The Role of Oxygen in Determining Upper Thermal Limits in *Lottia digitalis* under Air Exposure and Submersion

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Accepted 5/6/2015; Electronically Published 6/19/2015

Online enhancements: supplementary figures.

ABSTRACT

Oxygen limitation of aerobic metabolism is hypothesized to drive organismal thermal tolerance limits. Differences in oxygen availability in air and water may underlie observed differences in upper thermal tolerance of intertidal limpets if oxygen is limiting in submerged environments. We explored how cardiac performance (heart rate, breakpoint temperature [BPT], flat-line temperature [FLT], and temperature sensitivity) was affected by hyperoxia and hypoxia in the finger limpet, *Lottia digitalis*, under air exposure and submersion. Upper thermal tolerance limits were unchanged by increasing availability of oxygen, although air-exposed limpets were able to maintain cardiac function to higher temperatures than submerged limpets. Maximum heart rate did not increase with greater partial pressure of oxygen (P_{O_2}), suggesting that tissue P_{O_2} levels are likely maximized during normoxia. Hypoxia reduced breakpoint BPTs and FLTs in air-exposed and submerged limpets and accentuated the difference in BPTs between the two groups through greater reductions in BPT in submerged limpets. Differences in respiratory structures and the degree to which thermal limits are already maximized may play significant roles in determining how oxygen availability influences upper temperature tolerance.

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Physiological and Biochemical Zoology 88(5):483–493. 2015. 1522-2152/2015/8805-4146$15.00. DOI: 10.1086/682220

Keywords: heart rate, intertidal limpet, oxygen limitation, temperature tolerance, environmental hypoxia and hyperoxia.

Introduction

Inhabitants of rocky intertidal zones experience a highly variable habitat, with routine submersion and aerial exposure leading to potentially dramatic fluctuations in desiccation, temperature, and oxidative stress. The potential for extreme and frequent thermal fluctuations is greater during air exposure, whereas during submersion the reduced oxygen (O_2) availability and rate of O_2 diffusion (approximately 30 × and 10,000 × lower, respectively; Truchot 1990) may limit aerobic respiration potential (Verberk et al. 2011). Physiological mechanisms that organisms use to tolerate environmental stress in such shifting environmental conditions are not fully understood. Terrestrial and marine habitats are predicted to experience increases in mean temperature and in frequency of extreme temperatures (Meehl et al. 2007). Hence, understanding how intertidal species respond to environmental stress and how this response may differ between submerged and air-exposed conditions is of fundamental importance in predicting impacts of climate change on rocky intertidal communities.

Organismal thermal tolerance has been hypothesized to be related to oxygen availability and supply to tissues, a conceptual framework referred to as the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis (Frederich and Pörtner 2000; Pörtner 2001, 2010, 2012; Pörtner and Knust 2007; Pörtner and Farrell 2008). The OCLTT hypothesis states that, at temperatures outside an optimal range, organisms experience a reduced capacity for respiratory and circulatory systems to deliver oxygen to respiring tissues. The mismatch between oxygen supply and demand, which is due to an exponential increase in O_2 need with increasing temperature, progressively reduces aerobic scope and overall organismal aerobic performance (Pörtner et al. 2007). Ultimately, as temperatures continue to increase or decrease, performance declines to such an extent (due to oxygen limitation at the level of the respiring tissues) that death is imminent. Consequently, the capacity to maintain adequate O_2 levels at the tissue level during warming and cooling is hypothesized to determine thermal tolerance, and factors that influence this capacity, including availability of environmental O_2, may set thermal tolerance limits.

Much of the thermal physiology research on intertidal organisms has focused on the mechanisms underlying thermal tolerance during submersion (Read and Cumming 1967; Still-

man and Somero 1996, 2000; Stenseng et al. 2005; Diederich and Pechenik 2013), despite recognition that thermal stress is more typically experienced during air exposure (Sokolova and Pörtner 2001; Sorte et al. 2011; Bjelde and Todgham 2013). The fingered limpet, *Lottia digitalis* (Rathke 1833), one of only a few species within the Lottidae family found at the highest edges of the intertidal zone (Lindberg and Pearse 1990), can experience large daily fluctuations in temperature (20°–30°C) during low tide. *Lottia digitalis* exhibited higher breakpoint temperatures of heart rate during aerial exposure as well as distinct differences in the metabolic response of submerged and air-exposed limpets to increasing temperatures (Bjelde and Todgham 2013). Submerged limpets demonstrated a 25%–50% depression in aerobic metabolic rate ($\dot{M}_{O_2}$) during heating that was not experienced by limpets exposed to aerial heating, resulting in air-to-water $\dot{M}_{O_2}$ ratios of 2–4 at elevated temperatures. In contrast, *Mytilus californianus* decreased heart rate and accumulated metabolite end products of anaerobiosis when exposed to aerial conditions (Connor and Gracey 2012). Air-to-water $\dot{M}_{O_2}$ ratios of gastropods and other intertidal organisms range from 1 to 5 (Wolcott 1973; Branch and Newell 1978; McMahon 1988; Truchot 1990), indicating that there are species-specific differences in relative ability to respire at an equal rate in air and water. Air-breathing terrestrial taxa are thought to have an enhanced ability to regulate oxygen relative to their aquatic counterparts, balancing oxygen toxicity against risk of asphyxiation (Verberk and Bilton 2013), in part because of higher ventilatory effort associated with oxygen supply when breathing water compared with air (Verberk and Atkinson 2013; Giomi et al. 2014). This may explain why terrestrial taxa or aquatic taxa with good respiratory control may exhibit less oxygen limitation at thermal extremes (Verberk and Bilton 2013).

Lottiid limpets are thought to use two distinct structures for respiration, a single gill (ctenidium) and vascularized mantle/pallial folds (Kingston 1968; Branch 1981; Lindberg and Ponder 2001). During submersion, high intertidal limpets are thought to primarily utilize their single gill for respiratory gas exchange, while the mantle fold is compressed. During air exposure, the expanded mantle fold is thought to serve as the primary respiratory structure (Wolcott 1973; Branch 1979). Differences in ventilatory efficiency between these structures are unknown, but if the mantle fold exhibits greater efficiency, the associated differences in respiratory strategies during air exposure and submersion may have the potential to differentially influence cardiac performance and temperature tolerance limits, an aspect of intertidal physiology that is currently underexplored.

Here we test the hypothesis that reduced O_2 availability in water causes a reduced capacity for submerged limpets to maintain adequate delivery of O_2 to the tissues during acute thermal ramping and lowers the upper critical thermal limits of cardiac performance under submerged conditions. Limpets were exposed to increasing temperatures in air-exposed and submerged conditions while altering (increasing and decreasing) environmental O_2 levels. Limpet cardiac activity (heart rate) was monitored to assess the effect of partial pressure of oxygen (P_{O_2}) and air versus water on upper breakpoint temperature (BPT), the temperature that caused the heart to stop (i.e., flat-line temperature [FLT]), and the temperature sensitivity of average and maximum heart rate (V_{max}).

Material and Methods

Limpet Collection

Lottia digitalis ($n = 180$) were collected on June 20, 2012, from the upper intertidal zone during low tide at Fort Ross, California (38°30′45.8″N, 123°14′45.5″W). Limpets (mean length ± SD, 18.9 ± 0.1 mm; mean weight ± SD, 1,130 ± 29.4 mg) were removed without harm from rocks using small spatulas and transferred to the laboratory within 3 h. Summer rock surface temperatures at the collection site may fluctuate daily from 10° to 40°C depending on weather, tides, and the orientation in relation to the sun (for more details, see Bjelde and Todgham 2013). Limpets were acclimated for at least 14 d in temperature-controlled recirculating seawater tables under stable, submerged conditions of 12°C, 34 ppt salinity, and a 14L:10D photoperiod, representing common summer coastal ambient ocean conditions at the collection site. Algal-covered rocks collected from Fort Ross were provided as food, and limpet feeding was observed during the acclimation period.

Role of Oxygen Availability in Cardiac Thermal Performance

To determine the effect of oxygen availability on the upper temperature tolerance limits of *L. digitalis* under air-exposed and submerged conditions, heart rates were recorded while limpets were exposed to one of three oxygen levels during a thermal ramp. The thermal ramp involved an increase in temperature in water or air from 13° to 43°C at a constant rate of 6°C per hour, a rate representing a natural ramping rate of temperature during summer low-tide periods (Bjelde and Todgham 2013). During the temperature ramp, the P_{O_2} of the air or water was held at three target levels: hypoxia (5 kPa O_2), normoxia (20 kPa O_2), and hyperoxia (35 kPa O_2; see fig. A1; figs. A1, A2 available online), as described below.

Limpets were prepared for cardiac trials as in Bjelde and Todgham (2013). Briefly, 24 h before each thermal tolerance trial, two small holes (~36 gauge) were made in the apex cavity of the limpet shell, and the animals were returned to the sea tables overnight. There were no observable effects of this procedure on limpet survival or behavior. The day of the ramping experiment, in-shell, wet weight (W_i) of each limpet was measured, and 40-gauge ceramic-coated copper wire electrodes (Belden, Richmond, IN) were inserted through the previously made holes and glued in the air cavity between the shell and the limpet, directly above the heart. Care was taken not to blot or dry the limpet tissues during transfer so that they maintained any internal water stores (pallial water). Limpets ($n = 15$) were placed in the air-exposed or submerged conditions and given 30 min at 13°C before the thermal ramp began. During this time, the limpets firmly attached their foot to the

substrate. Each limpet was used in a single treatment combination (i.e., O_2 level and air-exposed or submerged condition).

Air-exposed heat ramps were conducted using an aluminium, temperature-controlled heat block in direct contact with the limpet's foot following Bjelde and Todgham (2013). Preliminary measurements confirmed that limpet foot temperature closely follows heat block temperature (±0.5°C; B. E. Bjelde and A. E. Todgham, personal observation). Submerged heating was performed in a circulating and aerated water bath. Different oxygen levels were achieved in air and water using Wösthoff mixing pumps (Bochum, Germany) mixing pure O_2 and N_2 gases (Praxair). During the air exposure experiments, gases were introduced through a tightly secured acrylic lid placed on the aluminium heating block. In submerged conditions, the mixed gases flowed into a Venturi intake of a recirculating pump, which generated numerous micro gas bubbles and rapidly equilibrated the water bath O_2 levels with the mixed gas. Gas equilibration and stability were monitored for the duration of the experiment using a fiber-optic microsensor as described in Carter et al. (2013); the microsensor was attached to a PreSens Microx TX2 (Precision Sensing, Regensburg, Germany).

Limpets were introduced into the experimental setup, and during the subsequent 30-min period at 13°C, the mixed gases were introduced and gradually adjusted to treatment levels (see fig. A1). Limpet cardiac performance was monitored by changes in impedance, as in Bjelde and Todgham (2013), and was converted to heart rate in beats per minute (bpm) using PowerLab Chart 5 (ADInstruments, Colorado Springs, CO). Following the cardiac trial, whole-limpet mass (W_f) was measured, and limpets were extracted from their shells and dried at 60°C for 24 h to attain dry weight (DW). Shell weight (SW) was also determined for each limpet. Percentage body water (%BW) was calculated to determine amount of tissue water before (%BW_i) and after (%BW_f) the thermal ramp as

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

For each oxygen treatment, air-exposed and submerged temperature ramps were conducted on a single day, and each oxygen treatment was run in duplicate (30 limpets per O_2 per exposure [submersion/air exposure] = 180 limpets total). Upper thermal limits of cardiac performance were calculated from the breakpoint temperature (BPT) in heart rate following Bjelde and Todgham (2013). Two linear regressions, one on the ascending heart rate and one on the descending heart rate, were plotted against temperature. The intersection of the lines was considered the BPT. A limpet's FLT was found by determining the temperature at which heart rate had completely ceased. FLTs were calculated for limpets that reached FLT before the heart rate trial was terminated. FLTs are missing for five air-exposed limpets in the hyperoxia trial, one in the normoxic trial, and two in the hypoxic trial and are missing for one submerged limpet each in the hyperoxia and normoxia trials ($n \geq 13$ limpets for each trial), because these limpets never experienced total cessation in heart function before the trial ended. BPT and FLT are common indices of upper critical thermal maxima of intertidal species (Stillman and Somero 1996; Stillman 2003; Stenseng et al. 2005), including limpets (Bjelde and Todgham 2013).

To assess maximum cardiac performance under hypoxia, normoxia, and hyperoxia during air exposure and submersion, the maximum heart rate (V_{max}) was calculated from individual limpet heart traces under each oxygen exposure. Additionally, we generated performance curves of average limpet heart rate across a temperature range of 13°–40°C. Performance curves were created by determining the limpet heart rates every 1°C (10-min period). Average heart rates at each temperature were calculated by determining the average heart rate in 30-s intervals (taken every 30 s) in the interval ±0.5°C around each performance temperature (i.e., for 25°C, average heart rates from 24.5° to 25.5°C).

Variation in heart rate with increasing temperature (potentially indicating subcellular causes of heart failure, such as ion leakage; Vornanen et al. 2014) was assessed using methods that were similar to those for generating cardiac performance curves. Standard deviations in heart rate for each individual limpet were calculated at 1°C intervals for the entire temperature exposure. Our cardiac performance setup does not allow us to accurately measure stroke volume, and therefore we are unable to quantify changes in cardiac output under the different experimental conditions.

Statistical Analyses

Statistical analyses were conducted in R (ver. 2.15.0, R Development Core Team 2012). Data were first tested for normality and heteroscedasticity visually by plotting quantile-quantile, density, and residual plots of the linear model to ensure parametric analysis assumptions were met. Upper thermal limits in heart function, measured as BPT and FLT in heart rate, were both transformed and analyzed separately with a two-way ANOVA, followed by a Tukey highly significant difference (HSD) test with P_{O_2} and air exposure or submersion as the independent factors. Maximum heart rates experienced during the temperature ramp were analyzed with a two-way ANOVA, followed by a post hoc Tukey HSD test with air exposure or submersion and P_{O_2} as independent factors. Limpet %BW was analyzed using a three-way ANOVA, followed by a Tukey HSD with time (i.e., before and after the experiment), P_{O_2}, and air-exposed or submerged condition as independent variables. Linear regressions were used to identify the role of temperature in heart rate variability across treatment groups (P_{O_2} and air exposure or submersion combined). Differential responses to temperature (differences in the slope of heart rate variability vs. temperature curves) between the individual treatment groups were assessed using linear regressions of heart rate variability as a function of temperature with exposure and P_{O_2} as covariates, followed by a post hoc Tukey HSD procedure.

We used generalized additive mixed modeling to test for differences in thermal sensitivity of heart rate between P_{O_2}

exposures during submersion and air exposure following Zuur et al. (2009) and Angilletta et al. (2013). To account for repeated measures, the identity of each limpet was included as a random factor. Analyses were performed with the mgcv (Wood 2004) and nlme (Pinheiro et al. 2013) libraries in R.

Results

Role of Oxygen Availability in Upper Thermal Limits of Cardiac Performance

Air-exposed limpets were able to maintain elevated heart rates at significantly higher temperatures than submerged limpets under all oxygen exposures (fig. 1; two-way ANOVA, $F_{1,94} = 43.92$, $P < .001$). BPTs of air-exposed limpets were 2.6°C higher than those of submerged limpets under normoxia ($P = 0.007$, Tukey HSD test), 2.3°C higher than those of submerged limpets under hyperoxia ($P = 0.010$), and 6.2°C greater than those of submerged limpets under hypoxia ($P = 0.001$; fig. 1). BPTs were not increased in either submerged ($P = 1.000$, Tukey HSD test) or air-exposed ($P = 0.999$) limpets exposed to 35 kPa O_2 in comparison with 20 kPa O_2. However, under hypoxia (5 kPa O_2), there was a significant decrease in BPT in both air-exposed (2.9°C decrease, $P = 0.001$) and submerged (6.5°C decrease, $P < 0.001$) limpets compared with normoxic limpets under the same conditions.

Similar to BPTs, air-exposed limpets experienced cessation of heart function (FLT) at significantly higher temperatures than submerged limpets under all oxygen exposures (fig. 1; two-way ANOVA, $F_{1,84} = 80.44$, $P < 0.001$). FLTs of air-exposed limpets were 2.47°C greater than those of submerged limpets under normoxia ($P < 0.001$, Tukey HSD test), 2.37°C greater under hyperoxia ($P < 0.001$), and 3.18°C greater under hypoxia ($P < 0.001$). FLTs did not change between normoxia and hyperoxia exposures under air exposure ($P = 0.954$) or submersion ($P = 0.921$). Under hypoxia, limpets had significantly earlier FLTs compared with limpets under normoxia while under air-exposed (decrease of 2.25° ± 0.46°C, $P < 0.001$) and submerged (decrease of 2.97° ± 0.36°C, $P < 0.001$) conditions. The effect of hypoxia was similar under submersion and air exposure, such that air-exposed limpets did not respond less strongly to hypoxia than did submerged animals.

Role of Oxygen Availability in Maximum Heart Rate

Maximum heart rates (V_{max}) reached by limpets under oxygen exposures of 5, 20, and 35 kPa were significantly affected by oxygen level (fig. 2; two-way ANOVA, $F_{2,92} = 84.58$, $P < 0.001$). There were no differences between overall air-exposure and submersion V_{max} ($F_{1,92} = 0.09$, $P = 0.763$); however, there was an interaction between P_{O_2} and air-exposed and submerged condition on V_{max} ($F_{2,92} = 7.44$, $P = 0.001$). During air exposure, the V_{max} of limpets exposed to 5 kPa O_2 was 26 bpm lower than the V_{max} of limpets at 20 kPa O_2 ($P < 0.001$, Tukey HSD test) and 25 bpm lower than at 35 kPa O_2 ($P < 0.001$). There was no significant difference between the V_{max} of air-exposed limpets at 20 and 35 kPa O_2 (mean bpm of 75.3 and 73.1, respectively; $P = 0.994$). A similar pattern was present for submerged limpets, such that V_{max} was 36 bpm lower at 5 kPa O_2 than at 20 kPa O_2 ($P < 0.001$) and 47 bpm lower at 5 kPa O_2 than at 35 kPa O_2 ($P < 0.001$). V_{max} measured at 20 and 35 kPa O_2 during submersion was not statistically different, but limpets at 35 kPa O_2 were able to achieve higher V_{max} (mean bpm of 75.1 at 20 kPa to 86.8 at 35 kPa; $P = 0.09$). At 35 kPa O_2, submerged limpets achieved greater average V_{max} in comparison with air-exposed limpets ($P = 0.023$).

A comparison of V_{max} versus BPT demonstrated that, for a given V_{max}, BPT may vary ~3°C between submersion and air exposure (fig. 3). During hypoxia, the pattern is further accentuated (~6°C) such that air-exposed limpets at 5 kPa, despite lower V_{max},

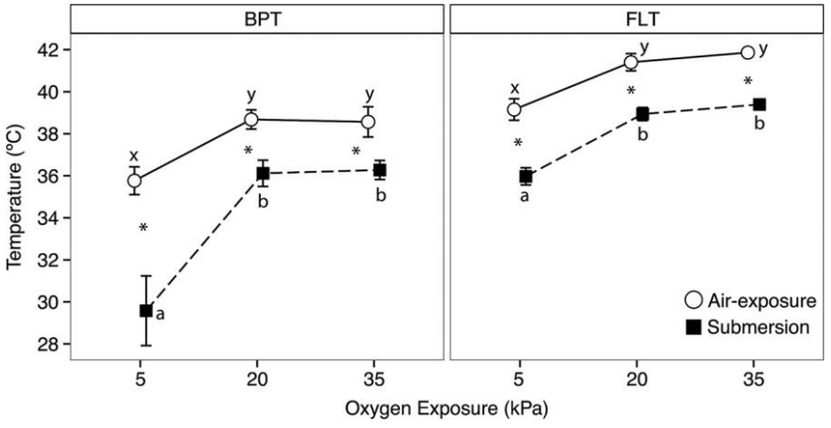

Figure 1. Final breakpoint temperature (BPT) and flat-line temperature (FLT) in heart rate of submerged (filled squares) and air-exposed (open circles) limpets under hypoxia (5 kPa), normoxia (20 kPa), and hyperoxia (35 kPa). Data are presented as mean values ± SEM for $n = 13–20$ limpets per point. Different letters indicate statistical differences ($P < 0.05$) between oxygen levels within air exposure or submersion. Asterisks represent significant differences ($P < 0.05$) between submerged and air-exposed conditions at a particular oxygen level.

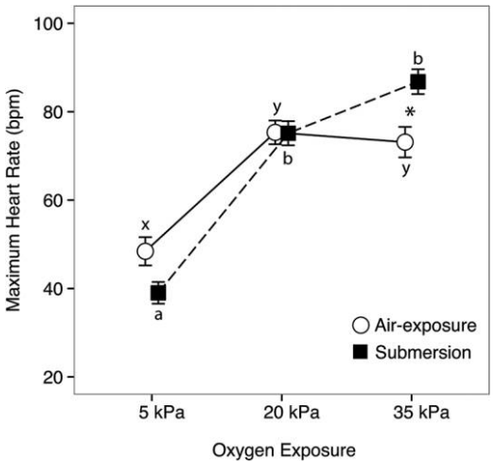

Figure 2. Maximum heart rates in beats per minute (bpm) experienced in submerged (filled squares) and air-exposed (open circles) limpets under hypoxia (5 kPa), normoxia (20 kPa), and hyperoxia (35 kPa). Data are presented as mean values ± SEM for $n = 14–20$ limpets per point. Different letters indicate statistical differences ($P < 0.05$) between oxygen levels within air exposure or submersion. Asterisks represent significant differences ($P < 0.05$) between submerged and air-exposed conditions at a particular oxygen level.

have BPTs equivalent to those of submerged limpets at 20 kPa (fig. 3).

Role of Oxygen Availability in Cardiac Performance

Limpet heart rates showed the expected nonlinear responses to warming under normoxia and hyperoxia during both submersion and air exposure (fig. 4). However, during submersion, the performance curves of 35 kPa–exposed limpets were significantly different from and higher than those of 20 kPa–exposed limpets (fig. 5; table 1). During air exposure, performance curves for 20 kPa–exposed limpets were significantly different from and higher than those of 35 kPa–exposed limpets (fig. 5; table 1). Performance curves under hypoxic conditions were relatively flat and temperature insensitive, with submerged limpets showing even greater reductions in heart function as temperatures were increased (fig. 5).

Role of Oxygen Availability in Temperature-Dependent Heart Rate Variability

Variability in heart rate, represented as the mean standard deviation in heart rate of individual limpets at 1°C increments, visually increased with increasing temperatures under 20 and 35 kPa O_2 (fig. 6). In all groups except the submerged 5 kPa O_2 group, there was a significant positive relationship between temperature and heart rate variability (table 2); however, regression slopes were not significantly different between groups (P values for all comparisons >0.05, ANOVA, Tukey HSD test).

Role of Oxygen Availability and Increasing Temperature in Body Water Content

Air exposure increased the potential for desiccation, and air-exposed limpets (under all oxygen exposures) experienced 3%–4.3% water loss during the 5 h of aerial exposure (fig. A2). A three-way ANOVA showed a significant effect of time ($F_{1,307} = 21.55$, $P < 0.001$; before and after thermal ramp) and treatment ($F_{1,307} = 21.95$, $P < 0.001$; air exposure and submersion) on % BW but no effect of P_{O_2} ($F_{2,307} = 0.89$, $P = 0.413$) on %BW. There was an interaction between time and air-exposure and submersion treatments ($F_{1,307} = 45.88$, $P < 0.001$) as well as between time and P_{O_2} ($F_{2,307} = 4.15$, $P = 0.017$); however, there were no significant interactions between treatment and P_{O_2} ($F_{2,307} = 1.20$, $P = 0.303$) or between time, treatment, and P_{O_2} ($F_{2,307} = 1.62$, $P = 0.199$). Air-exposed limpets experienced significant water loss after thermal ramps at 5 kPa O_2 ($P = 0.003$, Tukey HSD test), 20 kPa O_2 ($P = 0.001$), and 35 kPa O_2 ($P < 0.0001$). Submerged limpets exposed to temperature increases showed no significant change in %BW content at 5 kPa O_2 ($P = 1.000$), 20 kPa O_2 ($P = 0.083$), and 35 kPa O_2 ($P = 0.987$).

Discussion

The OCLTT hypothesis postulates that O_2 limitation of aerobic metabolism drives organismal thermal tolerance (Pörtner 2001), suggesting that aquatic and terrestrial organisms may differ in thermal tolerance given differences in O_2 availability in air and water and the increased ventilatory effort required

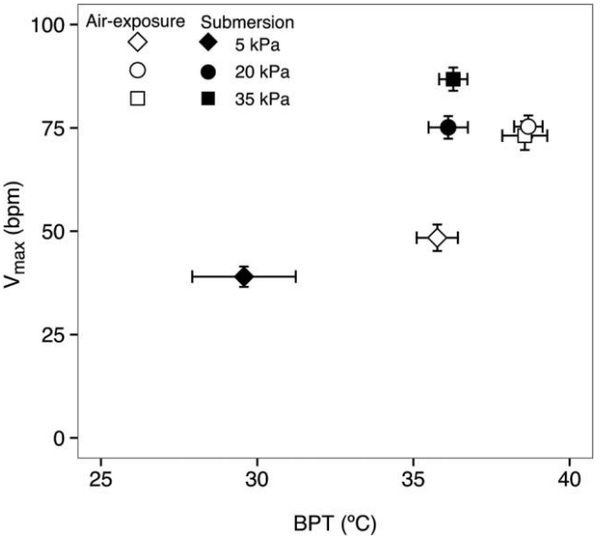

Figure 3. Comparison of maximum heart rate (V_{max}) in beats per minute (bpm) and breakpoint temperature (BPT) of air-exposed (open) and submerged (filled) limpets under hypoxia (5 kPa; diamonds), normoxia (20 kPa; circles), and hyperoxia (35 kPa; squares). Data are presented as mean values ± SEM for $n = 14–20$ limpets per point.

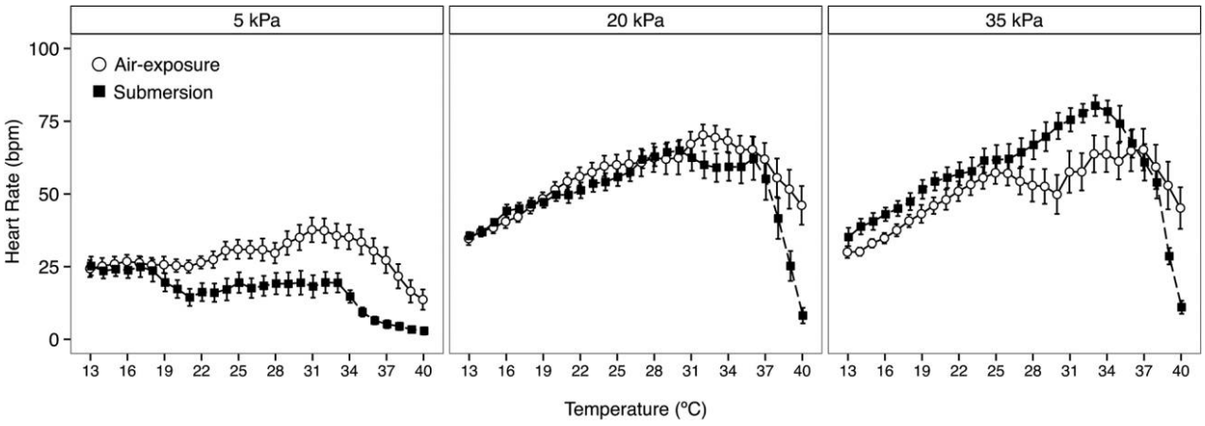

Figure 4. Cardiac performance curves of submerged (filled squares) and air-exposed (open circles) limpets in response to increasing temperatures under hypoxia (5 kPa), normoxia (20 kPa), and hyperoxia (35 kPa). Data are presented as mean values ± SEM for $n = 14–20$ limpets per point for heart rate of limpets at every 1°C increase when exposed to increasing temperature at a rate of 6°C per hour from 13° to 40°C.

to move water across gills (Verberk and Atkinson 2013; Giomi et al. 2014). This may be an important consideration in intertidal zone organisms, which transition between aquatic and terrestrial habitats, especially given recent evidence that acute upper thermal tolerance limits in the limpet *Lottia digitalis* were higher during air exposure than during submersion (Bjelde and Todgham 2013). We hypothesized that lower O_2 availability in water compared with air accounted for previously observed differences in upper critical thermal limits of cardiac performance and that increasing O_2 content would increase upper thermal tolerance in water. In contrast, under both submersion and air exposure, upper thermal limits of cardiac performance were unchanged by hyperoxia, and therefore, under normoxia, the upper thermal limit of cardiac performance does not appear to be oxygen limited (fig. 1). Within submerged and air-exposed treatments, V_{max} also did not increase with hyperoxia, although submerged limpets did have significantly higher V_{max} than air-exposed animals under hyperoxia (fig. 2). Thus, increased O_2 availability increased cardiac performance, reflecting a greater exploitation of capacity to deliver O_2, but not upper

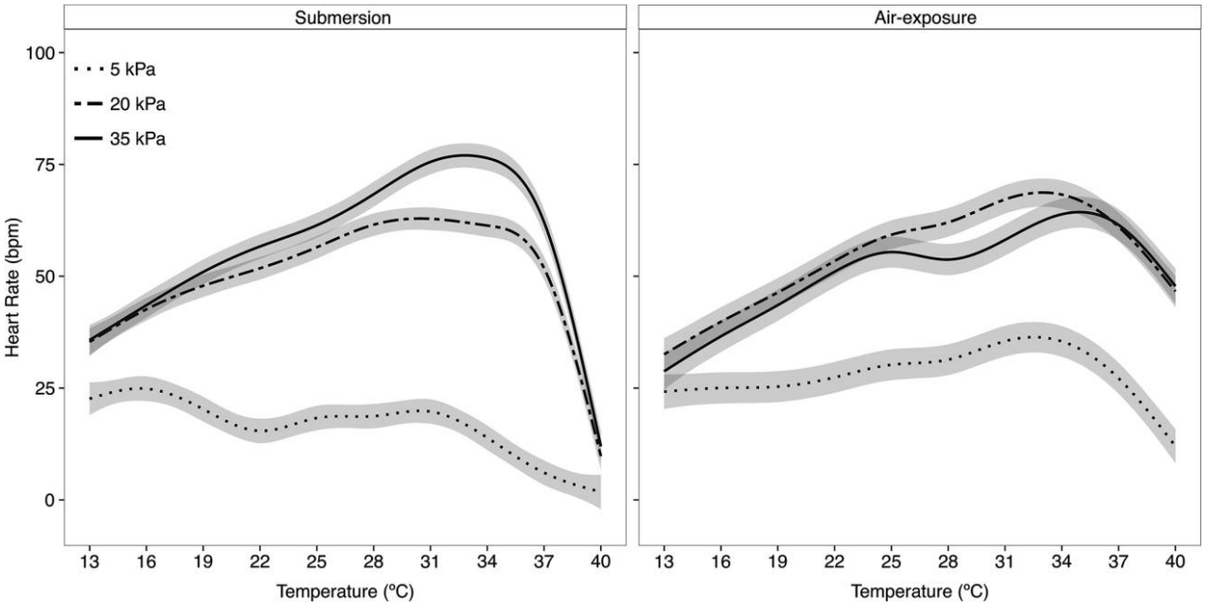

Figure 5. Thermal performance curves testing for differences in curve shape for submerged and air-exposed limpets at 5, 20, and 35 kPa partial pressure of oxygen as estimated by the generalized additive mixed model. Shaded regions represent standard errors of the fit.

Table 1: Comparisons of generalized additive mixed models of heart rate as a function of temperature, $f(T)$, referenced to the curve at 20 kPa in both air-exposed and submerged conditions

Condition	edf	F	P
Air exposure:			
$f(T)$ for 20 kPa treatment	6.63	57.04	<.0001
Deviation from $f(T)$ for 5 kPa	4.056	30.81	<.0001
Deviation from $f(T)$ for 35 kPa	5.886	3.27	<.01
Submersion:			
$f(T)$ for 20 kPa treatment	8.157	67.84	<.0001
Deviation from $f(T)$ for 5 kPa	8.599	40.73	<.0001
Deviation from $f(T)$ for 35 kPa	5.903	6.67	<.0001

Note. edf = effective degrees of freedom.

thermal tolerance limits in submerged limpets. Perhaps 35 kPa was not enough to alleviate baseline aerobic costs under warming, allowing no flexibility to increase upper thermal limits.

Reduced O_2 also played a role in upper thermal limits of cardiac performance, although submerged and air-exposed animals responded similarly with reduced BPTs and FLTs during hypoxia. Additionally, hypoxia appeared to accentuate the difference in upper thermal limits of cardiac performance, as measured by BPT, between the two groups (from 2° to 6°C) through greater reductions in BPT in submerged limpets (figs. 1, 3). This reduced thermal tolerance was likely due to the combined effect of reduced P_{O_2} as well as the subsequent reduction in circulation, because maximal heart rate under hypoxia was reduced by more than 50% in air-exposed and submerged limpets. Consequently, although submerged limpets may exhibit O_2-influenced circulatory limitations, our results suggest such performance limitations do not account for the difference in upper thermal limits of cardiac performance between submerged and air-exposed limpets under natural conditions. However, overall thermal limits under both tidal conditions are influenced by reduced environmental P_{O_2}, suggesting an asymmetry in P_{O_2}/thermal tolerance relationships.

The finding of equivalent reductions in V_{max} with hypoxia in air-exposed and submerged limpets, coupled with a greater reduction in BPT only in submerged animals, further suggests that V_{max} and upper thermal limits of cardiac performance are uncoupled under hypoxia (fig. 2; also compare submerged 5 kPa O_2 group limpets vs. air-exposed 5 kPa O_2 group limpets in fig. 3). Environmental stress can result in temperature insensitivity of physiological parameters, including metabolic and heart rates (Davies and Tribe 1969; Marshall and McQuaid 1991; Fangue et al. 2008; Marshall et al. 2011). Cardiac performance of air-exposed limpets was similar under normoxia and hyperoxia and exhibited the expected temperature-dependent increases in heart rate (fig. 5). However, the positive effect of hyperoxia on thermal performance during submersion suggests the potential for O_2 limitation at high temperatures under this condition (fig. 5). As temperatures approached the BPT, variation in heart rate also increased, which suggests that increasing temperatures are associated with greater cardiac malfunction (fig. 6), potentially through differential thermal sensitivity of heart muscle ion channels (Vornanen et al. 2014). When exposed to hypoxic conditions, submerged and air-exposed limpets had heart rates that exhibited thermal insensitivity. Most notably, the performance curves of submerged limpets were extremely flat and even negative in some parts, which suggests potential metabolic depression (figs. 4, 5). Additionally, limpets under hypoxic conditions did not exhibit temperature-dependent increases in heart rate variability (fig. 6). The reduced thermal sensitivity of hypoxic hearts may serve a protective role to preserve heart function, albeit in a much reduced state by decreasing the variation in heart rate until ultimate heart failure. Additionally, changes in heart rate are likely of little consequence if oxygen uptake represents the limiting step under hypoxia.

Hyperoxia did not increase CT_{max} in aquatic insects (Verberk and Calosi 2012) or in some fishes (Berschick et al. 1987; Rutledge and Beitinger 1989; Healy and Schulte 2012). Reduced thermal tolerance during hypoxia has been reported for aquatic organisms (Rutledge and Beitinger 1989; Verberk and Bilton 2011; Verberk and Calosi 2012; Healy and Schulte 2012) and terrestrial arthropods (Klok et al. 2004; Stevens et al. 2010). These findings suggest that the delivery of O_2 may already be maximized under normoxic conditions in many animals, and thus upper thermal limits cannot be further increased by increasing oxygen availability. The situation is more complex, however, because there are instances in which greater environmental P_{O_2} results in increased thermal tolerance, which suggests oxygen limitation under normoxic conditions (Pörtner et al. 2006; Gardeström et al. 2007; Verberk and Bilton 2011). We found that, for an intertidal limpet, although upper thermal limits (in this case of cardiac performance, not CT_{max}) were not influenced by greater environmental P_{O_2}, circulatory performance during submersion was. Such findings suggest that variation in respiratory structures (Verberk and Bilton 2013) and their efficiency may influence the role that oxygen limitation plays in upper thermal limits and the coupling between cardiac performance and thermal limits. To confirm this possibility, additional information regarding oxygen permeability of the respiratory tissues and the oxygen carrying capacity of the hemolymph will be necessary. Limpets and other mollusks are known to maintain relatively low P_{O_2} within their mantle cavity under both submerged and air-exposed conditions (Abele et al. 2010).

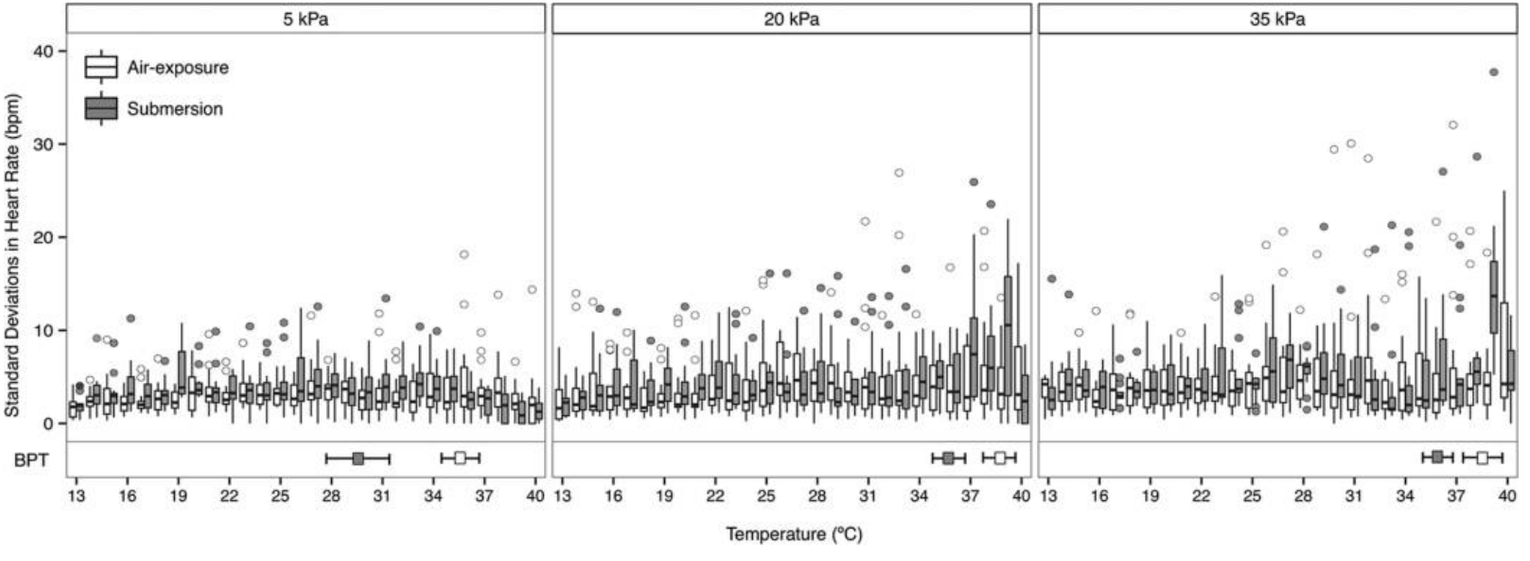

Figure 6. Variation in heart rate of submerged (filled symbols) and air-exposed (open symbols) limpets in response to increasing temperature under hypoxia (5 kPa), normoxia (20 kPa), and hyperoxia (35 kPa). Data are presented as boxplots of standard deviation in heart rate in beats per minute (bpm) every 1°C during a temperature ramp from 13° to 40°C (n = 14–20 limpets per boxplot). Outliers are 1.5 × the interquartile range. Breakpoint temperatures (BPTs) in heart rate of submerged (filled symbols) and air-exposed (open symbols) limpets are presented as mean values ± SEM in the lower panel of each partial pressure of oxygen treatment to visually assess variability before and after the average BPT.

Table 2: Regression parameters for linear regressions of standard deviation of heart rate (heart rate variability) as a function of temperature, grouped by partial pressure of oxygen (P_{O_2}) and submersion versus air exposure

P_{O_2}, treatment	Intercept	Slope	R^2	df	F	P
5 kPa O_2:						
Air exposure	2.3162	.0295	.0106	1, 427	4.59	<.05
Submersion	4.3947	−.0327	.0116	1, 396	4.64	<.05
20 kPa O_2:						
Air exposure	2.1888	.0751	.0247	1, 494	12.5	<.001
Submersion	1.3418	.1215	.0623	1, 434	28.8	<.0001
35 kPa O_2:						
Air exposure	2.2548	.0932	.0261	1, 457	12.1	<.001
Submersion	1.5251	.1398	.0574	1, 360	21.9	<.0001

In high intertidal zone Antarctic limpets, mantle P_{O_2} was routinely <1 kPa during normoxic air exposure and <13 kPa during submersion in normoxic (21 kPa) water (Weihe and Abele 2008). The active regulation of constant, low P_{O_2} values near tissues through ventilatory control is thought to be widespread in water breathers and serves as protection against oxidative stress (Massabuau 2003). Given that limpets regulate ventilation and reduce mantle cavity P_{O_2} under normoxic conditions (i.e., not exposing tissues to maximum P_{O_2} available), they likely utilize similar strategies during exposure to hyperoxia. Consequently, that thermal tolerance does not increase with increased environmental P_{O_2} is not all that surprising, because these animals routinely exclude much of the available oxygen as a defense mechanism against oxidative stress. The ability to maintain low oxygen values within the mantle cavity may become problematic during hypoxia, because mechanisms for increasing tissue-level P_{O_2} may be less developed.

Despite the possibility of oxygen limitation during normoxic submersion (figs. 1, 5), differences in thermal tolerance between air-exposed and submerged limpets do not appear to be solely due to differences in O_2 availability in air and water. What then may be driving this pattern? One possibility is that the differences in thermal tolerance are due to differential baseline costs in ventilation effort associated with breathing water compared with air (Giomi et al. 2014). A greater basal metabolic rate could decrease overall aerobic scope for performance. Differential costs during submersion might be suggested by reduced BPT at all P_{O_2} levels, similar heart rate curves, and greater V_{max} under hyperoxia compared with air-exposed limpets. During air exposure, lowered cardiac performance and V_{max} under hyperoxia may reflect a cost alleviation in cardiocirculation, potentially suggesting lowered baseline costs for thermal exposures, which may ultimately allow for a greater aerobic scope and potential upward shift in pejus temperatures in comparison to submerged limpets. Our findings of lower cost under hyperoxia have been observed previously in fishes (Mark et al. 2002) and terrestrial arthropods (Stevens et al. 2010). Another possibility is that aerial exposure serves as a signal to upregulate anticipatory mechanisms at the cellular level to protect against thermal stress that may occur at low tide during the subsequent hours (Gracey et al. 2008; Connor and Gracey 2011). If this is the case, one would expect that air exposure alone (no heating) would initiate a cellular stress response that is not present during submersion, resulting in the submerged limpets in our experiments experiencing high temperatures without the appropriate defense mechanisms in place. Intertidal organisms are adapted to respond to acute temperature stress during low tide, and therefore one might expect that mechanisms of stress tolerance are triggered by aerial exposure. The response to chronic heat stress was unexplored in the current study, because it may not represent the natural environment, where animals experience acute fluctuations in environmental temperatures; however, we would expect that responses to chronic repeated heat stress might differ from responses to single acute heat stress, such as those observed here (Rezende et al. 2014).

Our results provide evidence that intertidal organisms may exhibit variation in respiratory control, with greater control within an aerial environment, which is a result more broadly suggested by Verberk and Atkison (2013). Air-exposed limpets may have evolved to sustain cardiac function over a wide range of acutely increasing temperatures, such that they are able to meet all oxygen demands while air exposed until near-lethal temperatures are reached. The observation that cardiac activity in limpets was higher in submerged limpets under hyperoxic conditions (fig. 2), without an associated increase in upper thermal tolerance, suggests that other biochemical and physiological processes fail earlier in submerged limpets, ultimately leading to lowered upper thermal limits. Such processes might include maintaining protein and membrane integrity as well as coping with oxidative stress. Future studies should explore suborganismal measures of thermal sensitivity (e.g., antioxidant defence and ion channel stability) in limpets to better understand the factors that underlie differences in thermal physiology of limpets exposed to increases in temperature in water and air.

Acknowledgments

We would like to thank Drs. Nann Fangue and Bob Kaufman for use of and assistance with the Wöstoffs gas-mixing equipment and Erin Flynn for help with limpet sampling. This work

was supported by funds from San Francisco State University to A.E.T. and the University of California Agricultural Experiment Station (CA-D-ASC-2252-H to A.E.T.), and this material is based on work supported by the National Science Foundation (1041225 to J.H.S.).

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