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Intertidal copepod *Tigriopus californicus* displays multilevel variation in tolerance to extended bouts of hypoxia

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ABSTRACT: Environments with fluctuating oxygen are intense challenges for organisms both on land and in the water. Aquatic organisms can be exposed to especially stressful bouts of hypoxia that come on rapidly and to extreme levels. The copepod *Tigriopus californicus* inhabits supralittoral rocky pools and appears tolerant of hypoxia levels considered lethal for other aquatic organisms despite lacking molecular components typically used by animals to detect and respond to low environmental oxygen. Here, we quantified the natural regime of dissolved oxygen (DO) pools inhabited by *T. californicus* via deployment of continuous oxygen sensors in copepod pools in Oregon, USA. Using wild-derived cultures from northern (Oregon) and southern (Californian) populations, we exposed copepods to hypoxia and anoxia and assayed loss of equilibrium (LOE) and survival. We also quantified respiratory regulation via critical oxygen tension, oxygen supply capacity, and regulation index. The pools underwent extreme daily cycles of DO, and near anoxia often persisted for up to 6 h. Respiratory statistics indicated individuals could regulate oxygen consumption even near anoxia, predicting a species with hypoxia tolerance ranking high among aquatic taxa. Copepods survived hypoxia below 0.3 mg O_2 l^{-1} for up to 72 h with some individuals not showing any LOE. Survival was high following even 6 and 15 h exposure to anoxia. We observed sex and population differences in lethality and LOE, with southern populations exhibiting higher resilience. Intraspecific variation in tolerance makes this system a candidate for future studies to investigate alternative molecular and physiological pathways of hypoxia response.

KEY WORDS: Anoxia · Loss of equilibrium · Survival · Oxygen supply capacity · P_{crit} · Regulation index · HIF · Hypoxia-inducible factor

1. INTRODUCTION

Exposure to hypoxic conditions is one of the most physiologically difficult challenges for organisms that rely on aerobic respiration. In exclusively terrestrial systems, environmental hypoxia occurs as a function of altitude. For aquatic organisms, the challenge of hypoxia is more widespread, and periods of low levels of available dissolved oxygen (DO) may be common, can vary in frequency and magnitude, and can impact fauna of shallow or deep waters (Childress & Seibel 1998, Richards 2011, Seibel 2011, Wishner et al. 2018). For many aquatic organisms, exposure to even partially hypoxic conditions is enough to present a significant challenge to metabolism or even survival (Vaquer-Sunyer & Duarte 2008), and a myriad of physiological and behavioral adaptations have evolved to maintain basic cellular function (Bell et al. 2003, Sokolova et al. 2019). The need for dealing with hypoxia has been exacerbated as a result of rapidly changing conditions in oceans due to changes in the global climate (Chan et al. 2008). Even highly mobile

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coastal animals like fish and crabs are being affected by the increased intensity and breadth of such hypoxia 'dead zones' (Altieri & Diaz 2019).

As a result of the immediate challenges presented by hypoxia, animals have evolved a robust and highly conserved genetic pathway devoted to detecting and responding to low oxygen. This hypoxia-inducible factor (HIF) pathway is well described (Gorr et al. 2010, Graham & Presnell 2017) and is widely conserved among organisms as the molecular machinery responsible for activating a cellular response to reduced oxygen availability. However, the resulting physiological response to hypoxia, with regards to organismal phenotypes such as respiration and energy metabolism, is quite diverse among animal groups (Vaquer-Sunyer & Duarte 2008, Hughes et al. 2022). Physiological strategies for dealing with hypoxia involve adjustments aimed at maintaining sufficient O_2 supply for aerobic metabolism or reducing O_2 demand via regulatory switches to anaerobic metabolism (Gorr et al. 2010, Fuhrmann & Brüne 2017). The relative role of these mechanisms varies by taxonomic group (Gorr et al. 2010) but can also diverge among related species as a result of adaptation to different DO regimes (Borowiec et al. 2020). The combination of unique evolutionary trajectories and rapid environmental change has resulted in considerable variation in hypoxia tolerance among aquatic species. Despite its presumed importance in initiating a physiological response to hypoxia, recent findings have shown that some aquatic taxa appear to have lost or modified key components of the HIF pathway, including HIF-1α, a transcription factor subunit required for activation of several genes (Graham & Barreto 2020, 2023). This may be evidence for lability in the physiological response to hypoxia across taxa (Graham & Barreto 2023), but it is unclear at this stage why or how some species possess altered molecular responses to hypoxia, and, critically, we do not understand the consequences of these molecular alterations on physiological tolerance and fitness during periods of minimally available oxygen.

The marine copepod *Tigriopus californicus* (Baker, 1912) is an organism that has lost the 2 most important regulators of the HIF pathway, namely HIF-1α, which initiates the hypoxia response cascades, and its inhibitor, prolyl hydroxylase (EGLN), which is an oxygen-sensing protein that maintains HIF-1α inactive during normoxia (Graham & Barreto 2019). This invertebrate is found in the supralittoral (or splash zone) of the rocky intertidal along most of the western coast of North America. It lacks a circulatory system, gills, and respiratory pigments and is thought to engage in respiration through the diffusion of oxygen across its external tissues (McAllen et al. 1999). The pools inhabited by *T. californicus* are in the spray zone of incoming waves but are not directly connected to the flow of water as the tides rise. Therefore, *T. californicus* experiences both diel environmental fluctuations and long-term isolation in stressful conditions. As a result, *T. californicus* usually comprise the sole fauna inhabiting these pools; the other primary residents are photosynthetic micro- and macro-algae (Powlik 1999) that consume or release oxygen depending on the time of day (Truchot & Duhamel-Jouve 1980). Recent evidence suggests *T. californicus* may be tolerant of hypoxia challenges despite lacking *HIF-α* and *EGLN*, with hypoxia-exposed individuals showing high rates of survival and only mild delays in offspring development rate (Graham & Barreto 2019, Deconinck & Willett 2022). In the field, snapshot sampling of DO during daytime hours in *T. californicus*-inhabited pools in locales of the Californian coastline indicate their environment does reach levels of hypoxia (~1–2 mg O_2 l^{-1}; Deconinck & Willett 2022) that are considered lethal for other aquatic taxa. However, considering the high degree of survivorship to much lower DO levels in laboratory experiments, as reported by both Deconinck & Willett (2022) and Graham & Barreto (2019), we predict that these populations have evolved under more intense levels of hypoxia stress in the field than the 1–2 mg O_2 l^{-1} so far reported. Therefore, a fine-scale survey of daily and nightly oxygen levels in this habitat over an extended period is necessary for a thorough description of this species' environment and selective pressures.

To quantify hypoxia tolerance, many studies measure survival following exposure to hypoxia or the onset of loss of equilibrium (LOE) (Speers-Roesch et al. 2013, McArley et al. 2020, Ern et al. 2020). Onset of LOE during stress, often recorded as the time it takes for an individual to lose the ability to right oneself or retain coordinated motor function in the water column, may capture how long an organism can maintain the ability to perform life history functions, such as acquiring food or mating, during periods of environmental hypoxia. Quantifying the probability of survival following LOE and the establishment of full reoxygenation can capture tolerance to both the lack of sufficient oxygen and the burst of resupplied oxygen in the mitochondria during recovery. Indeed, rebounding after extended periods of LOE may require the capacity to safely relieve metabolic suppression to transition back to aerobic processes via preparation for oxidative stress (Giraud-Billoud et al. 2019, 2024). However, prior to resorting to aerobic suppression and, presumably, prior to the onset of LOE, most hypoxia-tolerant organisms attempt to maintain respiration rates as available oxygen is depleted (Cobbs & Alexander 2018). The ability to do so is described as the ability to 'regulate' oxygen consumption and is thought to allow them to maintain normal behaviors (Mueller & Seymour 2011). When an animal can no longer independently regulate a steady metabolic rate, it becomes an oxyconformer (Mueller & Seymour 2011), and its metabolic rate decreases steadily with decreasing available oxygen. During this phase and leading into LOE, metabolic expenditure is expected to be channeled towards survival.

Several metrics have been developed to capture the scope of respiratory regulation and conformity as a means of quantifying an individual's tolerance to hypoxia based on metabolic parameters instead of fitness-level phenotypes such as LOE or survival. Perhaps the most widely used respiratory statistic of this kind is critical oxygen tension (P_{crit}), which is the partial pressure of oxygen (P_{O_2}) at which an organism can no longer actively regulate its respiratory rate. Low P_{crit} is interpreted to suggest a high tolerance to hypoxia. Another metric is the oxygen supply capacity (known as alpha), which approximates the supply of oxygen needed to support a given metabolic rate. It is closely related to P_{crit}, as P_{crit} can be inversely interpreted as the P_{O_2} at which oxygen supply reaches its capacity for a given metabolic rate and any amount of oxygen below this breakpoint becomes a limiting factor for the respiration rate (Seibel et al. 2021). Alpha is interpreted on the same scale as P_{crit}, where lower values of alpha indicate a lower required supply of oxygen to support respiration and, therefore, greater tolerance to hypoxia. An alternative or complementary statistic to P_{crit} and alpha has been proposed in the form of the regulation index (RI). The RI statistic captures an organism's ability to regulate oxygen consumption across all oxygen values from normoxia to anoxia instead of quantifying a single breakpoint or maxima like P_{crit} or alpha, respectively (Mueller & Seymour 2011). Higher RI values are descriptive of higher oxyregulation capacity. Importantly, while survival and development following periods of hypoxia have been assessed in *T. californicus* (Graham & Barreto 2019, Deconinck & Willett 2022), LOE and respiratory statistics like P_{crit}, alpha, and RI have yet to be quantified.

Tigriopus californicus is an excellent model with which to begin understanding hypoxia tolerance in organisms with altered hypoxia-response genetic pathways, which include at least 3 taxonomic orders of Copepoda, 3 orders of Cirripedia (barnacles), and some myxozoan Cnidaria (Graham & Barreto 2020, Graham & Barreto 2023). This copepod species will also help us contextualize the physiological limits of the taxa facing increased risk of elevated and prolonged hypoxia exposure due to our planet's rapidly changing climate. Moreover, distinct populations of *T. californicus* experience a wide variation in temperature, sunlight, and rainfall regimes along latitudes, with copepod pools in the southern Californian coastline exposed to warmer days with less rain and higher UV indexes compared to populations in the Pacific Northwest in the USA and Canada (Edmands & Deimler 2004, Willett 2010, Healy et al. 2019). This provides an opportunity to test whether there is variation in hypoxia tolerance among populations that occupy different environmental regimes. In this study, we show high-resolution and prolonged DO data from northern *T. californicus* splash pools collected during summer months to characterize the intensity and frequency of hypoxia acting on natural populations of this species. In the laboratory, we quantified the fitness limits of *T. californicus* copepods via LOE and survival assays in prolonged hypoxia and anoxia while comparing phenotypic divergence among 4 populations from southern and northern latitudes. In these assays, we included sex comparisons within each population to account for previously described sex-specific stress responses for this species (Willett 2010, Foley et al. 2019, Powers et al. 2021). Finally, we quantified P_{crit}, alpha, and RI to describe the breakpoint and scope of the regulation of aerobic respiration in this species for comparison to other hypoxia-tolerant taxa.

2. MATERIALS AND METHODS

2.1. Field DO measurements

The DO and temperature in pools inhabited by *Tigriopus californicus* were recorded during 3 mo of 2022 in 2 Oregon sites: Strawberry Hill (SH, 44.254454° N, 124.112880° W) and Boiler Bay (BOB, 44.830704° N, 124.059398° W), from 28 June through 30 September (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m757p053_supp.pdf). One pool at each site was selected for data collection based on requirements of depth needed to submerge oxygen sensors, the presence of algae needed to feed typical and healthy *T. californicus* populations, minimum size to remain stable in support of *T. californicus* throughout the summer, and annual persistence of *T. californicus* based on previous year observations. In each pool, miniDOT DO and temperature loggers (Precision Measurement Engineering) were anchored to a flat rock at the bottom of the pool using 2 steel rods and were set to record DO and temperature every 10 min. Sensors were calibrated by the manufacturer and oxygen saturation values were automatically adjusted to real-time temperature while salinity was set to 35 ppt in the software. The placement depth of the sensor in the SH pool was approximately 13 cm while the depth in the BOB pool was approximately 9.5 cm. These pools represent average depths for a *T. californicus*-inhabited splash pool; however, this species can persist in shallower and deeper pools than the ones we selected. These pools contained typical blooms of both micro- and macroalgae needed to sustain healthy *T. californicus* populations, and these blooms expectedly increased in density over the summer in response to increased sunlight. During the data-collection window, we visited the sites every 2–3 wk to transfer data and monitor the pools. While it is not possible to know whether pools dried and re-flooded between our visits, the pools in both BOB and SH had sufficient water and healthy *T. californicus* stocks at every visit.

2.2. Copepod culture and husbandry

We used lab-cultured populations of copepods originally collected from field sites in SH and BOB in Oregon, as well as from San Diego (SD; 32.7333° N, 117.2500° W) and Bird Rock (BR; 32.4854° N, 11.7162° W), California. Each population was cultured as a large outbred population resupplied yearly with wild individuals. Cultures were kept in 400 ml beakers at an approximate density of 200 ind. beaker^{-1} for a minimum of 3 generations (~3 mo) prior to any data collection to remove effects of environmental carryover and ensure that fitness measurements were not affected by copepods still adjusting to laboratory conditions. All outbred cultures from each of the 4 populations were supplemented with new individuals captured in 2022 prior to use in this study. All cultures were maintained at 20°C in 35 ppt artificial saltwater (ASW; Instant Ocean) on a 12:12 h light-to-dark cycle in incubators and were fed an ad libitum mixture of *Nannochloropsis* and *Isochrysis* microalgae along with ground-up spirulina wafers and Tetramin fish flakes (Tetra). Partial water changes with fresh ASW were performed twice each month.

2.3. Respirometry for metabolic indices

We performed respirometry assays on age-matched individuals from each cultured population (SD, BR, BOB, and SH) to measure population- and sex-specific P_{crit}, alpha, and RI. From each outbred stock culture, well-developed egg sacs (those that were large, pigmented red, and with visible eyes) were removed from gravid females and hatched individually in 6-well plates containing ASW and food. When copepods reached 30 d post-hatch, pairs of full siblings, separated by sex, were used for the assays. From each clutch, which is always composed of full siblings (Burton 1985), only one pair of males and one pair of females were used, with each pair considered a replicate. Respiration for 2 copepod siblings at a time was chosen due to this species' small size (~1 mm in length at full maturity); the complete depletion of oxygen in the respirometer by single individuals often takes longer than 24 h, which risks sensor signal drift, according to the manufacturer. In total, we obtained the following sample sizes: 43 SD female, 57 SD male, 33 BR female, 38 BR male, 38 BOB female, 29 BOB male, 31 SH female, and 25 SH male (Table S1).

We captured oxygen consumption using a closed-chamber 24-well optical fluorescence microplate respirometry system with 80-µl wells (Loligo Systems). All uses of the respirometer were completed in a dark, climate-controlled incubator set to 20°C, including sensor calibrations (see Text S1 in the Supplement for details). Following overnight starvation, each replicate was transferred to a single well of the respirometer, and the plate was sealed using Whatman UniSeal polyester seal film (Cytiva). A rubber gasket and a heavy block were placed on top of the plate, which was immediately placed on the sensor reader inside the incubator and the data recording was started. We monitored oxygen depletion for 24 h or until the copepods' oxygen consumption plateaued at 0 mg O_2 l^{-1}. In each trial, 21 wells contained copepods, and 3 wells were used as blanks to monitor signal drift. Replicates that did not fully deplete the oxygen in their well were removed from the final data set prior to all statistical analyses (59 out of 353). Following each respirometry trial, every individual was photographed and their body lengths measured as the distance from the eyespot to the center branch of the caudal ramus using FIJI software (v.2.10.0; Schindelin et al. 2012). The lengths of both copepods in each replicate were combined and this value was used to standardize oxygen consumption to body size per well prior to the calculation of all respiratory statistics.

2.4. Calculation of respiratory statistics

All calculations were performed in R (v. 4.2.2; R Core Team 2023). All R packages used are listed in

Text S2 in the Supplement. Annotated R code used to calculate respiratory statistics and summary plots can be found in an online repository (see data availability statement). Prior to calculating respiratory statistics, we trimmed our 24 h DO data using the 'runner' package (v.0.4.4; Kałędkowski 2023) whereby all data points 1 h past the depletion of oxygen were removed to minimize the effect of zero-inflated data on the binning and calculation of oxygen consumption rate (MO_2) values when calculating respiratory statistics (see Text S3 in the Supplement for full parameters). We also included a 1 h burn-in at the start of the oxygen consumption data to remove the copepod acclimation period and the equilibration of water temperature to incubator conditions.

For all metrics below, MO_2 values were standardized by body length prior to calculations. We calculated P_{crit} and alpha using the 'respirometry' package (v.1.3.0; Birk 2021). We report these statistics in units generated from this package, where P_{crit} is in terms of oxygen partial pressure (kPa O_2) and alpha is in length-specific units of supplied oxygen for a given oxygen partial pressure (µmol O_2 mm^{-1} h^{-1} kPa^{-1}). To calculate RI, we used code adapted from Tremblay et al. (2020) and used the length-normalized MO_2 values exported from the 'respirometry' package. As demonstrated by Tremblay et al. (2020), we calculated the RI value in both positive and negative space, wherein positive values represent a degree of regulation between 0 (perfect conformity) and 1 (perfect regulation) and negative values represent the degree of metabolic suppression or hypoxia sensitivity (Alexander & McMahon 2004). Complete parametrization of the calculations can be found in Text S3.

2.5. LOE and survival in anoxia

Using the same microplate respirometry system as described above, we assayed population- and sex-specific LOE and survival after exposure to anoxia for 6 h, which represents the longest bout of anoxia we recorded in natural pools inhabited by this species. All assays were completed in an incubator set at 20°C in complete darkness. We used cultures matched by condition and approximate culture age by hatching multiple clutches in the same beaker with clean ASW and fresh food.

To measure LOE and survival over 6 h, we added groups of 10 copepods of the same sex, starved in clean ASW for 24 h, to microplate wells containing ASW that had been bubbled with nitrogen gas to lower DO to facilitate more rapid onset of anoxia. During bubbling, DO was measured using a handheld oxygen meter (YSI, Xylem Analytics). Wells were sealed individually with pre-cut film. For each population-by-sex grouping, we measured 6 replicates (Table S1). The wells were monitored continuously until the copepod-filled wells plateaued at a DO level of 0.0 mg O_2 l^{-1}. This time was recorded on a per-well basis as the start of anoxia exposure. At this point, wells were monitored hourly for 6 h. At each hourly check, we recorded the number of copepods that exhibited LOE. This was diagnosed as an inability to swim or remain in the water column following the plate being inverted and tapped. At the sixth hour for each well, LOE was recorded one final time, the wells were unsealed individually, and the copepods were moved to a 24-well plate with fresh normoxic ASW and food. The copepods were allowed to recover for a total of 72 h, at which point the number of surviving copepods was recorded.

We performed 2 additional survivorship assays following longer exposures to anoxia. We repeated the above assay but waited 15 and 24 h after the DO had been depleted by the copepods. As above, we tracked each well's oxygen consumption and adjusted the time at which wells were unsealed on a per-well basis. We chose 24 h to push the limit at which the species may be expected to survive without oxygen and chose 15 h as an intermediate time point. We used replicates of 10 copepods from the same condition-matched cultures described above, taking 12 replicates from each population (6 male and 6 female replicates).

We ran a nonsynchronous control assay to test for confounding effects of CO_2 and waste buildup in the 80-µl microplate wells that could have affected LOE and survival. We describe this in full detail in Text S4 in the Supplement. Briefly, we matched the experimental assay design, but only filled the wells with 70 µl ASW, leaving an oxygen-filled air bubble in the top of the sealed well. This maintained a normoxic environment while testing for the impact on copepods for being sealed for the length of our anoxia assays (6 and 24 h). In this control assay, we observed that waste buildup did not significantly affect the proportion of individuals that experienced LOE or the proportion of individuals that survived being sealed for the length of the anoxia trials (Table S2, Fig. S2). We also tested for any confounding effect of oxygen consumption rate on LOE and survival and found no significant effect of this variable, thereby excluding it from any statistical models (see Supplement Text S5 for full description).

2.6. The relationship between LOE and survival over prolonged hypoxia

To test whether LOE was predictive of survival over prolonged exposure to hypoxia, we quantified these traits at 3 time intervals over a 72 h hypoxia exposure using copepods from inbred lines of SD, SH, and BOB created via 3 generations of strict inbreeding. As individuals within each line have high genetic similarity, this assay also captures intrapopulation variation among genotypes. For detailed descriptions of inbred line generation, see Text S6 in the Supplement. We sampled from 12 BOB, 30 SD, and 66 SH inbred lines in total with 12 copepods (6 male and 6 female) per replicate (Table S1). Prior to the assay, sampled copepods from each inbred line were placed in 6-well plates with clean water and an identical amount of food. Copepods remained in these standardized conditions for 3 d to reduce environmental variation across lines in their stock culture conditions. For the assay, copepod replicates were moved to 25 ml scintillation glass vials filled with hypoxic water (DO between 0.15 and 0.3 mg O_2 l^{-1}; P_{O_2} between 0.43 and 0.86 kPa at 20°C), prepared via bubbling of nitrogen gas. The actual number of copepods added to the vials was recorded to account for some natural mortality that occurred in acclimation plates. Test vials were then kept in incubators at 20°C for 3 d (12:12 light-to-dark cycle). We recorded the number of copepods showing LOE at 3 intervals: 24, 48, and 72 h. After 72 h in the hypoxia vials, copepods were transferred to normal conditions in 6-well plates and allowed to recover for 72 h, at which point the number of surviving individuals was counted. We quantified the relationship between LOE at the intervals of 24, 48, and 72 h and survival proportion using a beta regression model with population included as a covariate to account for non-independence of data and pseudoreplication (see Text S7 in the Supplement for a detailed description of the models in this analysis).

2.7. Statistical analyses and data visualization

All statistical analyses and data visualizations were performed in R (R Core Team 2023). We provide the fully annotated R code used to complete the analyses and figures shown in this manuscript, a summary file of all model results and model formulas, and all data files used in these analyses on GitHub (see 'Data availability' section for the URL to access this material). We also provide a brief overview of the packages used and our statistical models.

We assessed statistical significance using 95% confidence limits (CL) on the estimated effect coefficients and a p-value of $\leqslant 0.05$. We provide confidence limits and estimates of effect (β values) to help provide context toward biological significance beyond a p-value alone. For each of the models described below, we analyzed pairwise comparisons using Tukey's post hoc correction via the 'emmeans' package (v1.10.1; Lenth 2024).

To maximize statistical power for estimating effect sizes of P_{crit}, alpha, and RI across populations and between sexes, we ran 2 mixed effects models: one comparing populations that included sex as a random variable and one comparing sexes that used population as a random variable. For completeness, we also include models that recode the independent variable into population by sex factor levels. To compare the proportion of surviving copepods among populations and between sexes, we similarly fit beta regression models with a logit link function to account for the use of proportion data. We used the 'emmeans' package to estimate among-group differences and report these on the response scale (i.e. the actual proportions of survivors) rather than the latent scale (the log odds difference in survival among groups). To model differences in the incidence of LOE among the groups, we used a beta regression model, again with a logit link function to account for our use of proportion data. We used the 'emtrends' function from the 'emmeans' package to estimate the effect of anoxia exposure over time and how this effect differed among populations or between sexes. We again report the results on the response scale as the actual increase or decrease in the proportion of copepods with LOE. Prior to modeling, all proportion data for LOE and survival variables were scaled to fit the interval (0, 1) using Eq. (1), as recommended by Smithson & Verkuilen (2006):

$$y = \frac{\left[y'\left(N-1\right) + 0.5\right]}{N} \tag{1}$$

where N is the sample size of observations in the variable of interest and y' and y are the original and scaled proportion values, respectively. All models were assessed using diagnostic plots generated using the 'plot'(model) function in R.

3. RESULTS

3.1. DO regime in splash pools

Oxygen levels in the copepod splash pools were cyclical, coinciding with diel light cycles on the

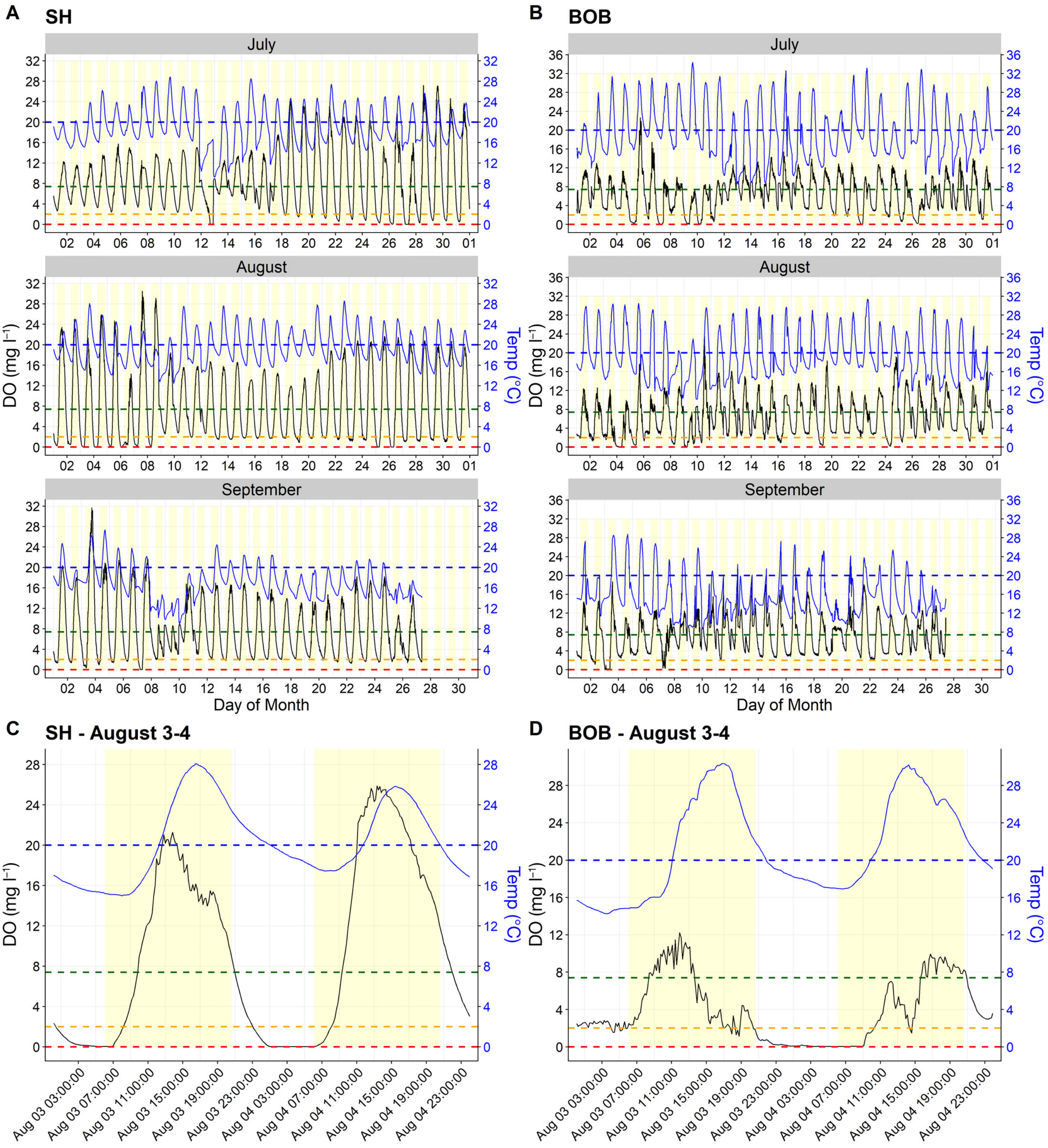

Fig. 1. Dissolved oxygen and temperature regimes in copepod-inhabited splash pools at Strawberry Hill (SH) and Boiler Bay (BOB), Oregon. Data collected at 10 min intervals from 1 July through 27 September 2022. (A,B) Daily values across the entire monitoring period for SH and BOB sites. (C,D) Zoom-in of a 2 d period for SH and BOB sites showing anoxia during the night. Black lines: dissolved oxygen; blue line: temperature; red dotted line: 0 mg O_2 l^{-1} (anoxia); orange dotted line: 2 mg O_2 l^{-1} (severe hypoxia for most organisms); green dotted line: 7.4 mg O_2 l^{-1} (normoxic conditions at 20°C); blue dotted line: the incubator temperature of 20°C used in our laboratory assays. Yellow shading: sunrise and sunset times for each day

shoreline (Fig. 1, Figs. S3 & S4). The range of DO values in the splash pools over 24 h was wide, with many days showing DO values both near anoxia at night and extending into hyperoxia during the day (Fig. 1A,B). Daily DO minima occurred after midnight and maximum DO levels were recorded just after midday (Fig. 1C,D). The lowest DO measurements in the SH and BOB pools were 0.007 mg O_2 l^{-1} on 6 and 26 August and 0.023 mg O_2 l^{-1} on 3 September, respectively. The highest DO values were 31.67 mg O_2 l^{-1} on 3 September and 23.56 mg O_2 l^{-1} on 10 August for the SH and BOB sites, respectively. These pools also experienced high daily levels of hyperoxia, often reaching levels over 300% saturation (Figs. S3 & S4).

We quantified the frequency and duration of extreme hypoxia levels in each pool across the monitored period of 89 d. In the SH pool, DO levels dropped below 2 mg O_2 l^{-1} on approximately 66% of the measured days, below 0.5 mg O_2 l^{-1} on 20% of measured days, and below 0.1 mg O_2 l^{-1} on approximately 11% of measured days (Fig. 2). Across days in which DO fell below 0.1 mg O_2 l^{-1}, levels stayed that low for a mean of 215 consecutive minutes, and some days for as long as 360 min (Fig. 2). In the BOB pool, DO levels dropped below 2 mg O_2 l^{-1} on approximately 41% of the measured days, below 0.5 mg O_2 l^{-1} on 16% of measured days, and below 0.1 mg O_2 l^{-1} on approximately 8% of measured days (Fig. 2). On the days in which DO fell below 0.1 mg O_2 l^{-1}, levels remained this low for a mean of 206 min with a maximum duration of 430 min (Fig. 2).

3.2. Physiological metrics of hypoxia tolerance in *Tigriopus californicus*

Respirometry revealed that *Tigriopus californicus* was extremely tolerant to declining oxygen. Across all populations and sexes, we assayed 353 pairs of siblings; after removing replicates that did not deplete oxygen within the 24 h test window, we analyzed a total of 294 replicates in the final data set. Summary values for *T. californicus* as a species (across all 294 replicates) are shown in Table 1. Most replicates across all populations steadily depleted oxygen in their wells near or completely to 0 mg O_2 l^{-1} (Figs. S5 & S6A,C). When very low levels of oxygen were reached (usually around 1 mg O_2 l^{-1}, or 2.85 kPa at 20°C), we observed a rapid, but temporary, spike in oxygen consumption before then rapidly declining as available oxygen was depleted (Fig. S6B).

We observed that SD and BR, the 2 California populations, had statistically significantly lower P_{crit} values on average than the Oregon populations, BOB and SH (Table 2, Fig. 3A). Female SD had average P_{crit} that was significantly lower than all other groups except male SD copepods and both sexes from BR (Table S3, Fig. S7). When P_{crit} values were averaged across all populations, mean P_{crit} was lower in female copepods, but this was not statistically significant ($p = 0.08$, CL = −1.24 to 0.07; Table S2, Fig. S8).

The populations did not differ significantly in alpha or RI values (Table 2, Fig. 3B,C). However, the estimated alpha for the SH population was the highest and its difference from the BR and BOB averages was statistically marginal ($p = 0.078$ and 0.081, respectively; Table 2). Alpha and RI values across all populations showed no statistically significant difference between males and females on average (Table S4, Fig. S8). When averaging alpha and RI values by sex within each population, male SH copepods had the highest alpha (Table S3, Fig. S7B). Female SH had the lowest RI of all groups (Table S3, Fig. S7C).

3.3. LOE and survival of *T. californicus* during anoxia

We observed significant variation among the populations in the rate at which individuals displayed LOE over the course of 6 h exposure to anoxic conditions. The proportion of individuals with LOE from the northern BOB and SH populations increased at a faster rate than individuals from the southern SD and BR populations throughout anoxia exposure. The hourly increase in the proportion of copepods displaying LOE was significantly lower in the BR population compared to the SD, SH, and BOB populations (Table 3, Fig. 4A). Copepods from SD also showed lower rates of LOE than the Oregon populations, but significantly so only relative to BOB ($p = 0.009$). There was a statistically significant difference in LOE between the 2 sexes, with female copepods experiencing a slower average increase in LOE per hour of anoxia than male copepods (Table S5, Fig. 5A). Males and females from the BR population and females from SD population had the lowest increase in LOE per hour of anoxia compared to each other sex-by-population grouping (Table S6, Fig. S9A).

Following 6 h of exposure to anoxia, the average proportion of surviving copepods was still high for each population, and only BOB copepods exhibited a statistically lower mean survival relative to SD and BR copepods (Table 3, Fig. 4B). When averaging the proportions of surviving copepods by sex (across all pop-

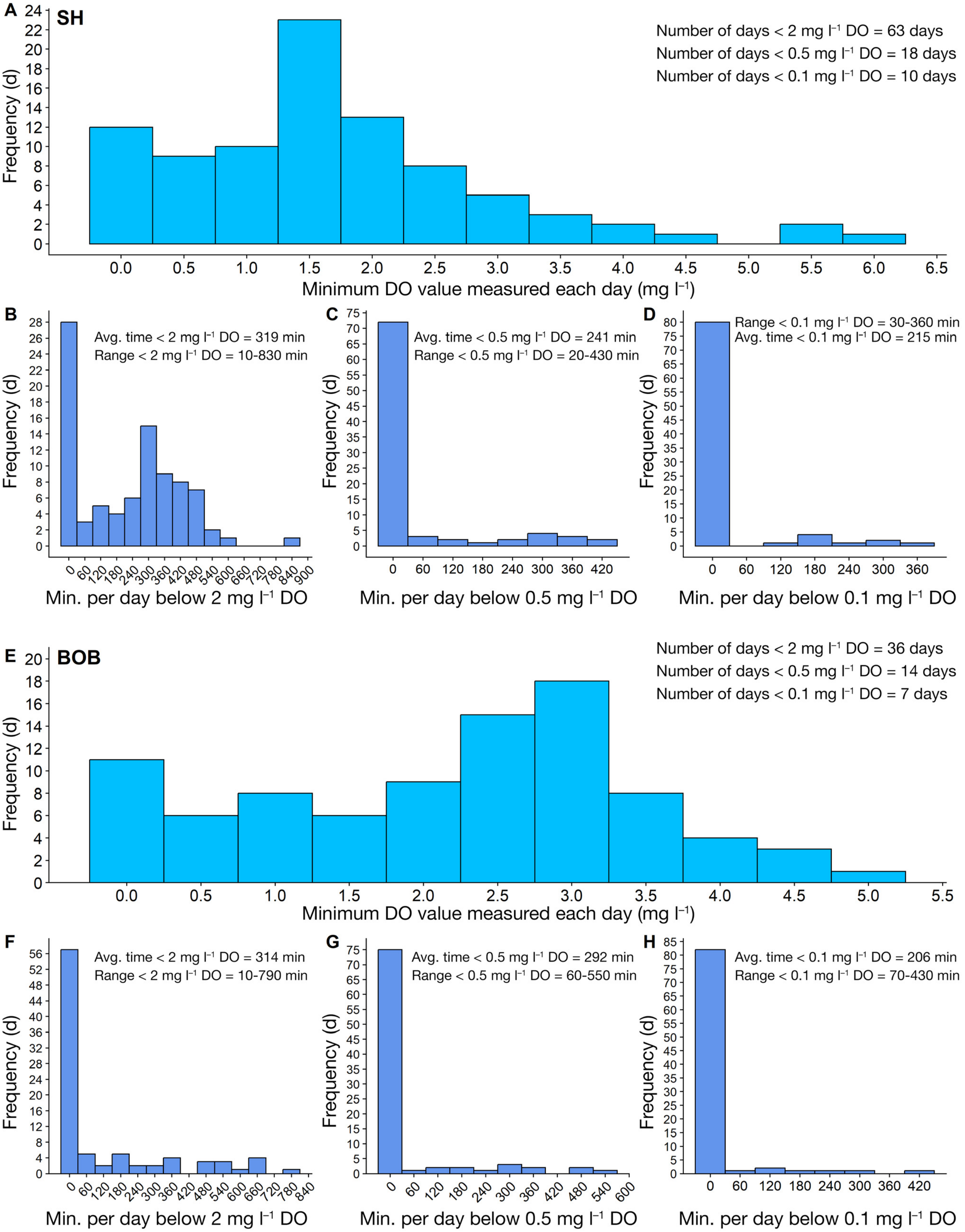

Fig. 2. Frequency and duration of hypoxia in copepod-inhabited pools in (A–D) Strawberry Hill, OR and (E–H) Boiler Bay, OR. Values calculated from data presented in Fig. 1. (A,E) Histograms of total number of days reaching different dissolved oxygen (DO) minima; text provides number of days reaching 3 thresholds of hypoxia. (B–D & F–H) Amount of time spent below 2, 0.5, and 0.1 mg O_2 l^{-1} thresholds. Text provides average and range (in minutes) spent below each threshold. These values were calculated only from days in which the respective threshold was reached

Table 1. Respirometry metrics of hypoxia tolerance for *Tigriopus californicus* across all samples (n = 294). IQR: interquartile range

	Mean	IQR	Range
P_{crit} (kPa O_2)	3.425	4.082	0.097 to 12.796
Alpha (µmol O_2 mm^{-1} h^{-1} kPa^{-1})	5.20×10^{-3}	8.40×10^{-4}	2.00×10^{-4} to 0.585
Regulation index (−1 to +1)	0.614	0.138	−0.139 to 0.856

Table 2. Statistical comparisons of respiratory statistics among populations of *Tigriopus californicus*. The effect size estimate (β) is interpreted as the difference between the mean P_{crit}, alpha, or regulation index of the first group compared to the second group in the comparison column. SE: standard error for estimate; CL: confidence limits; *t*-ratio and p-value reported from post hoc Tukey's correction for multiple comparisons. **Bold** p-values and confidence limits indicate a statistically significant result. SD: San Diego; BR: Bird Rock; BOB: Boiler Bay; SH: Strawberry Hill

Comparison	Estimate (β)	SE	Lower CL	Upper CL	*t*	p
P						
SD–BR	−1.337	0.440	**−2.473**	**−0.200**	−3.040	**0.014**
SD–BOB	−2.601	0.453	**−3.773**	**−1.429**	−5.737	**<0.0001**
SD–SH	−2.839	0.477	**−4.071**	**−1.607**	−5.954	**<0.0001**
BR–BOB	−1.264	0.487	**−2.522**	**−0.007**	−2.598	**0.048**
BR–SH	−1.502	0.509	**−2.817**	**−0.188**	−2.954	**0.018**
BOB–SH	−0.238	0.515	−1.568	1.092	−0.463	0.967
Alpha (µmol O_2 g^{-1} h^{-1} kPa^{-1})						
SD–BR	2.66×10^{-3}	5.58×10^{-3}	−0.012	0.017	0.477	0.964
SD–BOB	2.77×10^{-3}	5.78×10^{-3}	−0.012	0.018	0.478	0.964
SD–SH	−0.013	6.01×10^{-3}	−0.028	0.003	−2.114	0.151
BR–BOB	1.03×10^{-4}	6.17×10^{-3}	−0.016	0.016	0.017	1.000
BR–SH	−0.015	6.39×10^{-3}	−0.032	0.001	−2.405	0.078
BOB–SH	−0.015	6.47×10^{-3}	−0.032	0.001	−2.391	0.081
Regulation index (−1 to +1)						
SD–BR	0.028	0.020	−0.023	0.078	1.412	0.493
SD–BOB	−0.019	0.020	−0.072	0.033	−0.944	0.781
SD–SH	0.026	0.021	−0.029	0.081	1.240	0.602
BR–BOB	−0.047	0.022	−0.103	0.010	−2.146	0.141
BR–SH	−0.001	0.023	−0.060	0.057	−0.056	1.000
BOB–SH	0.046	0.023	−0.013	0.105	1.995	0.192

ulations), males and females also did not significantly differ after 6 h of anoxia exposure (Table S5, Fig. 5B). There were also no significant differences in survival between the sexes within each population after the 6 h anoxia exposure (Table S6, Fig. S9B).

After the 15 h exposure to anoxia, we observed more variation in survival among the populations. Overall, copepods from southern sites (SD and BR) showed consistently higher survival than northern copepods (Fig. 4C), although mixed-effects models estimated that this difference was statistically significant for BR and not for SD (Table 3). Females had a statistically significant and higher proportion of surviving individuals compared to male copepods overall (Table S5, Fig. 5C). Female BR copepods had the highest survival after 15 h of anoxia exposure, followed by females from the SD population (Table S6, Fig. S9C).

Even after a full 24 h of exposure to anoxia, all populations still had one or more replicates with non-zero survival proportion (Fig. 4D). The copepods from BR had the highest proportion of survivors after 24 h, and this population's average survival was significantly higher than that of the BOB and SH populations (Table 3, Fig. 4D). Between the sexes, females continued to show significantly higher survival proportion than males after the 24 h exposure (Table S5, Fig. 5D). When comparing all sex-by-population groups, female copepods from BR had survival proportion that was significantly higher than all groups except SD females (Table S6, Fig. S9D).

3.4. LOE and survival of *T. californicus* during long-term hypoxia

LOE was significantly predictive of survival among replicates over extended periods of hypoxia (Fig. 6). After just 24 h, a 0.1 increase in the proportion of individuals with LOE was predicted to result in a 0.09 (CL: 0.06–0.12) decrease in the proportion of surviving individuals (*z*: 6.46; $p < 0.0001$). After 48 h exposure, a 0.1 increase in LOE proportion resulted in a 0.11 (CL: 0.09–0.13) decrease in the proportion of surviving individuals (*z*: 10.84; $p < 0.0001$). After 72 h exposure to hypoxic water in vials, we found that for every 0.1 increase in the proportion of individuals with LOE, the proportion of surviving individuals decreased by 0.09 (CL: 0.07–0.11; *z*: 8.64; $p < 0.0001$).

Across all 3 populations exposed to 72 h of hypoxia, 585 out of 1218 copepods survived with the proportion of individuals with LOE often low in many replicates (Fig. 6). Despite an overall pattern reflecting an inverse relationship between LOE and survival, there were surprising cases of low mortality and minimal

LOE. For example, after 72 h of hypoxia, one group of copepods still had 0 individuals experiencing LOE, and another group with 60% of its individuals experiencing LOE still had a survival proportion of 100% (Fig. 6C).

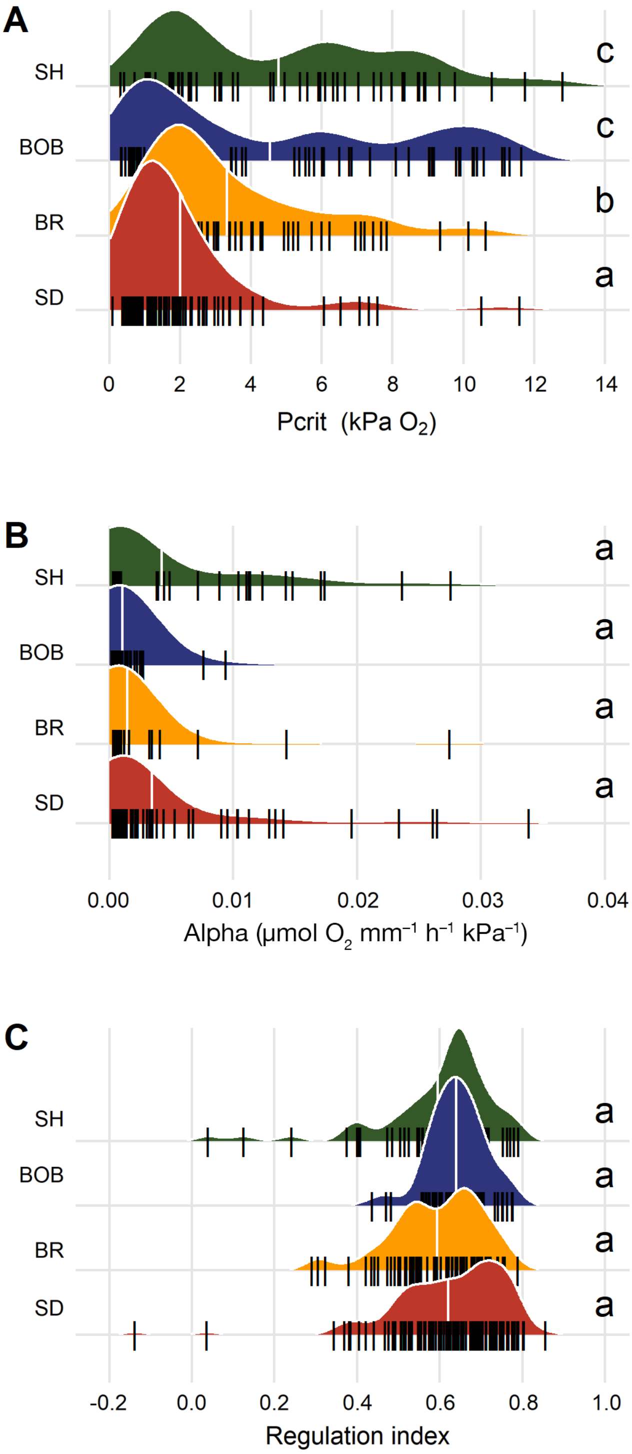

Fig. 3. Ridgeline plots showing the distribution of (A) P_{crit}, (B) alpha, and (C) regulation index values measured across *Tigriopus californicus* populations. Ridgeline plots indicate the density of values per group; vertical white line: mean per group. Black dashes: individual replicates of copepod siblings (n = 100 SD, 71 BR, 57 BOB, 56 SH). Lowercase letters reflect post-hoc pairwise comparisons with a significance cutoff of $\alpha = 0.05$ using a linear mixed-effects model with a random effect of 'sex' included. Site abbreviations as in Table 2

4. DISCUSSION

This study was motivated by the recent discovery that *Tigriopus californicus*, a widespread and abundant species of crustacean, appears tolerant to hypoxia despite having secondarily lost the genetic machinery that typically drives the response to hypoxia in animals (Graham & Barreto 2019). This evolutionary trajectory is now known to be shared at least among many copepod and barnacle taxa (Graham & Barreto 2020), indicating that molecular and physiological mechanisms of hypoxia tolerance not driven by the HIF pathway must exist. Our results indicated that *T. californicus* is highly tolerant of hypoxia, even though this species lost key components of the HIF pathway, and that this tolerance varied among genetically distinct populations and between sexes.

4.1. Strong selective pressure in splash pools

Intertidal zones provide an often volatile and dynamic environment in which oxygen fluctuations become the norm for any species capable of surviving in this habitat. In pools in contact with the tides, near-anoxic conditions are much less frequent, and hypoxic conditions do not last the entire night thanks to incoming tides (Huggett & Griffiths 1986, Richards 2011). This observation is consistent with the suggestion that oxygen availability is a major driver of the decrease in species distribution and diversity in pools of increased elevation (Huggett & Griffiths 1986). *T. californicus* can persist at high intertidal pools where no fish or other crustacean species are known to persist (Powlik et al. 1997, Powlik 1999). Moreover, the only other primary residents of *T. californicus*-inhabited pools are photosynthetic algae species that are known to contribute heavily to available oxygen in intertidal pools (Truchot & Duhamel-Jouve 1980, Powlik 1999).

Deconinck & Willett (2022) recently sampled DO in *T. californicus* pools across 6 sites along the West Coast of the USA, including San Diego, and found the lowest DO values of 1–2 mg O_2 l^{-1}, which is indeed stressful for most taxa. Their sampling, however, was restricted to a few daylight hours and was aimed at

Table 3. Statistical comparisons of *Tigriopus californicus* survival and loss of equilibrium (LOE) averaged by population. For LOE, the effect size estimate (β) is shown as the difference in the mean increase in LOE from one time point to the next between the 2 groups in the comparison column (i.e. a difference in rate of increase in LOE proportion). For survival, the effect size estimate is interpreted as the difference between the mean survival proportion of the first group compared to that of the second group in the comparison column. **Bold** p-values and confidence limits indicate a statistically significant result. Abbreviations as in Table 2

Comparison	Estimate (β)	SE	Lower CL	Upper CL	*t*	p
LOE over 6 h anoxia						
SD–BR	0.064	0.015	**0.027**	**0.102**	4.396	**<0.0001**
SD–BOB	−0.055	0.018	**−0.101**	**−0.010**	−3.148	**0.009**
SD–SH	−0.043	0.018	−0.088	0.003	−2.416	0.074
BR–BOB	−0.120	0.016	**−0.160**	**−0.079**	−7.553	**<0.0001**
BR–SH	−0.107	0.016	**−0.148**	**−0.066**	−6.714	**<0.0001**
BOB–SH	0.013	0.018	−0.034	0.059	0.699	0.898
Survival over 6 h anoxia						
SD–BR	−0.005	0.025	−0.069	0.060	−0.189	0.998
SD–BOB	0.117	0.045	**0.002**	**0.232**	2.605	**0.045**
SD–SH	0.034	0.032	−0.048	0.117	1.076	0.704
BR–BOB	0.122	0.044	**0.007**	**0.236**	2.736	**0.032**
BR–SH	0.039	0.031	−0.041	0.120	1.248	0.596
BOB–SH	−0.082	0.049	−0.207	0.042	−1.699	0.324
Survival over 15 h anoxia						
SD–BR	−0.396	0.097	**−0.644**	**−0.148**	−4.106	**2.36×10^{-4}**
SD–BOB	0.103	0.067	−0.068	0.274	1.541	0.413
SD–SH	0.095	0.067	−0.078	0.269	1.414	0.491
BR–BOB	0.499	0.086	**0.279**	**0.719**	5.815	**<0.0001**
BR–SH	0.492	0.087	**0.269**	**0.714**	5.675	**<0.0001**
BOB–SH	−0.007	0.052	−0.141	0.127	−0.139	0.999
Survival over 24 h anoxia						
SD–BR	−0.084	0.035	−0.174	0.006	−2.397	0.078
SD–BOB	0.010	0.021	−0.044	0.063	0.466	0.967
SD–SH	0.014	0.020	−0.037	0.066	0.717	0.890
BR–BOB	0.094	0.034	**0.006**	**0.182**	2.743	**0.031**
BR–SH	0.099	0.034	**0.012**	**0.186**	2.915	**0.019**
BOB–SH	0.005	0.018	−0.043	0.052	0.258	0.994

illustrating geographic differences instead of capturing DO minima. Our continuous oxygen sensor data indicated that hypoxic conditions reach more extreme levels well after sunset. In organisms that inhabit open waters, oxygen below 2.28 mg O_2 l^{-1} (6.50 kPa at 20°C) is the benchmark threshold at which hypoxia severely affects respiration and survival (Vaquer-Sunyer & Duarte 2008), although this threshold is often higher (Gray et al. 2002). In the pools inhabited by *T. californicus*, oxygen values regularly dipped far below this threshold on most summer nights for several hours at a time until sunrise (Figs. 1 & 2). In comparison, hypoxia-tolerant animals in lower intertidal pools, such as sculpins, become stressed when DO reaches 1.2 mg O_2 l^{-1} (Sloman et al. 2008), while fish in coral reef systems have adapted to hypoxic conditions routinely between ~1.47 and 2.21 mg O_2 l^{-1} (Nilsson & Östlund-Nilsson 2004). DO regimes experienced by the SH and BOB *T. californicus* during the summer months when microalgae are dense are comparable to oceanic oxygen minimum zones (OMZ), where persistent hypoxia levels are reported to reach as low as 0.13 mg O_2 l^{-1} (Childress & Seibel 1998, Seibel 2011). However, these data represent a limited sample size (n = 1 sensor per locale), and oxygen data from other pools and additional years will make it clearer whether these extreme hypoxic conditions are widespread and expected annually. Moreover, oxygen sensor data that explain whether conditions become less or more volatile in *T. californicus* pools during winter months is still lacking; previous work has shown that macroalgae grasses increase in density in *T. californicus* pools during winter months in populations from British Columbia, Canada (Powlik 1996). Those populations exhibit decreased clutch sizes, population sizes, and increased offspring development during winter months (Powlik 1996), but it is not clear whether this is the result of elevated hypoxia stress or changes in copepod nutrition due to altered algal species composition.

4.2. *T. californicus* is tolerant to extreme hypoxia

Our survival and LOE assays describe *T. californicus* as a highly hypoxia-tolerant animal, which is concordant with the intense selective pressure we measured with oxygen sensors in their natural habitat. Overall patterns across our data sets illustrated robust tolerance for *T. californicus* as a species, with remarkable instances of resilience: nearly one-quarter of *T. californicus* copepods exposed to 15 h of complete anoxia survived and, even after 24 h of anoxia, 33 of 480 copepods survived across all populations, with one group of copepods from BR having 50% survival (Fig. 4).

The general pattern in the 72 h hypoxia assays indicated that LOE and survival proportions were inversely related (Fig. 6). In hypoxia-tolerant species,

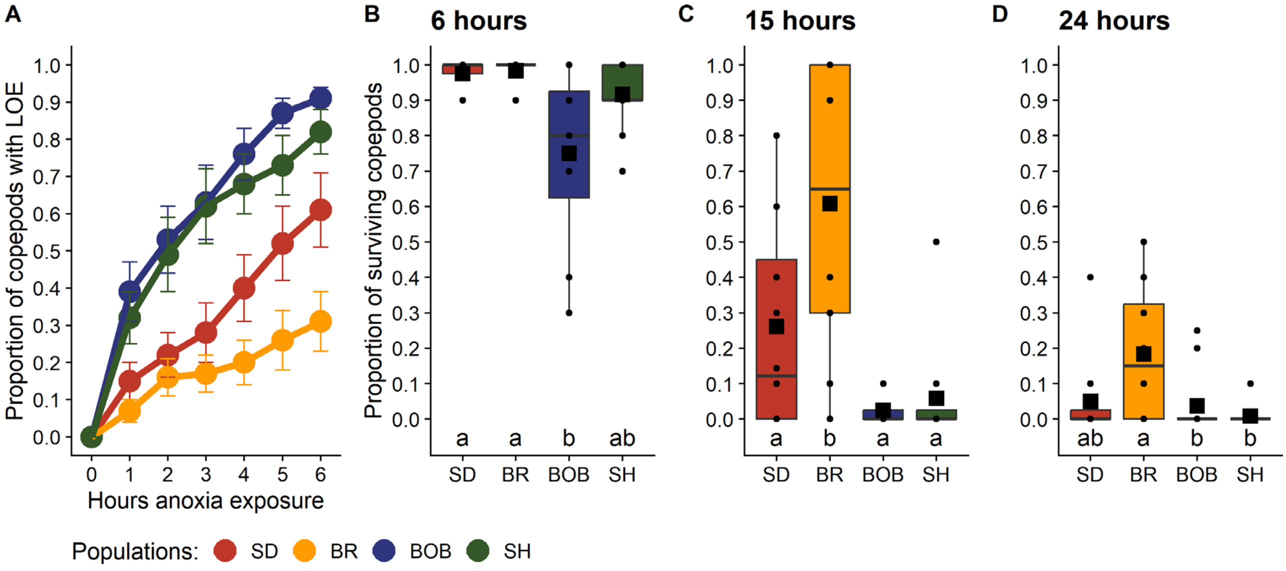

Fig. 4. Population variation in (A) loss of equilibrium (LOE) of *Tigriopus californicus* during 6 h anoxia exposure, and survival after exposure to (B) 6, (C) 15, and (D) 24 h anoxia. In (A), dots and error bars represent the means (±SE) of all 12 replicates of 10 copepods. In (B–D), boxplots indicate the median, lower quartile, and upper quartile; black squares are group means; whiskers represent 1.5× the interquartile range. Black dots are individual replicates of 10 copepods ($n = 12$ for each population). Lowercase letters reflect post-hoc pairwise comparisons with a significance cutoff of $\alpha = 0.05$ using a linear mixed-effects model with a random effect of sex included. Location abbreviations as in Table 2

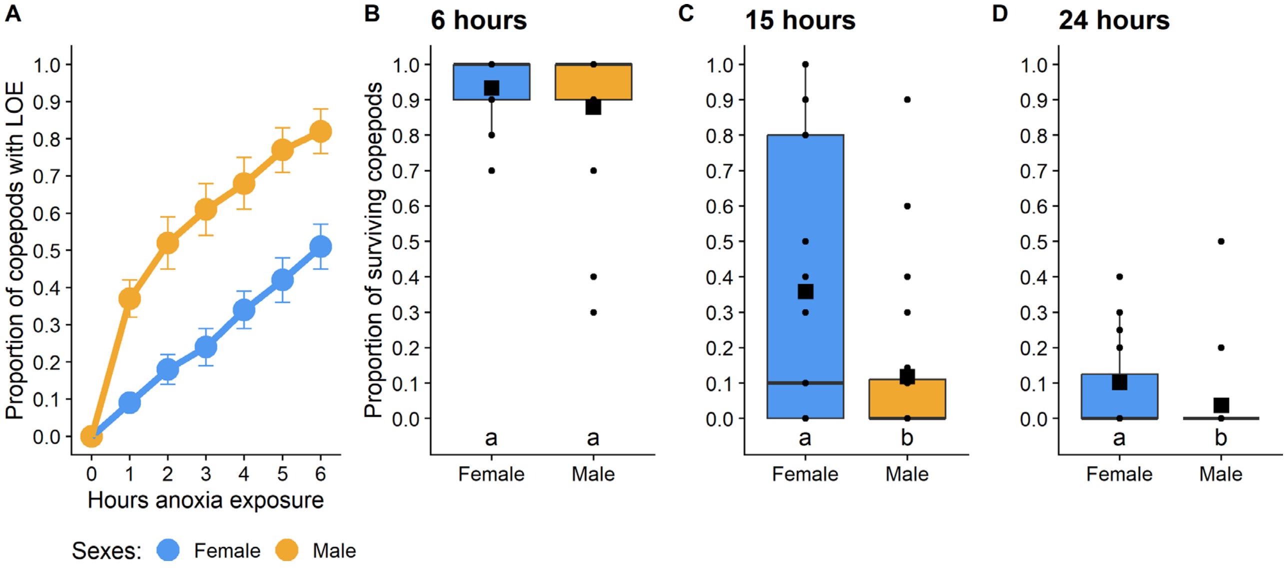

Fig. 5. Sex-specific variation in (A) loss of equilibrium of *Tigriopus californicus* during 6 h anoxia exposure, and survival after exposure to (B) 6, (C) 15, and (D) 24 h anoxia. In (A), dots and error bars represent the means (±SE) of all 24 replicates (10 copepods in each) per sex. See Fig. 4 for boxplot parameter definitions. Black dots are individual replicates of 10 copepods in each ($n = 24$ per sex). Lowercase letters reflect post hoc pairwise comparisons with a significance cutoff of $\alpha = 0.05$ using a linear mixed effects model with a random effect of population included

LOE may be a consequence of reaching the limit of oxygen uptake or it may be the end consequence of exhausting metabolic suppression (Boutilier & St-Pierre 2000). It is possible that *T. californicus* has not evolved metabolic suppression as a strategy for dealing with low environmental DO and instead may rely on maintaining aerobic activity for as long as possible. This is consistent with patterns of gene expression in SD copepods, which showed no evidence of metabolic suppression (Graham & Barreto 2019). In *Drosophila melanogaster*, which evolved to tolerate perpetual hypoxia, dozens of genes of the oxidative

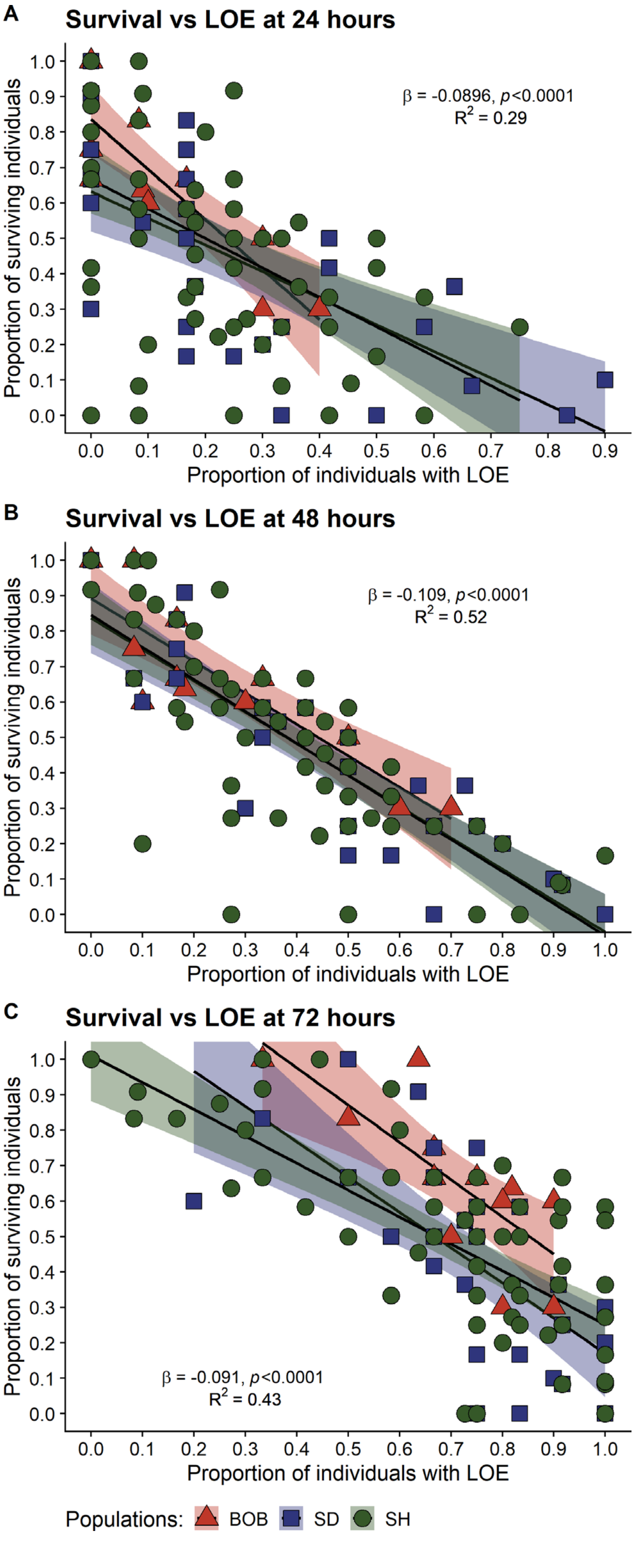

Fig. 6. Relationship between the ratio of copepods displaying loss of equilibrium (LOE) and the ratio of copepods that survived after recovering from (A) 24, (B) 48, and (C) 72 h exposure to hypoxia (0.15–0.30 mg l^{-1} O$_2$). Colored shading: 95% CI. Dots are individual replicates of 12 copepods (n = 12 BOB, 30 SD, and 66 SH). See Table 2 for location abbreviations

phosphorylation (Oxphos) and tricarboxylic acid (TCA) pathways were strongly down-regulated relative to flies in normoxia (Zhou et al. 2008). In contrast, in *T. californicus*, transcriptional depression did not occur in any gene of Oxphos or TCA, and 3 were actually up-regulated (Graham & Barreto 2019). If *T. californicus* is utilizing aerobic suppression, it may not employ this tactic until very late during oxygen depletion. The mechanism by which this copepod could suppress its oxygen consumption is still unclear. However, previous research examining the transcriptomic response of *T. californicus* to extreme hypoxia has implicated cuticle genes as candidates for further study (Graham & Barreto 2019), as regulation of cuticle composition may provide a mechanism to control oxygen flow across external tissues in other species (Tang et al. 2023).

Despite *T. californicus* demonstrating a high tolerance of hypoxia as a species, we observed marked differences among populations in fitness-level phenotypes. Populations from the southern range in California were robustly more tolerant than Oregon copepods in the north, both at resisting LOE and surviving long periods in anoxia. This trend contrasts with the patterns reported by Deconinck & Willett (2022), who found that populations from Northern California and Washington showed higher survival after 24 h hypoxia exposure than copepods from the SD population in Southern California. They did not, however, sample copepods from Oregon, so direct comparisons to our BOB and SH populations are not currently possible. Tolerance to high temperatures has been well studied in this system, and it has been shown repeatedly to be inversely associated with latitude, with SD and BR populations consistently being the most tolerant (Willett 2010, Kelly et al. 2012, Leong et al. 2018). This association is easily explained by the natural temperature gradient across latitudes. Without DO data from SD and BR pools, we cannot directly assess possible differences in selective pressures between northern and southern sites. However, we speculate that warmer regimes in SD and BR may promote increased algal densities in splash pools with already lower natural levels of DO due to elevated temperatures and hence more extreme and prolonged nighttime hypoxia relative to northern, cooler pools. In addition, we hypothesize that some evolution to more extreme hypoxia regimes may have occurred as a byproduct of increased thermal tolerance in warm latitudes, assuming the 2 stressors share some genetic and physiological mechanisms (Graham & Barreto 2019). Moreover, Graham & Barreto (2019) found candidate genes differentially expressed during hypoxia stress that are also involved with response to heat stress, including the temperature-synchronizing gene *pyx* and 4 heat shock protein chaperones: 3 from the *Hsp70* family and one small *Hsp*. We suspect that there may be overlap in other stress response pathways involved with both responses to heat and hypoxia, such as those related to unfolded protein response and mediation of oxidative damage.

We also report a strong sex-specific effect whereby females show higher survival and lower LOE than males, which agrees with findings from Deconinck & Willett (2022) in other populations. Female-biased stress tolerance has now been reported by several studies and occurs with regard to high temperature (Willett 2010, Kelly et al. 2012), high and low salinity (DeBiasse et al. 2018, Foley et al. 2019), and water pollutants (Foley et al. 2019). The mechanisms that explain the superior stress tolerance in female *T. californicus* may involve multiple possibilities. At the proximate level, Foley et al. (2019) found that females can maintain high proteolytic activity during increased heat and copper stress, while that mechanism was reduced in males. Proteasome function is an important pathway for the degradation of cellular proteins that are damaged by stress (DeMartino & Gillette 2007). Natural selection may favor different energetic investment across biological functions in each sex. While males of this species mate multiple times and in short succession, females only mate once yet must make a large energetic investment to sustain and supply nutrients to multiple clutches spread over their adult lives (Burton 1985, Powers et al. 2020). It is possible that such differences in timing and rates of reproductive investment between the sexes could explain differences in stress tolerance in mature copepods.

4.3. Comparison of *T. californicus* to other hypoxia-tolerant species

The application of statistical metrics of hypoxia tolerance revealed estimated P_{crit}, alpha, and RI values that predict *T. californicus* is highly capable of regulating its respiration at even very low levels of oxygen and offers some explanation for the high survival and low rates of LOE observed in our assays. Our estimates of P_{crit} in *T. californicus* copepods further suggest that this animal is highly capable of regulating its oxygen consumption down to very low levels of $P_{\mathrm{O_2}}$. Our species-wide average for P_{crit} in *T. californicus* was below that of many aquatic species reported to be tolerant of hypoxia (Ern et al. 2016, Wood 2018, Seibel et al. 2021). The SD population of *T. californicus* had the lowest average P_{crit} of the populations we tested, and this was lower than most of the taxa reviewed by Seibel et al. (2021). This population's average is outmatched only by the goldfish *Carrasius auratus* (Fu et al. 2011), the krill *Euphasia mucronata* (Seibel et al. 2021), the lophigastrid *Gnathophausia ingens* (Childress & Seibel 1998), and the copepod *Lucicutia hulsemannae* (Wishner et al. 2018). Notably, the latter 3 in this comparison inhabit the oceanic OMZ (Childress & Seibel 1998, Wishner et al. 2018). We note also that our estimated P_{crit} values broadly captured variation in survival and LOE between northern and southern populations, providing support that P_{crit} is useful for predicting fitness-level phenotypes of tolerance (Ultsch & Regan 2019, Seibel et al. 2021). Nevertheless, comparisons of P_{crit} across studies should be done with the disclaimer that this metric has been criticized for being dependent upon experimental methodology. For example, Wood (2018) raises concerns over the fact that P_{crit} estimation can be influenced by how authors filter their data points and by which mathematical method is used to calculate P_{crit} (i.e. traditional broken-stick, midpoint, or segmented). Therefore, ranking and comparing hypoxia tolerance across species based on this metric alone should be done with caution.

Species average RI estimates for *T. californicus* indicated that this species can regulate oxygen consumption over a broad range of oxygen values. The average RI for *T. californicus* is higher than that of the 9 zooplankton species reviewed by Tremblay et al. (2020), including species inhabiting regions of the North American coast exposed to hypoxic upwelling events. Additionally, *T. californicus* RI was higher than the average RI reported for 15 of 17 species of hypoxia-tolerant corals by Hughes et al. (2022). In the same study, Hughes et al. (2022) also report average RI values across 6 other hypoxia-tolerant taxonomic groups, including Class Actinopterygii (ray-finned fishes), Class Bivalvia, Subphylum Crustacea, Class Gastropoda, Class Insecta, and Class Polyplacophora (chitons). The average RI for *T. californicus* was higher than the averages reported for each of these other 6 groups (Hughes et al. 2022).

The average alpha values we estimated for *T. californicus* populations were lower than any of the species reviewed in the literature. This indicates that this copepod requires a very low level of oxygen to achieve a sufficient supply of oxygen for respiration within its tissues. Comparing the average alpha for *T. californicus* to other hypoxia-tolerant species indicates that this copepod requires a lower supply of oxygen to maintain respiration than species that inhabit oceanic depths greater than 1000 m, species that inhabit splash pools closer to the water line, and species that persist in OMZs. We speculate that this feat may be possible thanks to the process by which this animal diffuses oxygen across its tissues, as this means *T. californicus* does not require the use of a circulatory system, gills, or respiratory pigments to utilize oxygen (McAllen et al. 1999). The molecular, structural, and physiological mechanisms of these adaptations are still to be examined.

4.4. Conclusions

Compared to many other marine organisms, the splash-pool-dwelling copepod *T. californicus* appears to be highly resistant to even long-term exposure to hypoxic and even anoxic challenges. This is perhaps unexpected, given its lack of oxygen-sensing and oxygen-responding genes *EGLN* and *HIF*-α (Graham & Barreto 2019), which are so far the only known molecular drivers of hypoxia response in animals. For example, knockouts of *HIF*-α in zebrafish *Danio rerio* result in significant increases to P_{crit} and decreases in the time until the onset of LOE (Mandic et al. 2020). Indeed, across actinopterygian fish, *HIF*-α seems to be under positive selection for retention even after more than one round of gene duplication (Townley et al. 2022). Therefore, it is surprising that groups like *T. californicus* and several related crustaceans have apparently lost such a central gene in the hypoxia-response pathway. In addition to employing a novel genetic regulator of hypoxia response, we predict that *T. californicus* relies on a suite of morphological and physiological strategies still to be characterized, including structural modifications to its oxygen-diffusing cuticle and exoskeleton (Graham & Barreto 2019, Tang et al. 2023), altered metabolism of its plentiful stores of pigmentary antioxidants (Powers et al. 2022), and/or reliance on its mitochondrial alternative oxidase function to prolong stable respiration (Weaver & McDonald 2023). Whether mechanisms such as these could explain the apparent tolerance of *T. californicus* to hypoxia, the question remains as to how this organism initiates the molecular cascades needed to trigger these mechanisms without the use of HIF-α. Given that this problem also applies to any other organism lacking *HIF*-α, research into the genetic mechanisms that initiate the apparent tolerance of hypoxia by *T. californicus* may indeed be critical to reveal novel molecular responses common to other groups with modified hypoxia pathways.

Data availability. The data presented in this manuscript and the R scripts used to analyze the data can be found freely online in the GitHub repository at https://github.com/mjp0044/Intertidal-copepod-Tigriopus-californicus-displays-multilevel-variation-in-tolerance-to-extended-bou.

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