>xy</sup>	14
15 °C	Summer	40.51 ± 0.31	43.15 ± 0.26	2.73 ± 0.2 <sup>a</sup>	12
	Winter	33.48 ± 1.92	39.1 ± 0.81	5.35 ± 1.6 <sup>xy</sup>	13
20 °C	Summer	40.53 ± 0.77	43.29 ± 0.63	2.76 ± 0.37 <sup>a</sup>	10
	Winter	37.05 ± 0.78	39.24 ± 0.48	2.48 ± 0.37 <sup>x</sup>	22
25 °C	Summer	40.61 ± 0.40	43.34 ± 0.38	2.82 ± 0.33 <sup>a</sup>	14
	Winter	33.12 ± 2.01	39.29 ± 0.53	6.71 ± 1.46 <sup>xy</sup>	16
30 °C	Summer	40.72 ± 0.40	43.21 ± 0.34	2.49 ± 0.12 <sup>a</sup>	31
	Winter	31.83 ± 1.54	39.42 ± 0.62	7.82 ± 1.29 <sup>y</sup>	17
32 °C	Summer	41.72 ± 0.33	43.74 ± 0.21	2.02 ± 0.23 <sup>a</sup>	10
	Winter	31.62 ± 1.54	37.54 ± 0.61	6.24 ± 1.12 <sup>y</sup>	20
35 °C	Summer	40.76 ± 0.36	43.64 ± 0.32	2.88 ± 0.26 <sup>a</sup>	10

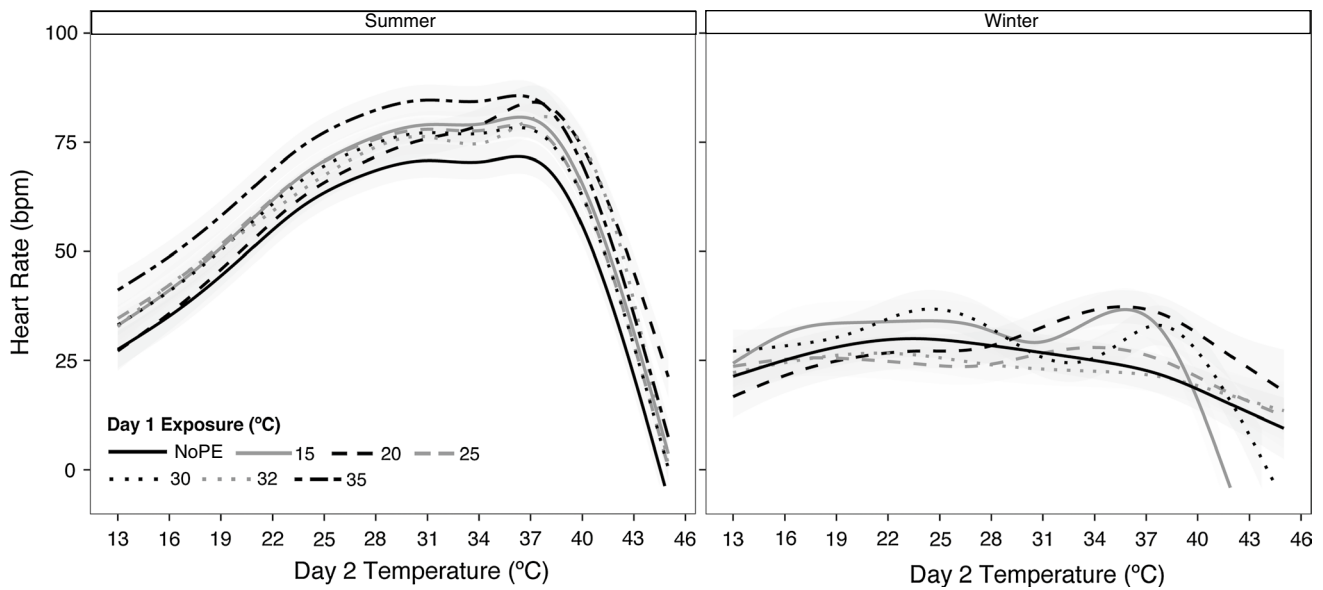
Limpets were subjected to different preliminary temperature exposures the day before (Day 1) exposure to a lethal increase in temperature. Letters indicate significant differences (ANOVA,  $P < 0.05$ ) in temperature difference between BPT and FLT within summer and winter preliminary heat exposure treatment groups independently

curves of winter laboratory-acclimated limpets displayed a more gradual and attenuated increase and decrease in heart rate during the temperature ramp compared to summer laboratory-acclimated limpets (Fig. 5).

### Ubiquitin-conjugated protein

Ubiquitin (Ub)-conjugated proteins were present in both summer and winter laboratory-acclimated limpets in all treatment groups ( $n = 8$  for each treatment group in both summer and winter) before the preliminary temperature

exposure on Day 1 and following the preliminary temperature exposure, immediately before exposure to a lethal temperature increase on Day 2 (i.e., two time points). There was no effect of previous exposure temperature (Two-way ANOVA, Summer:  $F(6,111) = 0.87$ ,  $P = 0.52$ ; Winter:  $F(5,95) = 1.33$ ,  $P = 0.26$ ), time point (Summer:  $F(1,111) = 0.002$ ,  $P = 0.96$ ; Winter:  $F(1,95) = 1$ ,  $P = 0.32$ ) or an interaction between temperature and time point (Summer:  $F(6,111) = 0.52$ ,  $P = 0.79$ ; Winter:  $F(5,95) = 1.29$ ,  $P = 0.28$ ) on levels of Ub-conjugated proteins in summer or winter laboratory-acclimated limpets (data not shown).



**Fig. 5** Cardiac performance curves of limpets in response to increasing temperatures in summer (panel 1) and winter (panel 2) laboratory-acclimated limpets. NoPE indicates limpets with no preliminary aerial or heat exposure. Heart rate curves were generated using

GAMM analysis when limpets were exposed to increasing temperature at a rate of 6 °C h<sup>-1</sup> from 13 to 48 °C during a lethal temperature ramp in summer (*n* = 8–31 per treatment) and winter (*n* = 16–28 per treatment) acclimation trials

**Table 4** Evaluations of generalized additive mixed models (GAMM) of heart rate as a function of temperature, *f(T)*, that is referenced to the curve of the NoPE group in both summer and winter seasons

	<i>edf</i>	<i>F</i> value	<i>p</i> value
<b>Summer</b>			
<i>f(T)</i> for NoPE	8.601	690.604	<0.0001
Deviation from <i>f(T)</i> for 15 °C	2.001	2.167	0.115
Deviation from <i>f(T)</i> for 20 °C	4.042	13.097	<0.0001
Deviation from <i>f(T)</i> for 25 °C	2.001	1.224	0.294
Deviation from <i>f(T)</i> for 30 °C	2.001	1.187	0.305
Deviation from <i>f(T)</i> for 32 °C	7.596	4.616	<0.0001
Deviation from <i>f(T)</i> for 35 °C	2.001	3.735	0.024
<b>Winter</b>			
<i>f(T)</i> for NoPE	4.497	5.716	<0.0001
Deviation from <i>f(T)</i> for 15 °C	7.412	4.331	<0.0001
Deviation from <i>f(T)</i> for 20 °C	5.338	9.855	<0.0001
Deviation from <i>f(T)</i> for 25 °C	5.168	3.613	<0.01
Deviation from <i>f(T)</i> for 30 °C	7.719	3.997	<0.001
Deviation from <i>f(T)</i> for 32 °C	3.418	1.6	0.179

*edf* effective degrees of freedom

**Percentage body water**

*Summer laboratory acclimation*

On Day 1, summer laboratory-acclimated limpets exposed to a 30 °C preliminary temperature exposure experienced

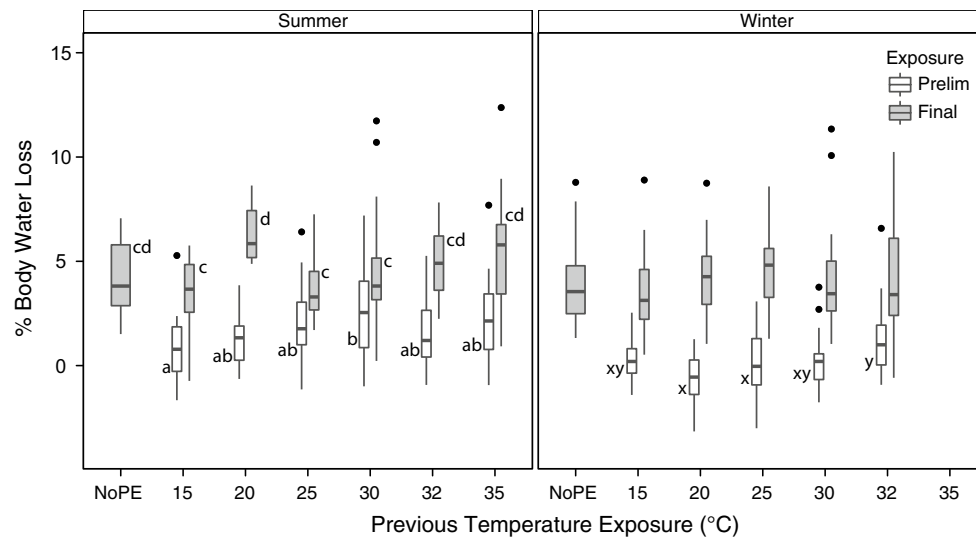
a significant increase in percentage body water loss (%BW<sub>Loss</sub>) compared to limpets exposed to a 15 °C preliminary temperature exposure, while differences in all other treatment groups were not significant (Kruskal–Wallis test, H5 = 16.44, *P* < 0.01). After exposure to a lethal temperature increase on Day 2, there was a significant increase in %BW<sub>Loss</sub> in summer laboratory-acclimated limpets pre-exposed to a 20 °C preliminary temperature exposure compared to limpets pre-exposed to 15, 25, and 30 °C preliminary temperature exposure (Kruskal–Wallis test, H6 = 27.19, *P* < 0.001) (Fig. 6a).

*Winter laboratory acclimation*

On Day 1, winter laboratory-acclimated limpets exposed to a 32 °C preliminary temperature exposure experienced a significant increase in %BW<sub>Loss</sub> compared to limpets exposed to 20 and 25 °C preliminary temperature exposures, while differences in all other treatment groups were not significant (Kruskal–Wallis test, H4 = 27.97, *P* < 0.001). After exposure to a lethal temperature increase on Day 2, there were no significant differences in %BW<sub>Loss</sub> between any of the treatment groups (Kruskal–Wallis test, H5 = 10.12, *P* = 0.072) (Fig. 6b).

**Glycogen content**

A two-sample *t* test was run to see whether there was an effect of season on basal glycogen content in limpet foot



**Fig. 6** Percentage body water loss (%BW<sub>Loss</sub>) after a preliminary heat exposure (white, “Prelim”) and a lethal heat shock (gray, “Final”) ramped from 13 to 48 °C at a rate of 6°C h<sup>-1</sup> in summer (panel 1) and winter (panel 2) laboratory acclimation groups. Percentage body water (%BW<sub>Loss</sub>) calculated by  $(\%BW_i - BW_f)$  expressed as  $\{1 - [\text{dry mass}/(W_{i,f} - \text{shell mass})]\} \times 100$  for each preliminary temperature exposure and NoPE. The line of the *boxplots* represents

the median of the data. The *box* represents the inter-quartile range (IQR), and the *whiskers* extend  $1.5 \times \text{IQR}$ . Points beyond the *whiskers* are outliers and were included in the dataset. *Letters* indicate significant differences (Kruskal–Wallis,  $P < 0.05$ ) in %BW<sub>Loss</sub> between different treatment groups after a preliminary heat exposure and a final lethal temperature increase

tissue following laboratory acclimation before repeated elevated temperature exposures. Winter laboratory-acclimated limpets had significantly lower levels of glycogen ( $0.98 \pm 0.24 \mu\text{mol glucosyl units g}^{-1}$  wet tissue,  $n = 9$ ) in foot tissue compared to summer laboratory-acclimated limpets ( $12.93 \pm 3.13 \mu\text{mol glucosyl units g}^{-1}$  wet tissue,  $n = 9$ ) ( $t_{16} = 6.999$ ,  $P < 0.001$ ) (data not shown).

## Discussion

### Inducible thermal tolerance

Organisms inhabiting the intertidal zone experience repeated aerial exposure and some degree of temperature change with each low-tide period; however, much of our understanding of the thermal physiology of intertidal organisms come from single acute heat–shock experiments or much longer thermal acclimation trials. How an intertidal organism’s immediate thermal history (i.e., temperature increase during the previous day’s daytime low-tide period) modulates its response to a severe heat stress and its upper temperature tolerance is not well understood. Exposure to a preliminary sublethal increase in temperature increased upper critical thermal limits of cardiac performance of *L. digitalis* as determined by temperature at which cardiac function was drastically reduced (final breakpoint temperature, BPT) and ceased (flatline temperature, FLT) in both summer and winter laboratory-acclimated

limpets. Additionally, the magnitude of temperature increase in these preliminary exposures mattered and the priming temperatures that increased thermal tolerance differed in summer and winter. These results provide evidence that inducible stress tolerance may be an important aspect of the thermal physiology of *L. digitalis* in nature.

The magnitude of the preliminary or priming stressor has been found to have important implications for the degree of resistance gained by the organism to the secondary stressor (DuBeau et al. 1998; Hoffmann et al. 2003; Todgham et al. 2005). If the preliminary stressor is too low or too high, inducible stress tolerance may not be conferred (Krebs and Loeschcke 1994; Loeschcke et al. 1994). Limpets in the present study displayed a graded response in heat hardening during the summer, where routine maximum temperatures experienced during summer daytime low-tide periods (25 °C) at west-facing intertidal sites at Fort Ross, CA (Fig. 1; Tables 1, 2), conferred some level of protection by increasing both final BPT and FLT, with slightly higher protection at 32 °C (a temperature only measured in the field 7 days during summer 2012). Even a 35 °C preliminary temperature exposure, which occurs rarely at the intertidal collection site (maximum temperature recorded was 37.5 °C), conferred increased thermal tolerance to limpets during summer trials. In contrast, a 20 °C preliminary temperature exposure, which was unable to significantly increase upper thermal tolerance of limpets in the summer trials, increased the thermal tolerance



of winter laboratory-acclimated limpets, demonstrating that the temperature sensitivity of inducible thermal tolerance varies seasonally. In nature during the winter months, a 20 °C exposure during daytime low-tide periods can occur occasionally, with maximum temperatures recorded at the Fort Ross, CA, collection site during the winter of 2013 not exceeding 24.5 °C at the west-facing site (Fig. 1; Table 2). In neither the summer nor winter laboratory acclimation trials did we expose limpets to a preliminary heat stress that significantly decreased upper thermal tolerance below that of the limpets that experienced no preliminary exposure. In a pilot study, winter laboratory-acclimated limpets experienced some mortality after a 35 °C preliminary heat exposure, suggesting a potential upper temperature limit of inducible thermal tolerance. Intertidal organisms are capable of tolerating wide fluctuations in environmental temperature on a daily basis; therefore, relying on inducible thermal tolerance and the associated cellular defense mechanisms to extend thermal tolerance by a few degrees when exposed to average maximal low-tide emersion temperatures in nature may be most effective for survival. The results of the current study suggest that the upper thermal tolerance of *L. digitalis* is plastic and is likely influenced by low-tide exposures (i.e., aerial exposure and some degree of temperature change) experienced the day prior.

Daily exposure to emersion during low-tide periods may provide enhanced thermal tolerance under natural conditions in the intertidal zone, regardless of the accompanying increase in temperature (Bjelde and Todgham 2013; Huang et al. 2015) as a predictable cue to environmental change (Sinclair et al. 2007). Our study was not designed to determine whether aerial or heat exposure had a greater influence on inducible thermal tolerance in limpets since these factors are coupled during low-tide periods; however, it is possible that these stressors had an additive effect on limpets' thermal tolerance. In the current study, all limpets that experienced a preliminary temperature exposure, regardless of the magnitude of temperature increase, experienced a small increase in %BW loss (Fig. 6), and many temperature groups had higher BPTs and FLTs compared to limpets with no previous exposure in both seasons. Whether this correlation with slight desiccation has a mechanistic basis of cross-tolerance would require further investigation; however, there were no obvious relationships between %BW loss and the degree of inducible thermal tolerance (i.e., similar %BW loss between 15 and 25 °C preliminary exposed limpets but differences in capacity to induced enhanced thermal tolerance). This is not surprising given the high desiccation tolerance of *L. digitalis* (Wolcott 1973).

Aerial exposure and elevated temperatures during low tide may act to prime stress tolerance mechanisms to protect organisms from subsequent, more severe increases in temperature. In our laboratory trials, aerial exposure may

have been a cue for low tide to the limpet and therefore initiated cellular stress response mechanisms that better prepared them to respond to a more severe increase in temperature. Aerial exposure without an accompanying heat stress has been found to increase inducible isoforms of heat-shock proteins (Hsps) in the intertidal mussel, *Mytilus galloprovincialis* (Anestis et al. 2010), suggesting that maintenance of protein integrity during repeated low-tide periods might be an important mechanism of protection. This is in contrast to a previous study on a mid-intertidal limpet, *Cel-lana toreuma*, that found no Hsp70 response to aerial exposure alone at 20 °C (Huang et al. 2015). Although there is an accumulating body of evidence that suggests that the heat-shock response and the induction of Hsps is a primary cellular mechanism underlying heat hardening (Kregel 2002; Sørensen et al. 2003), several studies have found that heat hardening may be uncoupled with Hsp synthesis and concentrations (Easton et al. 1987; Dahlggaard et al. 1998). In the current study, Ub-conjugated proteins were measured directly as a measure of protein damage present in the limpets prior to exposure to the lethal temperature increase. There was no effect of preliminary temperature exposure on levels of Ub-conjugated proteins in foot tissue, suggesting that preliminary heat exposures were either not high enough to induce protein denaturation or if protein damage did occur, the 18-hour period in ambient conditions between trials was long enough to repair any thermally damaged proteins. Research on a high intertidal congener, *L. austrodigitalis*, demonstrated that this limpet has a strategy of maintaining high levels of constitutive Hsc70 in preparation for the unpredictable heat stress they may encounter during a daytime low-tide period (Dong et al. 2008). Further studies are needed to examine whether Hsp transcript or protein levels are upregulated in response to a preliminary temperature exposure that induces enhanced thermal tolerance and whether this potential front-loading of Hsps delays the onset of the thermal denaturation of proteins during a subsequent severe heat shock.

### Seasonal differences in cardiac performance and inducible stress tolerance

Results from the current study provide strong evidence that the mechanisms of inducible thermal tolerance are tailored to the thermal environment of a given season in *L. digitalis*. There were seasonal differences in the priming temperature of heat hardening in summer versus winter laboratory-acclimated limpets as well as the breadth of temperatures that conferred inducible thermal tolerance. These seasonal differences were present despite the laboratory acclimation to similar temperatures (13 °C) in our summer and winter trials. Upper critical limits of cardiac performance (BPT) and thermal tolerance (FLT) of summer laboratory-acclimated

limpets were increased in response to preliminary exposure to aerial temperatures of 25–35 °C, whereas during winter trials, only a 20 °C preliminary exposure increased BPT, but 15–30 °C preliminary exposures increased the temperature these limpets could tolerate before heart rate ceased (FLT) (Figs. 2, 3). Therefore, there are clear seasonal differences in the temperature sensitivity of mechanisms that contribute to inducible thermal tolerance in *L. digitalis* as would be predicted based on seasonal differences in thermal tolerance of intertidal organisms (Somero 2002). What is more unexpected is the narrow breadth of temperatures that increased BPT (20 °C but not 15 or 25 °C) but the wide breadth of temperatures that increased FLT (15–30 °C) during winter trials, suggesting seasonal differences in the temperature sensitivity of different aspects of cardiac performance. During the winter months at Fort Ross, CA, limpets would rarely see temperatures above 20 °C (Table 2) and yet they appear to defend the upper limits of cardiac function (i.e., FLT) to temperatures well above seasonal maximum low-tide exposures. The ecological relevance of degree of heat hardening in the winter requires further investigation.

In *L. digitalis*, final BPT was more strongly affected by season than FLT, similar to what was documented in another mid-intertidal limpet, *Cellana toreuma* (Han et al. 2013). Heat-related mortality in marine ectotherms can be a result of oxygen (O<sub>2</sub>) deficiency to the tissues, which prompts the transition to passive tolerance (Pörtner 2002, 2010). As a first line of defense during increased temperatures, limpets increase heart rate and circulation to compensate for the increased O<sub>2</sub> demand at tissues (Fig. 5). Once temperature stress reaches a threshold, O<sub>2</sub> demand can no longer be met and metabolism is depressed, which supports passive tolerance and can be observed after the dramatic decrease in heart rate (BPT) and until mortality sets in and heart rate completely ceases (FLT). Passive tolerance provides time-limited survival during adverse environmental conditions and may be exploited for short-term or long-term exposures depending on the nature and severity of the stress (Pörtner 2002, 2010). Winter laboratory-acclimated limpets displayed a larger temporal window between BPT and FLT, indicating an earlier transition to passive tolerance possibly due to reduced energy reserves reflected by lower glycogen stores and an earlier incapacity of the circulatory system to deliver sufficient O<sub>2</sub> to tissues.

Both summer and winter laboratory-acclimated limpets experienced breaks in heart rate during temperature ramps in which heart rate would decrease considerably, but recover and increase again before final BPT. Breaks in heart rate in the current study are consistent with previous studies in *L. digitalis* (Bjelde and Todgham, 2013), although not routinely reported in other studies of cardiac performance across increasing temperature. A growing

number of studies suggest that ectotherms and limpets specifically have the ability to regulate physiological activity to a remarkable extent (Segal 1956; Marshall and McQuaid 1993; De Pirro et al. 2001; Chelazzi et al. 2001). Limpets exposed to unfavorable conditions have been found to display both strong bradycardia and cessation of heart rate for extended periods, which can be reversed when the limpets are returned to ambient conditions (Marshall and McQuaid 1991; De Pirro et al. 1999, 2001; Chelazzi et al. 2004). Alterations in heart rate may be due to an adaptive response to extend survival by metabolic rate suppression, rather than from functional impairment of cardiac activity. The intertidal snail, *Echinolittorina malaccana*, was found to display depressed, temperature-insensitive metabolism as a means of energy conservation at high body temperatures (Marshall et al. 2011). It is possible that winter laboratory-acclimated limpets, with lower glycogen stores, recruited a similar strategy and entered periods of temperature insensitivity and reduced heart rate to extend the critical thermal limits of cardiac performance. An earlier transition to passive tolerance may also explain why some winter laboratory-acclimated limpets, with lower glycogen energy reserves, displayed no breaks in heart rate for the duration of the temperature ramp, while summer laboratory-acclimated only displayed 1, 2, or 3 breaks in heart rate (Fig. 4). Since oxygen consumption was not measured simultaneously with heart rate, it is not possible to know whether metabolic rate was decreased when heart rate decreased. Further research is warranted to understand the energy balance associated with breaks in cardiac activity and whether the energy savings outweigh the costs of replenishing any oxygen debt associated with metabolic suppression.

Differences in the physiological condition of limpets during the summer and winter months may be an important factor in addition to season acclimatization contributing to the differences in upper critical thermal limits of cardiac performance observed in the current study. Winter laboratory-acclimated limpets had depleted levels of glycogen in foot tissues that were significantly lower than levels measured in summer laboratory-acclimated limpets. The foot muscle of gastropods is known to store energetic reserves such as lipids and carbohydrates (Lawrence 1976; Voltzow 1994), and thus is a good index of seasonal differences in energy stores. Differences in glycogen levels may reflect the lower food supply and feeding rate of limpets during the winter (Santini and Chelazzi 1995; Santini et al. 2002). Additionally, *L. digitalis* spawn during the winter months, and therefore, more energy may be allocated to reproduction over stress tolerance mechanisms, including upper temperature tolerance (Sokolova et al. 2012). In fact, spawning was observed in the laboratory during winter trials. Spawning is energetically costly and can leave organisms with reduced energy reserves, loss of body weight,

compromised immune systems, and increased risk of mortality (Smith et al. 1990; Lambert and Dutil 2000; Li et al. 2007; Petes et al. 2008). Lipids and glycogen are the primary energetic source for gametogenesis in gastropods, including limpets (Blackmore 1969; Simpson 1982; Gabbott 1983; Lurman et al. 2010), so it is not surprising that limpets in the winter trial displayed depleted glycogen levels. Additional research is needed to better understand the potential trade-offs in thermal physiology and reproduction of *L. digitalis* during the winter.

## Concluding remarks

This study demonstrates the importance of considering serial increases in temperature when examining the thermal physiology of intertidal organisms. For intertidal limpets that inhabit highly variable environments that fluctuate in temperature and aerial exposure, these aspects of environment are critical for modulating an organism's capacity to respond to and tolerate severe heat stress. This study demonstrates that the upper thermal tolerance of *L. digitalis* is plastic and modulated by the previous day's low-tide period (immediate thermal history and aerial emersion), with temperature sensitivity of inducible thermal tolerance tailored to season (chronic thermal history). Future studies on intertidal thermal physiology should incorporate serial exposure to elevated temperatures under aerial conditions to more realistically predict the capacity of intertidal organisms to tolerate future warming scenarios (mean increases in temperature and heat waves). Results from our study suggest that if heat waves are accompanied by multiple above-average temperature days, that upper temperature tolerance could be extended by a couple of degrees due to previous low-tide exposures, likely until a point where the sublethal temperature increase is damaging at the cellular level. The current study only investigated two heat exposures in series. Future research should focus on multiple preliminary sublethal increases in temperature that are stochastically relevant metrics of environmental change that shape an intertidal organism's temperature sensitivity and upper temperature tolerance (Helmuth et al. 2014).

**Acknowledgments** We would like to thank Dr. Jonathon Stillman for his helpful input in heart rate analysis and use of his heart rate setup as well as Dr. Lars Tomanek for his helpful discussions on the manuscript. We would also like to thank Dr. Nate Miller for his assistance with the GAMM analyses and Erin Flynn for help with the statistical analyses. This work was supported by San Francisco State University to AET, the University of California Agricultural Experiment Station (Grant Number CA-D-ASC-2252-H to A.E.T.), and a CSU Council on Oceans Affairs, Science and Technology (COAST) student scholarship to CP.

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