Heat tolerance and thermal preference of the copepod *Tigriopus californicus* are insensitive to ecologically relevant dissolved oxygen levels

Khuong V. Dinh[1,*], Arani Y. Cuevas-Sanchez[1], Katherine S. Buhl[1], Elizabeth A. Moeser[2], W. Wesley Dowd[1]

[1]Washington State University, School of Biological Sciences, P.O. Box 644236, Pullman, WA 99164-4236, USA

[2]University of Southern California, Department of Environmental Studies

Corresponding author: Khuong V. Dinh

Emails: khuong.dinh@wsu.edu, khuongaquatic@gmail.com

Telephone number: +1 (509) 339-4903

Abstract

Shifting climate patterns may impose novel combinations of abiotic conditions on animals, yet understanding of the present-day interactive effects of multiple stressors remains under-developed. We tested the oxygen and capacity limited thermal tolerance (OCLTT) hypothesis and quantified environmental preference of the copepod *Tigriopus californicus*, which inhabits rocky-shore splashpools where diel fluctuations of temperature and dissolved oxygen (DO) are substantial. Egg-mass bearing females were exposed to a 5h heat ramp to peak temperatures of 34.1 - 38.0°C crossed with each of four oxygen levels: 22, 30, 100 and 250% saturation (4.7-5.3, 5.3-6.4, 21.2-21.3, and 50.7-53.3 kPa). Survival decreased at higher temperatures but was independent of DO. The behavioral preference of females was quantified in seven combinations of gradients of both temperature (11-37°C) and oxygen saturation (17-206% or 3.6-43.6 kPa). Females avoided high temperatures regardless of DO levels. This pattern was more pronounced when low DO coincided with high temperature. In uniform temperature treatments, the distribution shifted toward high DO levels, especially in uniform high temperature, confirming that *Tigriopus* can sense environmental pO_2. These results question the ecological relevance of OCLTT for *Tigriopus* and raise the possibility of microhabitat selection being used within splashpool environments to avoid physiologically stressful combinations of conditions.

Keywords: dissolved oxygen, climate change, copepod, temperature extreme, interacting stressors

Introduction

Marine organisms are facing increasing prevalence of multiple stressors, particularly extreme temperatures and heatwaves [1-5] and low dissolved oxygen [6-9]. Furthermore, stressors can interact and modulate each other's effects [10-12]. For example, extreme temperatures decrease the oxygen solubility while simultaneously increasing the organism's oxygen demand, hence intensifying hypoxic stress on marine species [7,13]. To locally survive, organisms may have to adjust their physiology [14-16] and/or behaviors [14,17,18] to cope with or avoid stressful conditions. However, we know relatively little about the prevalence and magnitude of the present-day interactions among stressors and their impacts on marine species [10-12].

A prevailing theory suggests that thermal tolerance, particularly of aquatic water-breathers, is dependent upon oxygen availability. Specifically, this theory posits that thermal tolerance is determined by the capacity for oxygen supply in relation to oxygen demand; these ideas are encapsulated in the Oxygen and Capacity Limited Thermal Tolerance hypothesis (OCLTT) [19-21]. There are physiological studies supporting OCLTT, particularly during acute thermal stress events. For example, the critical thermal maxima (CT_{max}) of fish increases under hyperoxia [22]. Yet, there is increasing evidence questioning the generality and ecological relevance of the OCLTT hypothesis [23-26]. For example, Lehmann et al. [27] found that CT_{max} of the pupae of the butterfly *Pieris napi* was 43.1 ± 0.5°C, and it did not change across the range of ambient pO_2 from 10 to 30 kPa. Similarly, the heat tolerance of grasshopper larvae (*Schistocerca americana*) was not decreased under hypoxic conditions [25]. These counter-examples often come from terrestrial systems, but recent work also questions the predictions of OCLTT in water-breathers such as fish [4].

By similar logic, it is also possible that oxygen levels influence organismal temperature preferences. Mobile species can employ behavioral adjustments to avoid stressful conditions by selecting specific microhabitats in spatially heterogeneous environments [14,18]. Indeed, spatial

heterogeneity is a widespread feature of natural habitats [28,29], even in small splashpools [29,30]. Behavioral adjustments can provide an alternative mechanism to rescue species from stressful conditions [14,17,18]. Many species can adjust their position to seek preferred thermal and oxygen zones [31,32]. For example, the American lobster (*Homarus americanus*) responded to changes in water temperatures by leaving their shelter and looking for temperatures within ~1.2°C of the acclimation temperature [31]. Similarly, *Cyclops vicinus* copepods showed their highest distribution (presence) within their optimal thermal conditions [33]. Calanoid copepod species prefer oxygen-rich water while cyclopoid *Oihona similis* and *Oncaea* sp. often aggregate in high abundance in the midwater hypoxic zone [32]. Fewer studies have examined preference behaviors in a multi-stressor context, and those showed that fish have a lower temperature preference in hypoxic conditions than in normoxia [34,35]. Overlooking the potentials of behavioral responses to multiple stressors such as extreme temperatures and low dissolved oxygen (DO) levels may fail to accurately quantify the real risk of environmental changes in natural populations [17,18].

Harpacticoid copepods in the genus *Tigriopus* are excellent species to study physiological and behavioral responses to single and covaring stressors. These species are commonly found in splashpools [36-38], where the diel fluctuations of temperatures and DO are substantial [30]. High temperatures (> 30°C) tend to coincide with high DO levels (> 200% saturation) during the day due to the elevated solar radiation and photosynthesis; low temperatures and low DO conditions are observed during the night [30]. *Tigriopus* spp. show a high tolerance to temperatures [39-41] and low DO [42-44] independently. However, the majority of these studies have investigated the impact of one stressor at a time, thereby leaving unexplored the potential for interactive effects that might impact organisms experiencing multiple stressors simultaneously in nature [15]. The behavioral responses of *Tigriopus* to temperature, dissolved oxygen, and their combination remain to be tested.

In this study, we combined physiological and behavioral tests to comprehensively assess whether (1) ecologically relevant high (low) oxygen levels may increase (decrease) heat tolerance of *Tigriopus californicus*, as predicted by the OCLTT hypothesis, and (2) copepods modulate their thermal preference depending on oxygen levels.

Results

Thermal tolerance

As expected, there was a significant main effect of temperature on survival of *T. californicus* (χ^2_1 = 51.20, $P < 0.001$). Specifically, survival decreased rapidly with increasing temperatures, from an average of approximately 90% at 34.1-34.9°C to < 10% at 36.3°C. No females survived at 37.2 and 38.0°C (Fig. 2). Survival did not differ among low, normal and high DO levels ($\chi^2_1 = 0.72$, P = 0.40). The interaction of temperature and DO also was not statistically significant ($\chi^2_1 = 0.69$, P = 0.41). Thus, the temperature-induced mortality of *T. californicus* was independent of oxygen at the levels tested.

Thermal and oxygen preferences

Overall, females avoided high temperatures regardless of the oxygen levels in the water (Fig. 3A-D). The distribution of females tended to shift more toward lower temperatures when the oxygen levels were high in this part of the chamber (Fig. 3B, C, pairwise comparisons between treatments 2, 3 and treatment 1, P = 0.075 and 0.051, respectively); this pattern was less clear when low DO occurred across the thermal gradient (Fig. 3D, pairwise comparisons of treatment 4 and treatments 1-3, all P-values < 0.05). Female distributions were skewed toward high oxygen levels when there was no thermal gradient (Fig. 3E, F, pairwise comparisons two groups of treatments 5-6 and 1-4, all P-values < 0.001), and this pattern was stronger at 36°C (Fig. 3F) than at 12°C (Fig. 3E, pairwise comparison between treatment 5 and treatment 6, $P < 0.001$). When there were no temperature and oxygen gradients, females were distributed evenly in the chambers, and no preferred positions were

observed (Fig. 3G, pairwise comparisons between treatment 7 and treatments 1-6, all *P*-values < 0.001). A full list of pairwise *P* values is provided in Table S1 in Supplementary information S2.

Distance traveled

Copepods were considerably more active in the uniform (no gradients) treatment of intermediate temperature and normal DO conditions relative to all other treatments. For every minute, they traveled 4.8 cm, ca. 2 - 3 times greater than the average distance traveled by copepods in all other treatments (Kruskal-Wallis, $H_{6,101} = 23.30$, $P < 0.001$, Fig. 4). There was no difference in distance traveled by copepods among the other treatments (all pairwise *P* values > 0.10, Table S2 in Supplementary information S2).

Discussion

There is extensive evidence that the splashpool copepods of the genus *Tigriopus* can tolerate extremely high-amplitude fluctuations of environmental conditions [36,37,45,46]. Our results showed strong mortality at temperatures higher than 34.9°C and only less than 10% of females survived after being exposed to 36.3°C; no surviving females were observed after exposure to 37.2 and 38.0°C. These results are comparable to published estimates of the thermal tolerance of *T. californicus* from the same climatic zone [37,38,47]. The thermal tolerance of *T. californicus* populations have been physiologically linked to the ATP synthesis capacity in the mitochondria; ATP synthesis declines at temperatures close to the knockdown temperatures [38]. The level of heat shock protein upregulation also has a positive correlation to thermal tolerance of *T. californicus* [41]. Suppression of a specific heat shock protein (HSPB1) has been shown to reduce the thermal tolerance of *T. californicus* to acute heat stress [17,48].

Importantly, the overall survival of *T. californicus* was not lower at two low, but ecologically relevant DO conditions relative to normal DO (~100% saturation), regardless of peak

126 exposure temperatures, nor did high DO level mitigate the effects of acute thermal stress. These
127 results appear to contradict predictions of OCLTT [19, 21]. A role for OCLTT, particularly during
128 acute heat stress such as imposed in our experiments, is supported by evidence from a range of taxa
129 in both aquatic and terrestrial ecosystems (reviewed in table 1 in reference [21]). However, it is likely
130 that insensitivity of thermal tolerance to low DO levels occurs in species with high capacity to
131 regulate oxygen intake and delivery (e.g., in the snail *Planorbis planorbis*) or when DO levels are
132 not lower than the critical levels (e.g., P_{crit} of 1.1-1.3 kPa for *T. californicus* [50]). For example, the
133 snail *Planorbis planorbis* has 2-4 times higher hemoglobin levels than its congeners [51]; under
134 hypoxic conditions the thermal tolerance of *P. planorbis* was not reduced, but the CTmax of *P.*
135 *carinatus* was lowered by 1.2 - 2.1°C [49]. *Tigriopus* species do not have gills, lack respiratory
136 pigments [50], and appear to have lost both the transcription factor HIF-1α and oxygen sensing prolyl
137 hydroxylase repressor, EGLN, from their genome [43]. It has been suggested that a high surface-area-
138 to-volume ratio of *Tigriopus* spp. may facilitate oxygen uptake from water [50]. This may allow them
139 to maintain their oxygen consumption rate independent of P_{O2} in the environment until the critical
140 P_{O2} of 1.1-1.3 kPa [50], which is approximately 4 – 5 times lower than the lowest P_{O2} of ca. 4.7-5.3
141 kPa in our experiment. Therefore, the insensitivity of thermal tolerance of *T. californicus* to low,
142 but ecologically relevant DO levels observed in our experiment was in line with previous studies;
143 reduced thermal tolerance of water-breathing species has been observed only at extremely low DO
144 levels [4,26]. As noted above, in the splashpool system, DO levels below P_{crit} of *T. californicus* are
145 exceedingly unlikely to coincide with high temperatures.

146 Therefore, the oxygen level may not be the limiting factor for thermal tolerance in
147 ecologically relevant scenarios for *Tigriopus* and in similar cases. Instead, the capacity to
148 physiologically cope with other consequences of temperature extremes may be the primary
149 determinant of their thermal tolerance. Mortality under extreme temperatures may be the result of

150 the dysfunction of a number of physiological processes such as the collapse of ATP synthesis [38],
151 membrane and protein structure instability [52,53], and suppressed expression of heat shock protein
152 (HSPB1) [17,48]. In light of our whole-organism survival data, we hypothesize that macromolecular
153 disruption, rather than any direct effect of oxygen availability, explains recently published patterns
154 of decreased ATP production capacity in *Tigriopus* mitochondria [38], particularly because those *in*
155 *vitro* experiments appear to have been run under normoxic conditions.

156 Irrespective of the mechanism(s) for the insensitivity of the thermal tolerance of *T.*
157 *californicus* to ecologically relevant DO levels, our results join a growing literature suggesting that
158 the OCLTT hypothesis may not be a universal principle for predicting the survivability of species in
159 ecologically relevant conditions in nature [4,26]. Whether the exceptional environmental covariation of
160 temperatures and DO levels in splashpools has selected for high thermal tolerance regardless of DO
161 levels remains to be seen; it is equally plausible that selection for surviving periods of extreme
162 night-time low DO may have coincidentally increased the ability to survive bouts of high
163 temperature using anaerobic ATP production pathways. Recent work reveals that *T. californicus*
164 can survive several days of anoxia [43]. It also is clear that the elevated DO levels that tend to
165 naturally coincide with high temperatures in splashpools [30] do little to alleviate the effects of high
166 temperature stress on these animals. Although beyond the focus of this study, it will be interesting
167 to explore whether low DO levels may also alter critical thermal minimum (CT_{min}) of *T. californicus*
168 and other species when low DO levels in their habitats occur during cold nights or the winter period
169 [54]. In terrestrial ecosystems, the CT_{min} values of some insects such as false codling moth
170 *Thaumatotibia leucotreta* [55], the beetle *Tenebrio molitor* [56] and crustaceans such as *Porcellio scaber*
171 [56] are independent of oxygen availability.

172 Overall, female *T. californicus* demonstrate a strong avoidance of elevated temperatures,
173 with oxygen playing a secondary role in influencing behavior. For example, their high distribution

174 in the low DO region in treatment 1 (Fig. 3A) was likely just to avoid the potentially lethal effects
175 of extreme temperatures in the oxygen-rich water. The distribution of *T. californicus* was
176 considerably more concentrated in oxygen-rich water only when low temperatures coincided with
177 high oxygen (Fig. 3B,C) or there was no thermal gradient in the chambers (Fig. 3E, F). These
178 results suggest that *T. californicus* can avoid low DO conditions.

179 Interestingly, the genome of *T. californicus* appears to lack prolyl hydroxylase and HIF-1α
180 [43], but our results clearly illustrate that these animals can sense the relative abundance of oxygen in
181 the water. This result suggests an alternative, extracellular (and perhaps superficial) mechanism(s)
182 for *T. californicus* to sense pO_2. Under uniformly extreme high temperatures, a shift in the
183 distribution of females toward oxygen-rich water was even stronger; none were found in the low
184 DO regions (Fig. 3F). This may be the result of higher basal metabolic demand, indicated by a
185 general higher oxygen consumption rate at higher temperatures in a congener [50]. Finally, *T.*
186 *californicus* did not show any preferred region within the chambers when they were in the control
187 treatment at room temperature of 19°C and the DO was maintained at 100% saturation. They also
188 traveled a longer distance in this control treatment than in all other treatments, an indication that our
189 observations of their distributions were consistent throughout the trial and uninfluenced by other
190 confounding factors such as light.

191 Lastly, the behavioral results complement the physiological results to suggest a potentially
192 novel explanation for how *T. californicus* can thrive in splashpools, where both temperature and DO
193 are highly fluctuating and often extreme. Specifically, the results for thermal tolerance suggest that
194 *T. californicus* may occasionally not be able to physiologically cope with extreme temperature. To
195 survive in splashpools with extremely high temperatures during the day, there must be an
196 alternative mechanism. Interestingly, field observations indicate that splashpools may be highly
197 stratified over their small spatial scales of a few 10s of centimeters or less (Fig. S1 in

198 Supplementary information S1, reference[30]). Indeed, our behavioral preference test showed that
199 females avoided near-lethal temperatures even if by doing so they had to deal with lower oxygen
200 levels at lower temperatures. This behavioral preference of *T. californicus*, which remains to be
201 demonstrated in a natural setting, supports a recent prediction that behavioral responses of natural
202 populations may enable them to exploit microclimatic variations in heterogeneous habitats as an
203 important mechanism to rescue species from rapidly changing environments [17,18].

204 Our study provides empirical evidence for the insensitivity of both heat tolerance and
205 thermal preference of the splashpool copepod *T. californicus* to the ambient DO level. These
206 patterns apply in ecologically relevant low and high DO conditions. It is likely that warming may be
207 more stressful for *T. californicus* than low DO levels. Our results are among a small but growing
208 collection of studies showing that the OCLTT may not be a universal tool for predicting the thermal
209 tolerance of species, particularly in ecologically relevant scenarios where environmental conditions
210 fluctuate dynamically across a small scale of space and time. The strong behavioral preference of *T.*
211 *californicus* suggests that together with physiological adjustments [39-41], microhabitat selection might
212 be used as an alternative mechanism for *T. californicus* to survive in highly fluctuating and often
213 extreme conditions in splashpools [30].
214
215 **Materials and methods**
216 ***Study population***
217 Female *Tigriopus californius* were collected in May 2019 from splashpools at Cattle Point
218 Lighthouse (+48°27'1.44''N, -122°57'48.6''W) on San Juan Island, WA, USA. The copepods were
219 acclimated to the laboratory condition at 17-18°C for 2 to 4 months (at least 1-2 laboratory-reared
220 generations). Copepods were fed *ad libitum* on fish flakes and an irradiated algae mixture (Shellfish
221 Diet 1800, Reed Mariculture), each provided once a week. They were kept under a photoperiod of

13L: 11D (light: dark cycle). Salinity values correspond to the practical scale of 32.5 - 42.0 and dissolved oxygen (DO) was maintained above 80% of the saturation level (> 6 mg L^{-1}) throughout the acclimation period. Salinity and DO were measured using a YSI digital meter (Pro 2030, Yellow Springs Instruments, USA).

Thermal tolerance assay at different dissolved oxygen levels

The thermal tolerance of *T. californicus* was quantified based on survival after exposing females to peak temperatures of 34.1, 34.4, 34.9, 35.6, 36.3, 37.2 and 38.0°C at different DO levels. Specifically, females carrying egg masses (380 individuals, n = 15-16 per temperature × DO combination) were randomly collected from the culture and exposed to a 5 h heat ramp at one of the peak temperatures (34.1-38.0°C) at each of four DO levels: 22.5, 30, 100 and 250% of the oxygen saturation level. Both DO and peak temperatures are ecologically relevant to the splashpools at the collection site. Individual females were placed in 0.2 mL PCR tubes (conical shape, h = 20.8 mm and d_{top} = 5.46, d_{bottom} = 2.8 mm) filled with 150 µL of the appropriate seawater (32 ppt) and DO level. Copepods could swim freely inside the tubes during the test, and they exhibited typical swimming behavior after the test.

To create different DO levels in the PCR tubes during the heat ramp, we prefilled tubes with seawater adjusted to one of the desired levels. Dissolved oxygen levels were manipulated in a 20-L water bath using a custom-built, Arduino microcontroller system that regulated DO (while maintaining a constant pH of ~ 8.05) by coordinating the opening/closing of solenoid valves connected to oxygen, carbon dioxide, and nitrogen gas cylinders. The systems included calibrated temperature, DO (Honeywell DL5000), and pH (Honeywell Durafet III) sensors connected to a Honeywell UDA1282 Universal Dual Analyzer. The milliamp outputs of this analyzer provided feedback to the Arduino on the current conditions in a header tank; after comparing the current conditions to the desired setpoints, the Arduino triggered brief (10s of milliseconds) pulsed

openings of the solenoid valves to regulate gas flow. This cycle continued on a continuous loop, constantly monitoring and maintaining the DO level. Target DO levels (in mm Hg) were confirmed in the PCR tubes using a fiberoptic oxygen sensor (Neofox, Ocean Optics) prior to the tubes being capped and sealed with parafilm. To sustain these DO levels during the heat ramp, the entire thermocycler used for the thermal tolerance assay was housed in a sealed incubator, in which we manipulated the atmospheric oxygen levels in parallel with the desired DO in the seawater by pumping nitrogen or oxygen into the incubator. This arrangement was necessary because in preliminary trials all DO levels equilibrated with the atmosphere by the mid-way point of the 5-h heat ramp. Due to safety concerns around high-amperage electrical equipment, we could only increase oxygen levels in the incubator to 150% saturation. Using the incubator, DO levels within the PCR tubes were 4.7-5.3, 5.3-6.4, 21.2-21.3, and 50.7-53.3 kPa (22, 30, 100 and 250% saturation), respectively, at the start of the thermal tolerance assay. We confirmed in preliminary trials that these target DO levels in PCR tubes (each containing one female copepod) were maintained during the heating phase until reaching the peak temperature, but they drifted by the end of the assay. At the completion of the heat ramp, DO levels in the PCR tubes were 12.7-13.3, 14.0-14.4, 21.2-21.3, and 40-42.7 kPa (60-62, 66-67.5, 100, 188-200% saturation, respectively). Thus, although DO conditions did not remain constant for the entire duration of the heat ramp, they remained different from each other. Both low DO treatments (22.5 and 30% saturation) remained within the ecologically relevant low DO ranges found in splashpools during the heating phase[30]. Experimental assays were run using identical methods as these preliminary trials, but we did not measure DO levels in PCR tubes for experimental copepods.

For each heat ramp, PCR tubes were placed in an Eppendorf Mastercycer gradient thermocycler, which was custom-programmed to generate a gradual rise and fall of temperature over a five-hour period. The start temperature for the heat ramp was 20°C. The thermocyler was

programmed to a new setpoint every 10 min. Over the first 25 min all columns increased by 1°C. Subsequent setpoints were programmed to increase 1°C every 10 min to a preset peak temperature of 37±3°C at 185 min. Using the gradient feature, each column reached a unique peak temperature of 34.1, 34.4, 34.9, 35.6, 36.3, 37.2 or 38.0°C. Upon reaching the peak, the temperature was maintained for 1 h. Following the 1-hour exposure, temperature was decreased to 20°C over the course of one hour. This protocol created a thermal profile more similar to the environmental temperature variation experienced by *T. californicus* in the wild [39]. Preliminary trials revealed that females exposed to low DO conditions and a peak temperature of 36°C suffered 100% mortality. In order to reduce unnecessary use of animals, it was determined that low DO levels, 22.5 and 30% saturation, combined with peak temperatures of 37.2 and 38.0 °C would not be tested as part of this experiment. Therefore, a total of 40 females were tested at each of the low DO levels of 22% and 30%, and 56 females were tested at the normal and high levels. This heat-ramp procedure was repeated twice at each dissolved oxygen level. DO levels were randomized and only one was examined per day. The survival of females was checked immediately after the ramp and daily for the following 4 consecutive days. Mortality was determined when females were unresponsive to mild shaking of the vial, changed colors to bright red, and the urosome was bent sharply at a right angle to the cephalothorax [57]. Statistical analyses were conducted using survival at day 4.

Behavioral preference assay in the presence of temperature and oxygen gradients

To test whether DO may impact thermal preference of *T. californicus*, we determined the positions and distance traveled by egg-mass bearing females (n = 14 - 15 individuals per treatment) in each of 7 treatment conditions (Table 1) in preference chambers (Fig. 1). Preference chambers were in-house designed based on the system for *Daphnia magna* described in Zeis *et al*. [58], with extensive modifications (Fig. 1). Specifically, 5 identical chambers were constructed of acrylic (L × W × H = 22.86 × 1.27 × 1.27 cm, volume = 36.87 ml). The chamber was sealed with a rubber gasket and

acrylic lid. It was divided into 18 equal intervals, 1.27 cm each, by marking the outside of the chamber. Copepods could swim freely throughout the chamber without any physical barriers. There are inlets at each end, which connect to two different header tanks (size: L × W × H = 28.58 × 31.12 × 40 cm; volume = 20.8 L) where the water temperature and DO were controlled by two programmable, recirculating water baths and the N_2 and O_2 gas systems. The pH in the water was controlled at around 8.05 ± 0.05 by the CO_2 gas system. Gas flows were automatically controlled by an in-house Arduino microcontroller system as described for the thermal tolerance assays above. Each preference chamber has 4 outlet ports distributed equally along one side (the distance between two adjacent outlet ports is 4.57 cm); inlet and outlet ports were fitted with a 50 μm mesh screen to prevent copepods swimming out of the chamber. The outflow rates were 0.8-0.9 ml/min for outlets. The resulted in a total of roughly 3.25 ml water outflow per min, approximately 9% of the volume of the preference chamber. This flow rate is comparable to an assay for *Daphnia magna* [58]. Copepods swam freely throughout the preference chambers, therefore the flow did not impede the swimming or behavioral preferences of *T. californicus*. Two outlet ports from each side of the chamber were connected to one sump tank and two others were connected to a second sump tank. The sump tanks have the same size and volume as the two header tanks. The water in each of the sump tanks was pumped up to the corresponding header tank to create two closed and recirculating water systems, which could be regulated independently for temperature and DO.

Prior to an experiment, the desired temperature (11 to 37°C) and DO levels (0 – 200% saturation) were established in the two header tanks using recirculating water baths and the Arduino system, respectively (see table 1). In the chambers, we confirmed the temperature and DO gradients at 6 positions; temperature fluctuated 34.5-35.1°C at the high end; 28.3-30.6, 25.5-27.1, 18.5-22.7, 15.7-18.4°C at the four outlet ports; and 13-13.4°C at the low end. Similarly, observed DO ranges from the high to low DO ends of the apparatus were 51.7-62.5, 26.4-53.3, 21.9-26.4, 9.2-14.7, 4.8-

8.0 and 0.7-3.2 kPa, respectively. These are also ecologically relevant for the temperature and DO levels observed in splashpools occupied by *Tigriopus* (Figure S1 – Supplementary information S1, [30]). For each experimental run, we randomly collected five egg carrying females from the culture (temperature of 17-18°C) and assigned one individual per preference chamber. In preliminary trials, we observed that copepods tended to modify their behavior in the presence of a con-specific, so our protocol isolated the effects of environment from any social factors.

Females were allowed 10 min to explore the chambers before beginning data collection. Our preliminary observations showed that females swam freely within the first three to five minutes, and subsequently showed a more stable position in the chamber. The female positions in each chamber were observed once per min for 29 min (30 observations). All preference assays were conducted in diffuse light to avoid stressing the animals, and the orientation of the gradients relative to the room was randomly reversed for some chambers to avoid systematic influence of the surroundings on copepod behavior.

The distance traveled by a female during the observation period was calculated by summing the distances from one observation to the next. This cumulative measure of distance traveled may not be accurate for two reasons. First, it captures movements in increments of 1.27cm; second, some females may move forward and back several times between consecutive observations. Nonetheless, this method provides a rough estimate of how active each female was within the observation chambers. The distance traveled was standardized to units of cm per minute, as not all females had all 30 observations due to the difficulty of observing them in the chambers under dim light. Specifically, 96/101 (95 %) behavioral assays had 27-30 observations. The other five behavioral assays (5%), each had 10, 18, 21, 24 and 26 observations.

Statistical analyses

To test for the effects of peak extreme temperatures, DO levels and their interaction on the thermal tolerance of *T. californicus* females, we ran a generalized linear model in R, using a binomial link function (0 = dead, 1 = alive). Temperature and DO were included as fixed factors. For thermal and oxygen preferences, the number of times that a female was observed in a specific position in the gradient (1 – 18 in the chamber) was counted to calculate the percentage of time (%) in each position. The distribution of *T. californicus* in each position in the preference chambers is the main indicator of thermal and oxygen preferences. A Chi-square test was employed to test for pairwise differences in the distribution of females among treatments. For the distance traveled by females per minute, data were initially checked for normality using a Shapiro-Wilk test and the homogeneity of variance using Levene's test; both assumptions for ANOVA were not met ($P < 0.05$). Therefore, we used a non-parametric Kruskal-Wallis test to examine differences in the distance that copepods traveled per minute. *P* values < 0.05 are considered statistically significant. All analyses were run in *R* (v.3.1.3).

Data deposition

Data for this study are available via the Research Exchange of Washington State University upon publication.

References

1 Perkins, S. E., Alexander, L. V. & Nairn, J. R. Increasing frequency, intensity and duration of observed global heatwaves and warm spells. *Geophys. Res. Lett.* **39**, doi:L2071410.1029/2012gl053361 (2012).

2 Oliver, E. C. J. *et al.* Longer and more frequent marine heatwaves over the past century. *Nat. Commun.* **9**, 1324, doi:10.1038/s41467-018-03732-9 (2018).

3 Frölicher, T. L., Fischer, E. M. & Gruber, N. Marine heatwaves under global warming. *Nature* **560**, 360-364, doi:10.1038/s41586-018-0383-9 (2018).

4 Hobday, A. J. *et al.* Categorizing and naming marine heatwaves. *Oceanography* **31**, 162-173, doi:10.5670/oceanog.2018.205 (2018).

5 Doan, X. N. *et al.* Extreme temperature impairs growth and productivity in a common tropical marine copepod. *Sci. Rep.* **9**, 4550, doi:10.1038/s41598-019-40996-7 (2019).
6 Wishner, K. F. *et al.* Ocean deoxygenation and zooplankton: Very small oxygen differences matter. *Sci. Adv.* **4**, doi:eaau518010.1126/sciadv.aau5180 (2018).
7 Roman, M. R., Brandt, S. B., Houde, E. D. & Pierson, J. J. Interactive effects of hypoxia and temperature on coastal pelagic zooplankton and fish. *Front. Mar. Sci.* **6**, doi:Unsp 13910.3389/fmars.2019.00139 (2019).
8 Ekau, W., Auel, H., Poertner, H. O. & Gilbert, D. Impacts of hypoxia on the structure and processes in pelagic communities (zooplankton, macro-invertebrates and fish). *Biogeosciences* **7**, 1669-1699, doi:10.5194/bg-7-1669-2010 (2010).
9 Breitburg, D. *et al.* Declining oxygen in the global ocean and coastal waters. *Science* **359**, 46-+, doi:eaam724010.1126/science.aam7240 (2018).
10 Darling, E. S. & Côté, I. M. Quantifying the evidence for ecological synergies. *Ecol. Lett.* **11**, 1278-1286, doi:10.1111/j.1461-0248.2008.01243.x (2008).
11 Crain, C. M., Kroeker, K. & Halpern, B. S. Interactive and cumulative effects of multiple human stressors in marine systems. *Ecol. Lett.* **11**, 1304-1315, doi:10.1111/j.1461-0248.2008.01253.x (2008).
12 Przeslawski, R., Byrne, M. & Mellin, C. A review and meta-analysis of the effects of multiple abiotic stressors on marine embryos and larvae. *Glob. Change Biol.* **21**, 2122-2140, doi:10.1111/gcb.12833 (2015).
13 Altieri, A. H. & Gedan, K. B. Climate change and dead zones. *Glob. Change Biol.* **21**, 1395-1406, doi:10.1111/gcb.12754 (2015).
14 Gunderson, A. R., Armstrong, E. J. & Stillman, J. H. in *Annual Review of Marine Science, Vol 8* Vol. 8 *Annu. Rev. Mar Sci.* (eds C. A. Carlson & S. J. Giovannoni) 357-+ (2016).
15 Todgham, A. E. & Stillman, J. H. Physiological responses to shifts in multiple environmental stressors: relevance in a changing world. *Integr. Comp. Biol.* **53**, 539-544, doi:10.1093/icb/ict086 (2013).
16 Chown, S. L. & Gaston, K. J. Macrophysiology for a changing world. *Proc. R. Soc. Lond., Ser. B: Biol. Sci.* **275**, 1469-1478, doi:10.1098/rspb.2008.0137 (2008).
17 Tangwancharoen, S., Moy, G. W. & Burton, R. S. Multiple modes of adaptation: regulatory and structural evolution in a small heat shock protein gene. *Mol. Biol. Evol.* **35**, 2110-2119, doi:10.1093/molbev/msy138 (2018).

18 Fey, S. B. *et al.* Opportunities for behavioral rescue under rapid environmental change. *Glob. Change Biol.* **25**, 3110-3120, doi:10.1111/gcb.14712 (2019).
19 Pörtner, H. O. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *J. Exp. Biol.* **213**, 881-893, doi:10.1242/jeb.037523 (2010).
20 Portner, H. O., Bock, C. & Mark, F. C. Oxygen- and capacity-limited thermal tolerance: bridging ecology and physiology. *J. Exp. Biol.* **220**, 2685-2696, doi:10.1242/jeb.134585 (2017).
21 Verberk, W. *et al.* Does oxygen limit thermal tolerance in arthropods? A critical review of current evidence. *Comp. Biochem. Physiol., A: Mol. Integr. Physiol.* **192**, 64-78, doi:10.1016/j.cbpa.2015.10.020 (2016).
22 Rutledge, C. J. & Beitinger, T. L. The effects o dissolved oxygen and aquatic surface respiration on the critical thermal maxima of three intermittent stream fishes. *Environ. Biol. Fishes* **24**, 137-143, doi:10.1007/bf00001283 (1989).
23 Clark, T. D., Sandblom, E. & Jutfelt, F. Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *J. Exp. Biol.* **216**, 2771-2782, doi:10.1242/jeb.084251 (2013).
24 Wang, T. *et al.* Anaemia only causes a small reduction in the upper critical temperature of sea bass: is oxygen delivery the limiting factor for tolerance of acute warming in fishes? *J. Exp. Biol.* **217**, 4275-4278, doi:10.1242/jeb.104166 (2014).
25 Youngblood, J. P., Silva, C. R. B. d., Jr., M. J. A. & VandenBrooks, J. M. Oxygen limitation does not drive the decreasing heat tolerance of grasshoppers during development. *Physiol. Biochem. Zool.* **92**, 567-572, doi:10.1086/705439 (2019).
26 Jutfelt, F. *et al.* Oxygen- and capacity-limited thermal tolerance: blurring ecology and physiology. *J. Exp. Biol.* **221**, doi:UNSP jeb16961510.1242/jeb.169615 (2018).
27 Lehmann, P., Javal, M. & Terblanche, J. S. Oxygen limitation is not the cause of death during lethal heat exposure in an insect. *Biol. Lett.* **15** (2019).
28 Pierson, J. J., Slater, W. C. L., Elliott, D. & Roman, M. R. Synergistic effects of seasonal deoxygenation and temperature truncate copepod vertical migration and distribution. *Mar. Ecol. Prog. Ser.* **575**, 57-68, doi:10.3354/meps12205 (2017).

29 Metaxas, A. & Scheibling, R. E. Spatial heterogeneity of phytoplankton assemblages in tidepools: Effects of abiotic and biotic factors. *Mar. Ecol. Prog. Ser.* **130**, 179-199, doi:10.3354/meps130179 (1996).

30 Morris, S. & Taylor, A. C. Diurnal and seasonal variation in physiochemical conditions within intertidal rock pools. *Estuar. Coast. Shelf Sci.* **17**, 339-355, doi:10.1016/0272-7714(83)90026-4 (1983).

31 Crossin, G. T., Al-Ayoub, S. A., Jury, S. H., Howell, W. H. & Watson, W. H. Behavioral thermoregulation in the American lobster *Homarus americanus*. *J. Exp. Biol.* **201**, 365-374 (1998).

32 Lucic, D. *et al.* The effect of temperature change and oxygen reduction on zooplankton composition and vertical distribution in a semi-enclosed marine system. *Mar. Biol. Res.* **15**, 325-342, doi:10.1080/17451000.2019.1655161 (2019).

33 Verbitsky, V. B. *et al.* Insights into thermal preferences of copepods in nature using the horizontal gradient method. *J. Plankton Res.* **39**, 849-859, doi:10.1093/plankt/fbx038 (2017).

34 Byrne, M., Foo, S. A., Ross, P. M. & Putnam, H. M. Limitations of cross- and multigenerational plasticity for marine invertebrates faced with global climate change. *Glob. Change Biol.* **26**, 80-102, doi:10.1111/gcb.14882 (2020).

35 Enders, E. C., Wall, A. J. & Svendsen, J. C. Hypoxia but not shy-bold phenotype mediates thermal preferences in a threatened freshwater fish, *Notropis percobromus*. *J. Therm. Biol.* **84**, 479-487, doi:10.1016/j.jtherbio.2019.08.001 (2019).

36 Damgaard, R. M. & Davenport, J. Salinity tolerance, salinity preference and temperature tolerance in the high shore harpacticoid copepod *Tigriopus brevicornis*. *Mar. Biol.* **118**, 443-449, doi:10.1007/bf00350301 (1994).

37 Foley, H. B. *et al.* Sex-specific stress tolerance, proteolysis, and lifespan in the invertebrate *Tigriopus californicus*. *Exp. Gerontol.* **119**, 146-156, doi:10.1016/j.exger.2019.02.006 (2019).

38 Harada, A. E., Healy, T. M. & Burton, R. S. Variation in thermal tolerance and its relationship to mitochondrial function across populations of *Tigriopus californicus*. *Front. Physiol.* **10**, doi:21310.3389/fphys.2019.00213 (2019).

459 39 Harada, A. E. & Burton, R. S. Ecologically relevant temperature ramping rates enhance the
460 protective heat shock response in an intertidal ectotherm. *Physiol. Biochem. Zool.* **92**, 152-
461 162, doi:10.1086/702339 (2019).
462 40 Tangwancharoen, S. & Burton, R. S. Early life stages are not always the most sensitive: heat
463 stress responses in the copepod *Tigriopus californicus*. *Mar. Ecol. Prog. Ser.* **517**, 75-83,
464 doi:10.3354/meps11013 (2014).
465 41 Schoville, S. D., Barreto, F. S., Moy, G. W., Wolff, A. & Burton, R. S. Investigating the
466 molecular basis of local adaptation to thermal stress: population differences in gene
467 expression across the transcriptome of the copepod *Tigriopus californicus*. *BMC Evol. Biol.*
468 **12**, doi:17010.1186/1471-2148-12-170 (2012).
469 42 McAllen, R. & Brennan, E. The effect of environmental variation on the reproductive
470 development time and output of the high-shore rockpool copepod *Tigriopus brevicornis*. *J.*
471 *Exp. Mar. Biol. Ecol.* **368**, 75-80, doi:10.1016/j.jembe.2008.10.013 (2009).
472 43 Graham, A. M. & Barreto, F. S. Loss of the HIF pathway in a widely distributed intertidal
473 crustacean, the copepod *Tigriopus californicus*. *Proc. Natl. Acad. Sci. USA* **116**, 12913-
474 12918, doi:10.1073/pnas.1819874116 (2019).
475 44 Goolish, E. M. & Burton, R. S. Energetics of osmoregulation in an intertidal copepod:
476 effects of anoxia and lipid reserves on the pattern of free amio acid accumulation. *Funct.*
477 *Ecol.* **3**, 81-89, doi:10.2307/2389678 (1989).
478 45 Liguori, A. L. Population level differences in life history responses to long-term pH stress in
479 *Tigriopus californicus*. *Integr. Comp. Biol.* **58**, E134-E134 (2018).
480 46 McAllen, R. & Taylor, A. The effect of salinity change on the oxygen consumption and
481 swimming activity of the high-shore rockpool copepod *Tigriopus brevicornis*. *J. Exp. Mar.*
482 *Biol. Ecol.* **263**, 227-240, doi:10.1016/s0022-0981(01)00308-2 (2001).
483 47 Kelly, M. W., Sanford, E. & Grosberg, R. K. Limited potential for adaptation to climate
484 change in a broadly distributed marine crustacean. *Proc. R. Soc. B Biol. Sci.* **279**, 349-356,
485 doi:10.1098/rspb.2011.0542 (2012).
486 48 Barreto, F. S., Schoville, S. D. & Burton, R. S. Reverse genetics in the tide pool: knock-
487 down of target gene expression via RNA interference in the copepod *Tigriopus californicus*.
488 *Mol. Ecol. Resour.* **15**, 868-879, doi:10.1111/1755-0998.12359 (2015).

489 49 Koopman, K. R., Collas, F. P. L., van der Velde, G. & Verberk, W. Oxygen can limit heat
490 tolerance in freshwater gastropods: differences between gill and lung breathers.
491 *Hydrobiologia* **763**, 301-312, doi:10.1007/s10750-015-2386-y (2016).
492 50 McAllen, R., Taylor, A. C. & Davenport, J. The effects of temperature and oxygen partial
493 pressure on the rate of oxygen consumption of the high-shore rock pool copepod *Tigriopus*
494 *brevicornis*. *Comp. Biochem. Physiol., A: Mol. Integr. Physiol.* **123**, 195-202,
495 doi:10.1016/s1095-6433(99)00050-1 (1999).
496 51 Alaykrinskaya, I. O. The functional role of hemoglobin in the hemolymph of some
497 representatives of the family Planorbidae (Gastropoda, Pulmonata). *Hydrobiol. J./Gidrobiol.*
498 *Zh.* **34**, 84-88, doi:10.1615/HydrobJ.v34.i6.120 (1998).
499 52 Pavillon, J. F., Menasria, R., Forget, J., Barka, S. & Institut, O. in *Recents Progres En*
500 *Chimie Et Ecotoxicologie Marines* Vol. 25 *Oceanis-Serie de Documents Oceanographiques*
501 609-650 (1999).
502 53 Somero, G. N. Proteins and temperature. *Annu. Rev. Physiol.* **57**, 43-68,
503 doi:10.1146/annurev.ph.57.030195.000355 (1995).
504 54 McAllen, R. & Block, W. Aspects of the cryobiology of the intertidal harpacticoid copepod
505 *Tigriopus brevicornis* (O. F. Müller). *Cryobiology* **35**, 309-317,
506 doi:https://doi.org/10.1006/cryo.1997.2046 (1997).
507 55 Boardman, L., Sorensen, J. G., Kostal, V., Simek, P. & Terblanche, J. S. Cold tolerance is
508 unaffected by oxygen availability despite changes in anaerobic metabolism. *Sci. Rep.* **6**,
509 doi:3285610.1038/srep32856 (2016).
510 56 Stevens, M. M., Jackson, S., Bester, S. A., Terblanche, J. S. & Chown, S. L. Oxygen
511 limitation and thermal tolerance in two terrestrial arthropod species. *J. Exp. Biol.* **213**, 2209-
512 2218, doi:10.1242/jeb.040170 (2010).
513 57 Finney, C. M. Salinity stress in harpacticoid copepods. *Estuaries* **2**, 132,
514 doi:10.2307/1351640 (1979).
515 58 Zeis, B., Pinkhaus, O., Bredebusch, I. & Rüdiger, J. P. Oxygen preference of *Daphnia*
516 *magna* is influenced by P_{O2} acclimation and biotic interactions. *Physiol. Biochem. Zool.* **78**,
517 384-393, doi:10.1086/430039 (2005).

518

519 **Acknowledgments**

520 We thank three anonymous reviewers for constructive feedback, which helped to improve the
521 manuscript. This research is supported by National Science Foundation IOS grant 1655822 to
522 W.W.D.

523 **Author contributions**

524 K.V.D., A.Y.C. and W.W.D designed the experiment; K.S.B. and E.A.M. contributed to developing
525 methods. K.V.D. and A.Y.C conducted the experiment and performed statistical analyses. K.V.D
526 wrote the first draft of the manuscript. All authors contributed to the later version of the manuscript;
527 all authors read and approved the manuscript for publication.

528 **Competing interests**

529 All authors declare no competing interests.

530

Table 1. The measured temperatures and dissolved oxygen in the experimental system for the preference behavior test. Data are means ± SD.

Treatments	Header tank 1		Header tank 2		Gradients
	Temperature (°C)	Dissolved oxygen (% saturation)	Temperature (°C)	Dissolved oxygen (% saturation)	
T1	36.7 ± 0.1	198.1 ± 1.9	11.8 ± 0.6	21.0 ± 4.3	Parallel temperature and DO gradients
T2	36.6 ± 0.2	26.4 ± 2.9	12.0 ± 0.3	193.7 ± 1.7	Inverse temperature and DO gradients
T3	36.5 ± 0.2	199.0 ± 2.3	11.7 ± 0.6	198.4 ± 1.9	Temperature gradient. No DO-gradient (high DO)
T4	36.7 ± 0.1	26.9 ± 0.4	11.3 ± 0.5	31.2 ± 2.6	Temperature gradient. No DO-gradient (low DO)
T5	12.2 ± 0.4	198.1 ± 0.4	11.7 ± 0.1	27.9 ± 8.2	No temperature gradient (cool temperature). DO-gradient
T6	36.5 ± 0.1	197.0 ± 9.5	36.3 ± 0.1	31.5 ± 1.2	No temperature gradient (warm temperature). DO-gradient
T7	19.6 ± 0.2	100.1 ± 1.3	19.4 ± 0.3	98.4 ± 2.6	No temperature gradient (intermediate temperature). No DO gradient, normoxic. (control treatment)

Figure legends

Figure 1. Experimental system for the behavioral preference assay, in the presence of oxygen and/or temperature gradients. H1 and H2 are header tanks where both temperatures and dissolved oxygen can be independently controlled within the ranges of 1-100°C and 0-200% saturation, respectively. WB1 and WB2 are water baths where temperatures were set up to control temperatures in the H1 and H2 tanks, respectively. Gases (O_2, N_2, and CO_2) were fed from cylinders to each header tank via airstones, and an Arduino-controlled system pulsed gas flows independently to each header tank through solenoid valves to maintain pH and pO_2 at desired levels. AS = Airstones and P = pump. Arrows indicate the directions of water flow.

Figure 2. The survival of *Tigriopus californicus* females in response to the lethal temperatures at different dissolved oxygen levels. Grey shaded areas indicate overlapping 95% confidence intervals.

Figure 3. The distribution of *Tigriopus californicus* females in response to gradients of temperature, oxygen, or both within assay chambers. Data are the cumulative percentages (%) of observations in which females stayed in each of the positions from 1 to 18 in the test chambers. The colored bars within each panel illustrate the ranges of temperature and dissolved oxygen presented in each treatment. Statistical differences ($P < 0.05$) among treatment groups are indicated by different lowercase letters.

Figure 4. The average distance (cm) traveled per minute by *Tigriopus californicus* females in response to the thermal and oxygen gradients in the test chambers. Data are means + 1 SE. Statistical differences ($P < 0.05$) among treatments are indicated by lowercase letters above the bars.