



# Effects of hypoxia and acidification on *Calanus pacificus*: behavioral changes in response to stressful environments

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**ABSTRACT:** Copepods, which play major roles in marine food webs and biogeochemical cycling, frequently undergo diel vertical migration (DVM), swimming downwards during the day to avoid visual predation and upwards at night to feed. Natural water columns that are stratified with chemical stressors at depth, such as hypoxia and acidification, are increasing with climate change. Understanding behavioral responses of copepods to these stresses—in particular, whether copepods alter their natural migration—is important to anticipating impacts of climate change on marine ecosystems. We conducted laboratory experiments using stratified water columns to measure the effects of bottom water hypoxia and pH on mortality, distribution, and swimming behaviors of the calanoid copepod *Calanus pacificus*. When exposed to hypoxic ( $0.65 \text{ mg O}_2 \text{ l}^{-1}$ ) bottom waters, the height of *C. pacificus* from the bottom increased 20 % within hypoxic columns, and swimming speed decreased 46 % at the bottom of hypoxic columns and increased 12 % above hypoxic waters. When exposed to low pH (7.48) bottom waters, swimming speeds decreased by 8 and 9 % at the base of the tanks and above acidic waters, respectively. Additionally, we found a 118 % increase in ‘moribund’ (immobile on the bottom) copepods when exposed to hypoxic, but not acidic, bottom waters. Some swimming statistics differed between copepods collected from sites with versus without historical hypoxia and acidity. Observed responses suggest potential mechanisms underlying *in situ* changes in copepod population distributions when exposed to chemical stressors at depth.

**KEY WORDS:** Hypoxia · Acidification · Swimming behavior · Copepod distributions · Sub-lethal stress

## 1. INTRODUCTION

Hypoxia (oxygen concentration  $< 2 \text{ mg l}^{-1}$ ) and acidification are 2 chemical stressors that are increasing in duration and extent in many coastal waters. Hypoxia and acidification can occur in productive coastal systems when excess organic matter sinks from the surface and is respired at depth, drawing down  $\text{O}_2$  and releasing  $\text{CO}_2$  (Doney 2010, Melzner et al. 2013, Isensee et al. 2016, Breitburg et al. 2018). Stratification of the water column due to differences in temperature and salinity limits mixing of oxygen-poor, more acidic bottom waters with surface waters, leading to stressful conditions that commonly occur at depth (Breitburg

et al. 2018). Hypoxia and acidification within deep coastal waters can also be caused by seasonal upwelling of cold, dense,  $\text{O}_2$ -poor, and  $\text{CO}_2$ -rich waters (Feely et al. 2010). Hypoxia and acidification have historically occurred in areas with high productivity and poor flushing, but can be exacerbated by anthropogenic-influenced processes such as development of coastal areas and increased nutrient runoff, as well as by increased atmospheric  $\text{CO}_2$  and warmer surface waters (Doney 2010).

Changes in bottom water chemistry are known to impact a range of marine species, including fish and zooplankton (Wu 2002). An important group of organisms impacted by changing bottom waters are

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copepods—crustaceous zooplankton that are ubiquitous in the world's oceans and which play major roles in marine food webs and biogeochemical cycling (Verity & Smetacek 1996). Hypoxia results in increased mortality rates for many species of copepods. Previous experiments have tested the mortality thresholds across a range of species, and while there is variation among species, many copepods have a steep mortality threshold of around  $1 \text{ mg O}_2 \text{ l}^{-1}$  (Auel & Verheye 2007, Grodzins et al. 2016). Adult copepods are thought to be fairly robust to moderate decreases in pH (Mayor et al. 2012, Weydmann et al. 2012). However, there is increasing evidence that mortality rates vary across life stages, with nauplii showing the largest lethal responses to decreased pH (Cripps et al. 2014). Adult copepods may exhibit sub-lethal responses to low pH, such as adverse effects on reproduction (Fitzer et al. 2012, Cripps et al. 2014).

Copepods exhibit a range of swimming behaviors to reposition themselves in the water column, feed, find mates, and avoid predation (Van Duren & Videler 1996). An important behavior for many species is diel vertical migration (DVM), in which animals most commonly swim downwards during the day into darker water to avoid visual predation and upwards at night to feed on phytoplankton near the surface (Frost 1988). The ability of copepods to perform DVM determines vertical population distributions and plays an important role in predator–prey interactions. However, in areas with stressful bottom waters, DVM can increase exposure to these stressful conditions, imposing a tradeoff between predation risk at the surface and adverse effects of chemical stress at depth.

*In situ* measurements of copepod distributions have shown that when bottom waters are stressful, the distributions of copepod populations often shift upwards. In Chesapeake Bay, the Gulf of Mexico, and the intermediate oxygen minimum layer in the northern Benguela Current upwelling region, copepod abundances are lower within hypoxic water, with distributions shifting upwards (Keister et al. 2000, Auel & Verheye 2007, Roman et al. 2012). Two potential mechanisms that could cause an upward shift in population distributions are increased mortality within stressful bottom waters, leading to an apparent upward shift, and behavioral avoidance of stressful layers, leading to upward movement of individual animals. The extent to which copepods modify their swimming behaviors to avoid stressful bottom waters is largely unknown.

Sub-lethal responses to chemical stress, such as behavioral avoidance, vary among stressors, species,

and populations. In one of the few laboratory studies quantifying behavior, the copepod *Calanus euxinus* spent more time swimming as oxygen concentrations declined from  $10$  to  $0.5 \text{ mg l}^{-1}$ , potentially to avoid sinking into anoxic bottom waters. (Svetlichny et al. 2000). In pH-stratified experimental water columns, larval sand dollars reversed the direction and shape of their swimming trajectory upon encountering the boundary between ambient and acidic water (Mabolo et al. 2020). Historical exposure to chemical stressors can also result in different behavioral responses between populations of the same species, through local adaptation or behavioral plasticity. Individuals from the copepod species *Acartia tonsa* avoided artificial hypoxic bottom waters when they were collected from an area that experiences hypoxia, but did not when they were collected from a non-hypoxic area (Decker et al. 2003). Behavioral avoidance has the potential to shift copepod distributions, but more work is needed to quantify its importance under changing chemical conditions.

The goals of this study were to assess the relative impacts of mortality versus behavioral avoidance in shaping vertical distributions of copepods when exposed to laboratory analogs of habitats with chemically stressful bottom waters. Puget Sound, a deep, glacially carved fjord connected to the Pacific Ocean by the Strait of Juan de Fuca, provides a useful region in which to measure effects of chemical stressors on copepods. The 4 basins of Puget Sound (Whidbey Basin, Main Basin, South Sound, and Hood Canal) vary in circulation patterns, deep water residence times, and flushing, resulting in different chemical conditions across the basins. Whidbey Basin, Main Basin, and South Sound typically do not experience episodes of hypoxia and acidification, whereas Hood Canal regularly experiences both. The duration and extent of hypoxia varies annually, but parts of southern Hood Canal can be hypoxic for 2–6 mo of the year (Newton et al. 2007). In the summertime, the deep waters of Hood Canal are also highly acidic, with pH values ranging from 7.3–7.8 and  $\text{pH} < 7.4$  reaching as shallow as 50 m (Feely et al. 2010).

We hypothesized that when exposed to stressful bottom waters, copepods would change their behavior in one or both of 2 ways: (1) avoid the stressful bottom waters or (2) descend into the stressful waters, and over time show increased mortality or increasing signs of sub-lethal stress such as changes in swimming speed. We hypothesized that copepods collected from areas that regularly experience hypoxia and acidification (such as Hood Canal) would experience lower rates of mortality and larger sub-

lethal stress responses. Without a practical means of observing copepod behaviors in the field, we tested these hypotheses using a laboratory study that aimed to replicate some aspects of naturally occurring behaviors. We chose the calanoid copepod *C. pacificus* as our study organism, because the species is ubiquitous in the Northeast Pacific and is an important food source for higher trophic levels due to its size and large lipid reserves. We designed laboratory experiments to quantify behavioral responses of *C. pacificus* collected from different basins of Puget Sound to bottom water hypoxia and acidification levels relevant to many coastal estuaries.

## 2. MATERIALS AND METHODS

### 2.1. Organism collection and handling

*Calanus pacificus* were collected from within Puget Sound, Washington, USA, between June and October of 2019 and 2020 (Table S1 in the Supplement at [www.int-res.com/articles/suppl/m697p015\\_supp.pdf](http://www.int-res.com/articles/suppl/m697p015_supp.pdf)). Collection sites (Fig. 1) were chosen for their differences in chemical histories.

Samples were collected using either a 60 cm diameter, 200  $\mu\text{m}$  mesh ring net with a non-filtering cod-end or a 60 cm diameter, 335  $\mu\text{m}$  mesh bongo net with non-filtering codends, lifted vertically from 10 m off the seafloor. Samples were stored in a cooler and air-

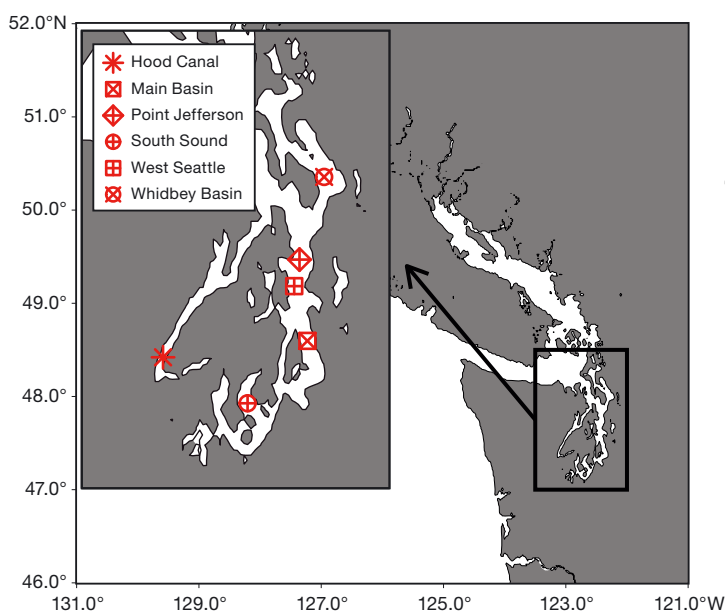


Fig. 1. Puget Sound, Washington, USA, and stations where *Calanus pacificus* were collected over the summers of 2019 and 2020

bubbled for <24 h until actively swimming adult female *C. pacificus* were manually sorted under a microscope into 1 l jars filled with 200  $\mu\text{m}$  filtered seawater. A total of 20 females were sorted into each jar; a single sex was used to exclude mate-seeking behaviors (Van Duren & Videler 1996). Copepods were kept at 14°C and fed a premade mixture of 5 marine microalgae (*Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira weissflogii* and *T. pseudonana*) daily, for no more than 2 wk until they were used in a single laboratory experiment. *C. pacificus* continued to produce fecal pellets and had visibly full guts throughout the culture period, indicating they were feeding.

### 2.2. Laboratory experiments and experimental design

*C. pacificus* behaviors and vertical distributions in response to either hypoxic or acidic bottom waters were observed in an array of 4 replicate 0.1  $\times$  0.1  $\times$  1 m acrylic tanks, installed in an environmental chamber set to 14°C (Fig. 2). Stressful water layers (or non-stressful controls) were placed at the bottoms of salinity-stratified tanks, modeled after conditions ex-

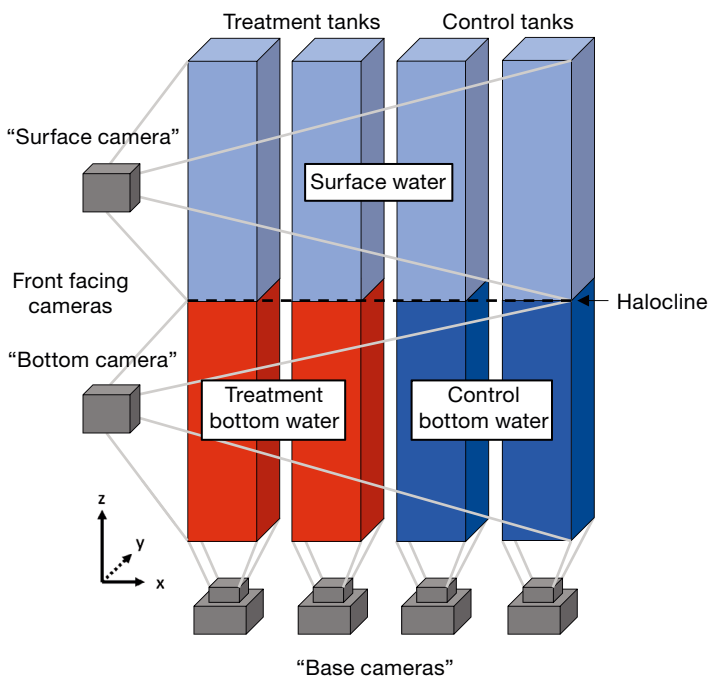


Fig. 2. Experimental schematic highlighting the 0.1  $\times$  0.1  $\times$  1 m 2-layer water columns, front-facing cameras (observing motion in X [left, right] and Z [up, down] directions), and base cameras (observing motion in X and Y [front, back] directions). Allocation of treatment and control tanks was randomized in every experiment. The same colors are used in later figures for continuity

perienced in the field. In preliminary daytime trial runs, we observed that *C. pacificus* consistently swam downwards when introduced to the top of experimental tanks, confirming that they continue behaviors consistent with natural DVM under these laboratory conditions. After being added to the top of the tanks, the first copepods reached the bottom in 2–5 min. Because we manipulated only one chemical characteristic at a time, acidification experiments were conducted after hypoxia experiments and therefore used *C. pacificus* collected later in the season.

Water treatments of 2 different salinities (29 and 31) were made using Instant Ocean (~36 and ~39 g l<sup>-1</sup>) and verified with a YSI Pro 2030 salinity probe. Replicate 2-layer water columns with stable haloclines were created by pumping light (low salinity) water into the bottom of the tanks using a peristaltic pump until the water level was 500 mm from the bottom. Then, heavy (high salinity) water was pumped slowly to avoid mixing into the bottom, displacing the light water upwards until the water columns were 780 mm deep with haloclines located 280 mm above the bottom.

In each set of experiments, 2 tanks were randomly selected to be treatment tanks and the other 2 were control tanks. In the 2 treatment tanks, the 'treatment bottom water' was heavy artificial seawater (ASW) bubbled either with N<sub>2</sub> (hypoxia experiments) or CO<sub>2</sub> (acidification experiments) (see Section 2.3). In the 2 control tanks, the 'control bottom water' was heavy, non-bubbled ASW to control for potential behavioral responses to the change in salinity across the halocline. The 'surface water' in all 4 tanks was light, non-bubbled ASW.

Before being added to the experimental array, 80 adult female *C. pacificus* were acclimated overnight in light ASW, identical to the surface water in the experimental tanks. To start each experiment, 20 animals were gently introduced to the top of each of the 4 tanks. Swimming behavior was then observed for 90 min using 5-megapixel IR USB cameras. Experiments were run during the day in the dark, with the tanks backlit with IR LED strips behind and around the base of each tank. Two front-facing cameras ('bottom camera', 'surface camera') recorded swimming in the X (left, right) and Z (up, down) directions, observing true vertical motion and projected horizontal motion (see Section 2.4). An upwards-facing 'base camera' was added to each tank in 2020 to improve tracking and behavioral analysis of copepods near the bottom. Base cameras recorded the bottom 2 cm of each tank in the X and Y (front, back) directions and therefore observed true horizontal motion but not vertical motion (Fig. 2).

## 2.3. Water chemistry

### 2.3.1. Hypoxia experiments

To create the different oxygen conditions, bottom water was split into 2 buckets of equal volume to use in treatment and control tanks. Treatment bottom water was then bubbled with pure N<sub>2</sub> gas until dissolved oxygen (DO) was <0.2 mg l<sup>-1</sup>, as measured by a Pre-Sens oxygen dipping probe. Surface water and control bottom water were not bubbled and were left at ambient oxygen concentrations (~10 mg l<sup>-1</sup>). At the end of each experiment, oxygen was measured at the surface, upper halocline, lower halocline, and bottom of each tank by lowering the oxygen probe slowly through the water column so as not to disrupt the halocline.

### 2.3.2. Acidification experiments

To manipulate pH conditions, bottom water was split into 2 buckets of equal volume to use in treatment and control tanks. Treatment bottom water was bubbled with CO<sub>2</sub> until it reached the desired pH. In the first 5 pH experiments, treatment bottom water was bubbled with a 2000 ppm CO<sub>2</sub>-air mixture for approximately 40 min. The final pH in this treatment was approximately 7.6, as measured by a Star A221 pH meter with a Ross ultra gel pH/ATC electrode that was calibrated daily prior to use. In the last 5 experiments, treatment bottom water was made using a feedback-control system that supplied mixed lab air and pure CO<sub>2</sub> gas at 3000 ppm at 4.1 l min<sup>-1</sup> through an airstone. The pH was measured with a Sunburst AFT (assumed constant salinity of 31) during bubbling to ensure a pH of 7.4 was maintained. The acidification method was altered halfway through experiments because 2000 ppm CO<sub>2</sub>-air mixture refills became unavailable in 2020 due to supply chain shortages. At that time, the CO<sub>2</sub> concentration was also altered to more closely match conditions experienced in conjunction with hypoxia *in situ*. Control bottom water and surface water were not bubbled and were left at ambient CO<sub>2</sub> concentrations.

At the end of each experiment, pH was measured above the halocline by dipping the pH probe into the top of each tank to measure the pH of the light ASW. The pH of the bottom water was measured by filling scintillation vials from ports at the bottom of each tank and measuring the pH of the water sample with the pH probe. Additionally, bottle samples were collected for laboratory analysis, first from the top port of one randomly selected control tank and one ran-

domly selected treatment tank, then from the bottom port of all 4 tanks. Bottle samples were poisoned with mercuric chloride and stored for less than 6 mo before analysis. Dissolved inorganic carbon (DIC) and alkalinity were measured from the bottle samples using a DIC VINDTA system with an electrochemical cell UIC Coulometer for DIC as well as a Dickson Total Alkalinity Titrator for alkalinity. Samples from all experimental trials were run in random order over the course of 5 d. An internal standard was run at the beginning and end of each day to check for accuracy and drift throughout the day. A replicate DIC measurement was taken for every other sample. Only one alkalinity measurement was taken for each sample. Three samples of laboratory seawater were analyzed each day to check instrument precision.

#### 2.4. Video processing and movement analysis

Videos were processed with the software Fosica (Wallingford Imaging) to distinguish moving copepods from stationary background and noise and to extract copepod pixel coordinates. Pixel coordinates were converted into physical space units and then assembled into individual swimming paths using the Matlab software package Tracker3D (Chan & Grünbaum 2010), neglecting parallax in the camera field of view (an example output is provided in Fig. S1). A smoothing spline was applied to remove features changing faster than 6 Hz, which were dominated by frame rate noise.  $X$  and  $Z$  (or, for the base cameras,  $X$  and  $Y$ ) pixel coordinates for each object and the total projected speed and velocities were calculated at every frame for each swimming path. Swimming paths included only animals actively moving in the tank, excluding motionless (moribund) animals at the bottom of the tank.

Copepod swimming paths were used to calculate the mean height from the bottom of the tank, mean number of copepod localizations per frame, and mean swimming speeds. The surface camera and bottom camera recorded velocity in the  $X$  and  $Z$  directions and therefore observed true vertical speed ( $VS_{tr}$ ), but only projected horizontal speed ( $HS_{proj}$ ); i.e. horizontal movement in the  $X$ - $Z$  plane but not the  $X$ - $Y$  plane. We assumed anisotropic swimming (no preferred direction) in the horizontal direction, implying true horizontal speeds ( $HS_{tr}$ ) were on average proportional to  $HS_{proj}$ . We calculated the constant of proportionality using Eq. (1), where  $HS_{proj}$  is measured and  $HS_{tr}$  is inferred from the equation:

$$HS_{proj} = HS_{tr} \times (1/2\pi) \times \int_0^{2\pi} |\cos(x)| dx = HS_{tr} \times (2/\pi) \quad (1)$$

Using the mean  $HS_{tr}$  and the measured mean  $VS_{tr}$ , we estimated the mean total speed ( $TS$ ) using Eq. (2):

$$TS = \sqrt{(HS_{tr})^2 + (VS_{tr})^2} \quad (2)$$

Raw vertical and projected horizontal speeds are provided in Figs. S2 & S3.

Base cameras recorded speed in the  $X$  and  $Y$  directions, observing  $HS_{tr}$  near the bottom of the tank. To ensure that copepod pixel coordinates were not double counted in both the bottom and base cameras, we determined the height above the bottom in the bottom camera ( $Z$  direction) at which copepods came into view in the base cameras, which was 26 mm. Any copepod coordinates in the bottom camera below 26 mm were dropped from the analysis so that those points were only stitched into swimming paths in the base camera analysis. The total copepod counts from the surface, bottom, and base cameras were calculated for a subset of frames and experiments to ensure that we were able to continually track individuals in each tank throughout the experiment.

Our video system could not distinguish between individuals that were dead and those that were lying immobilized on the bottom for extended periods (a behavior leading, at least in hypoxia, to a high likelihood of eventual mortality). Therefore, for 2020 experiments, we developed a video-based metric using the base cameras to classify copepods at the bottom of tanks that were 'moribund.' Remaining motionless on the bottom of the tank is an uncommon behavior for *C. pacificus*, and we conservatively estimated that copepods motionless for a 2 min threshold were in a 'moribund' or stressed state. To quantify moribundity, we calculated the mean brightness values for each pixel from frames in each of the last 1 min sections (89<sup>th</sup> and 90<sup>th</sup> minutes). Because the videos were recorded in a dark field with IR back-lighting, copepods appeared as bright spots in the videos. The brightness of pixels representing a copepod in these 1 min means was a direct function of the number of frames in which it remained stationary. We then used a brightness threshold to classify copepods as moribund if pixel brightness indicated they had not moved during the last 2 min of video observations.

In both 2019 and 2020 hypoxia experiments, the positions of living copepods were visually counted above the halocline, below the halocline but above the base, and at the base of each tank at the end of the 90 min runs. A visual count of the number of



moribund animals on the bottom of each tank was made. No visual counts were done for the acidification experiments because we assessed the video metric to be as good as or better than visual counts during the hypoxia experiments.

## 2.5. Statistics

Metrics from the processed videos were analyzed to test hypotheses about distribution changes and behavioral responses of *C. pacificus* when exposed to stressful bottom waters. From preliminary analysis, it was determined that copepod vertical position and behaviors approached a steady state within 30 min of being added to the experimental tanks. Therefore, to optimize processing time, only the first 30 min of video were analyzed. In 10 of the 23 experiments conducted in 2020, one of the 4 base cameras malfunctioned (for an equal number of experimental and control tanks across the 10 runs). In statistical analyses that required base camera video, those individual tanks were dropped. A single batch of zooplankton collected from South Sound in 2020, used in 3 hypoxia experiments, behaved differently from all other experimental replicates: this one batch of copepods did not swim downwards when added to the top of the experimental tanks, did not interact with the sub-halocline hypoxic water, and therefore did not test our set of hypotheses. Those 3 experiments were dropped from all further analysis. The 2 bubbling methods used for making acidified water (premixed CO<sub>2</sub>/air and a feedback control system) differed in their final pH value. However, results from the 2 sets of experiments were not significantly different from each other so they were pooled for all analyses. There was only one pH experiment using copepods collected from South Sound, so for pH experiments only, South Sound was dropped as a collection site in post hoc statistical analysis due to the insufficient replication for that site. All statistics were generated using the software R (version 4.2.0) (R Core Team 2022).

### 2.5.1. Moribund counts

To test for treatment effects on the video-based moribund counts from 2020, a 2-way ANOVA was performed to compare the effect of treatment, collection site, and a treatment  $\times$  collection site interaction. Visual inspection of residual plots, Shapiro-Wilk, and Levene's test did not reveal any deviations from normality or homoscedasticity. Visual counts of mori-

bund copepods from 2019 and 2020 were pooled. Visual counts did not meet the assumptions of normality or homoscedasticity, so a negative binomial regression (NB reg) was used to test for treatment and collection site effects using the R package 'MASS' (version 7.3-57) (Venables & Ripley 2002).

### 2.5.2. Mean height

The mean height above the bottom of copepod swimming tracks was calculated over 5 min intervals for the first 30 min of each experiment. Copepods observed by the base camera during this time interval were included in the mean height metric by assigning counts from the base camera a height of 0 mm. To test for treatment effects on the mean height, mixed-effects models were generated using the R package 'lme4' (version 1.1-29) (Bates et al. 2015). Nested models were generated using different combinations of treatment, experimental time (linear and quadratic term), and copepod collection site as main effects. Experiment was included as a random effect to account for differences between copepod collections and batches. The best model was identified using Akaike's information criterion (AIC) values (see Tables S2 & S3). A visual inspection of the Q-Q plots of model residuals and model residuals against the fitted values plots did not reveal any deviations from normality or homoscedasticity.

### 2.5.3. Swimming speeds

Calculated mean *TS*s observed by the surface and bottom cameras in 2019 and 2020 were pooled for analysis. *HS*<sub>tr</sub> at the bottom of the tanks was observed by the base cameras only in 2020. Mixed-effects models were generated using the 'lme4' package in R. Nested models were generated using different combinations of treatment, experimental time (linear and quadratic term), and copepod collection site as main effects and experiment as a random effect. The best models were identified using AIC values (see Tables S4–S9). A visual inspection of residual plots did not reveal any deviations from normality or homoscedasticity.

### 2.5.4. Differences among collection sites

Additional post hoc analyses were used to evaluate the effect of collection site on *TS*. A Tukey

pairwise comparison of treatment and collection site was generated using the R package ‘emmeans’ (version 1.7.3) (Lenth 2021). To visualize the effect of treatment among different collection sites, the mean *TS* from control tanks was subtracted from the mean *TS* from the treatment tanks, and a pooled standard error was calculated for each collection site, time interval, and camera view combination.

### 3. RESULTS

#### 3.1. Water chemistry

In the 20 hypoxia experiments, mean ( $\pm$ SE) DO concentration was  $0.65 \pm 0.03$  mg l<sup>-1</sup> in the treatment bottom water compared to  $10.2 \pm 0.04$  mg l<sup>-1</sup> in the control bottom water (Table 1).

In the 10 acidification experiments, treatment bottom water made using premixed CO<sub>2</sub>-air had a mean ( $\pm$ SE) pH of  $7.58 \pm 0.01$ , and treatment bottom water made using a feedback control system had a mean pH of  $7.39 \pm 0.01$ . Control bottom water had a mean pH of  $8.06 \pm 0.02$  and  $8.03 \pm 0.03$  during experiments using the premixed CO<sub>2</sub>-air and feedback control system methods, respectively (Table 2).

Table 1. Mean ( $\pm$ SE) realized conditions for *Calanus pacificus* during the 2019 and 2020 hypoxia experiments. DO: dissolved oxygen

Treatment	n	Temp (°C)	Salinity (ppt)	Density ( $\sigma_t$ )	DO (mg l <sup>-1</sup> )	Percent saturation
Surface—control	40	14	29.0	21.6	$10.1 \pm 0.05$	$117 \pm 0.6$
Surface—treatment	40	14	29.0	21.6	$10.0 \pm 0.05$	$116 \pm 0.6$
Bottom—control	40	14	31.0	23.1	$10.2 \pm 0.04$	$119 \pm 0.5$
Bottom—treatment	40	14	31.0	23.1	$0.65 \pm 0.03$	$7.7 \pm 0.9$

#### 3.2. Moribundity

The number of moribund animals was significantly higher in hypoxic tanks than in control tanks after 90 min, using both visual (NB reg;  $p = 0.001$ ) (Fig. 3a) and video (ANOVA;  $F = 10.4$ ,  $p = 0.003$ ) (Fig. 3b) metrics. A 118% increase in moribund individuals was observed using the video-based metric.

The number of moribund animals was not significantly different between acidic tanks and controls using the video-based metric (ANOVA; treatment:  $F = 0.088$ ,  $p = 0.77$ ; site:  $F = 0.703$ ,  $p = 0.5$ ). At the end of each experiment, a mean ( $\pm$ SE) of  $3.4 \pm 0.56$  and  $3.2 \pm 0.39$  moribund animals were counted in acidic and control tanks, respectively (Fig. 3c).

#### 3.3. Mean height

Swimming animals were significantly higher in the water column in hypoxic tanks compared to control tanks (Fig. 4a). Mixed effects model results indicated that treatment and time had significant effects on mean height (treatment:  $p < 0.0001$ ; time:  $p < 0.0001$ ; time<sup>2</sup>:  $p < 0.0001$ ). Over the full 30 min, the mean ( $\pm$ SE) height of animals in hypoxic tanks was  $36 \pm 8.7$  mm higher in the water column than animals in control tanks—a 20% increase. In the last 5 min only (25–30 min), animals in hypoxic tanks were  $59 \pm 39.9$  mm higher in the water column than animals in control tanks—a 25% increase. The mean position of copepods in hypoxic tanks during the last 5 min was only 10 mm below the estimated halocline (280 mm), whereas copepods in control tanks were 70 mm below. Including exper-

Table 2. Mean ( $\pm$ SE) realized conditions for *Calanus pacificus* during pH experiments for the 2 different bubbling methods. Pre-mix: premixed CO<sub>2</sub>-air; Feedback: feedback control system; DIC: dissolved inorganic carbon

Bubbling method	Treatment	n	Temp (°C)	Salinity (ppt)	Alkalinity ( $\mu$ mol kg <sup>-1</sup> )	DIC ( $\mu$ mol kg <sup>-1</sup> )	pCO <sub>2</sub> ( $\mu$ atm)	pH (total scale)
Pre-mix	Surface—control	5	14	29.0	$3081 \pm 24.7$	$2777 \pm 33.0$	$386 \pm 24.5$	$8.18 \pm 0.02$
	Surface—treatment	5	14	29.0	$3008 \pm 29.9$	$2703 \pm 34.1$	$367 \pm 22.2$	$8.19 \pm 0.02$
	Bottom—control	10	14	31.0	$3085 \pm 40.1$	$2840 \pm 26.5$	$537 \pm 29.7$	$8.06 \pm 0.02$
	Bottom—treatment	10	14	31.0	$3266 \pm 20.7$	$3239 \pm 19.8$	$1889 \pm 38.4$	$7.58 \pm 0.01$
Feedback	Surface—control	5	14	29.0	$3035 \pm 40.0$	$2735 \pm 42.1$	$378 \pm 15.9$	$8.18 \pm 0.01$
	Surface—treatment	5	14	29.0	$2985 \pm 36.4$	$2684 \pm 37.8$	$365 \pm 14.8$	$8.19 \pm 0.01$
	Bottom—control	10	14	31.0	$3035 \pm 53.7$	$2735 \pm 36.6$	$559 \pm 40.2$	$8.03 \pm 0.03$
	Bottom—treatment	10	14	31.0	$3236 \pm 28.6$	$3284 \pm 27.8$	$2936 \pm 42.9$	$7.39 \pm 0.01$

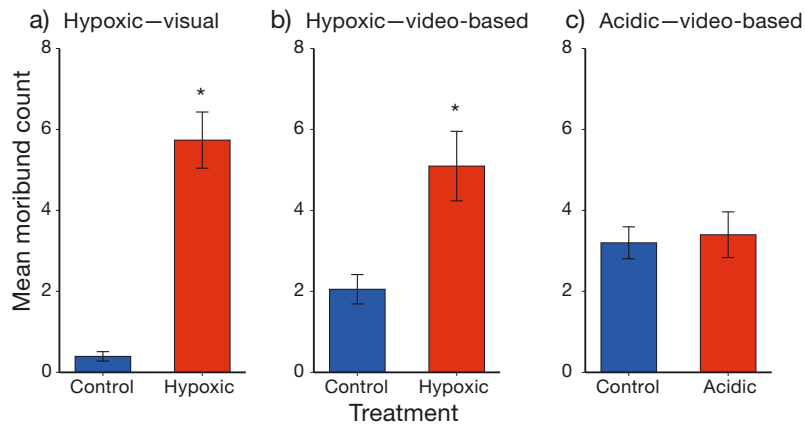


Fig. 3. Mean ( $\pm$ SE) number of 'moribund' (immobilized or dead) copepods in control and treatment tanks after 90 min (out of 20 copepods). (a) Visual moribundity metric at the end of all 2019 and 2020 hypoxia experiments. \*Significant differences between treatments ( $p < 0.0001$ ). (b) Video-based moribundity metric from 2020 hypoxia experiments. \*Significant difference between treatments ( $p = 0.003$ ). (c) Video-based moribundity metric from 2020 acidification experiments. There was no significant difference between treatments

iment as a random effect explained significant variability in the model.

There was no difference between the mean height of copepods in acidic versus control tanks. The mean height of copepods in each tank decreased with time (time:  $p < 0.0001$ ; time<sup>2</sup>:  $p < 0.0001$ ). During the last 5 min (25–30 min), the mean height of copepods was  $84.6 \pm 11.1$  mm in control tanks and  $93.0 \pm 14.7$  mm in acidic tanks, both well below the halocline height (Fig. 4b).

### 3.4. Swimming speed

Across all hypoxia experiments in 2019 and 2020, there was a general trend that animals exposed to hypoxic bottom waters swam slower in bottom and base waters but faster in surface waters than animals in control tanks (Fig. 5). Over the first 30 min of video, the mean  $HS_{tr}$  from copepod swimming paths at the base of the tanks (observed by base cameras during the 2020 experiments) varied with time and treatment (time:  $p < 0.0001$ ; treatment:  $p < 0.0001$ ; time<sup>2</sup>:  $p = 0.0004$ ). At the final time point, the mean  $HS_{tr}$  was  $0.04 \pm 0.007$  mm s<sup>-1</sup> slower at the base of hypoxic tanks—a 58% decrease relative to copepods at the base of control tanks (Fig. 5a). Within the bottom waters (observed by the bottom camera

and not including the base of the tanks) in 2019 and 2020, there was an 8% decrease in the mean  $TS$  from the first 30 min of video recorded in hypoxic tanks relative to controls, with a significant collection site effect (Fig. 5b). Within the surface waters (observed by the surface camera), mean  $TS$  increased gradually with time ( $p = 0.002$ ), and there was a significant interaction between treatment and collection site ( $p = 0.0002$ ) (Fig. 5c). Copepods in the surface water of hypoxic tanks showed a 12% increase in swimming

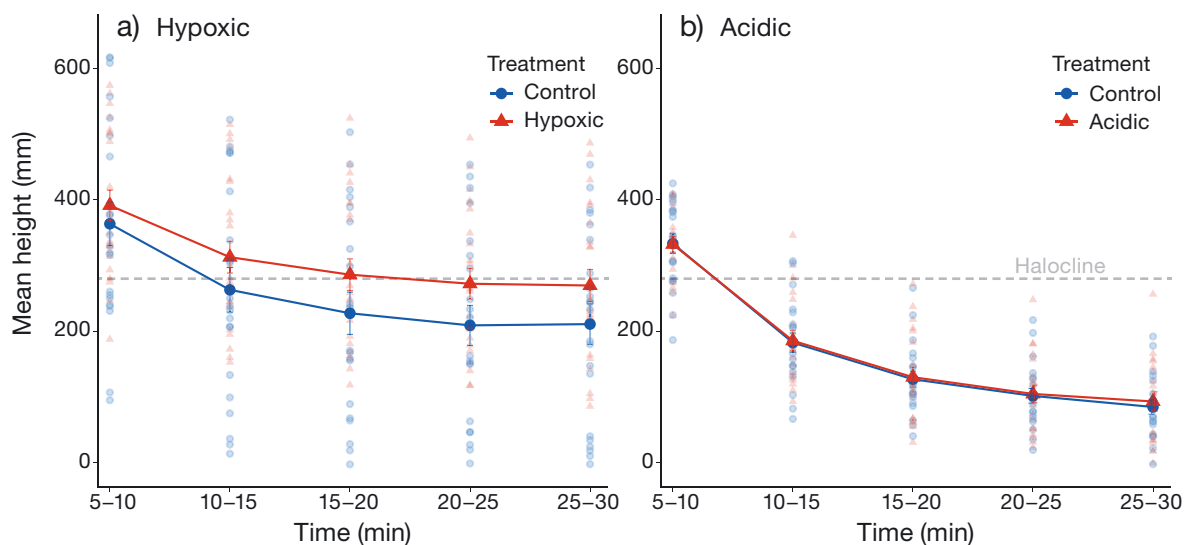


Fig. 4. Mean ( $\pm$ SE) copepod height above bottom over 5 min intervals from 2020 (a) hypoxia and (b) acidification experiments. Blue line: mean height for control tanks; red line: treatment tanks; grey dashed line: location of the halocline. The hypoxic treatment and control were significantly different from each other ( $p < 0.0001$ ); the acidic treatment and control were not significantly different



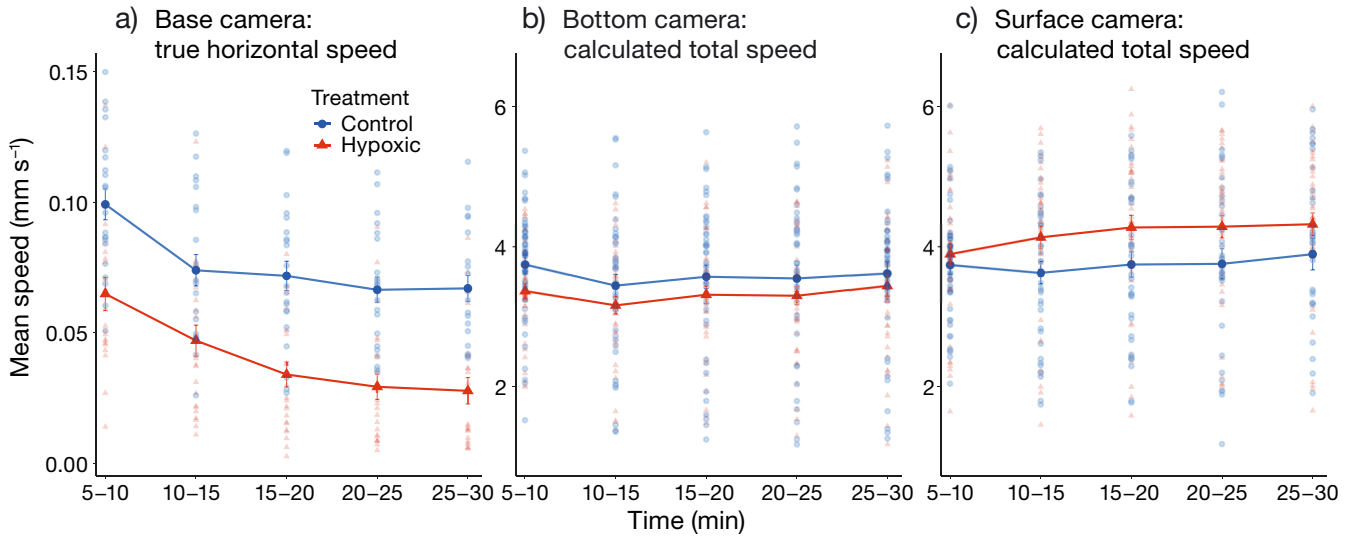


Fig. 5. Mean ( $\pm$ SE) copepod swimming speeds from different camera views during the hypoxia experiments. (a) True horizontal speed observed by the base camera during the 2020 hypoxia experiments. Treatment and control tanks differed significantly ( $p < 0.0001$ ). (b) Calculated total speed observed by the front-facing bottom camera (below the halocline and above the base) and (c) surface camera (above the halocline) during 2019 and 2020 hypoxia experiments. Swimming speeds significantly differed in the surface camera, and there was a significant treatment  $\times$  collection site interaction in both the bottom and surface cameras. Blue lines: mean speed in control tanks; red lines: mean speed in treatment tanks. Note the difference of scale in (a)

speed, moving  $0.60 \pm 0.10 \text{ mm s}^{-1}$  faster than copepods in control tanks ( $p < 0.0001$ ). The copepod counts per frame for each camera view can be found in Fig. S4, which approximates the number of swimming paths included in the above speed statistics.

Overall, copepods exposed to acidic bottom waters swam slower in surface waters and at the base of tanks compared to copepods in control tanks, with

some significant differences in the swimming speeds of copepods from different collection sites (Fig. 6). At the base of the tanks, the mean  $HS_{tr}$  was  $0.006 \pm 0.002 \text{ mm s}^{-1}$  slower in acidic tanks than in control tanks—an 8% decrease, with speeds decreasing over time (time:  $p < 0.0001$ ; treatment:  $p = 0.007$ ) (Fig. 6a). A majority of the total copepod counts were observed by the base cameras (see Fig. S5). Within

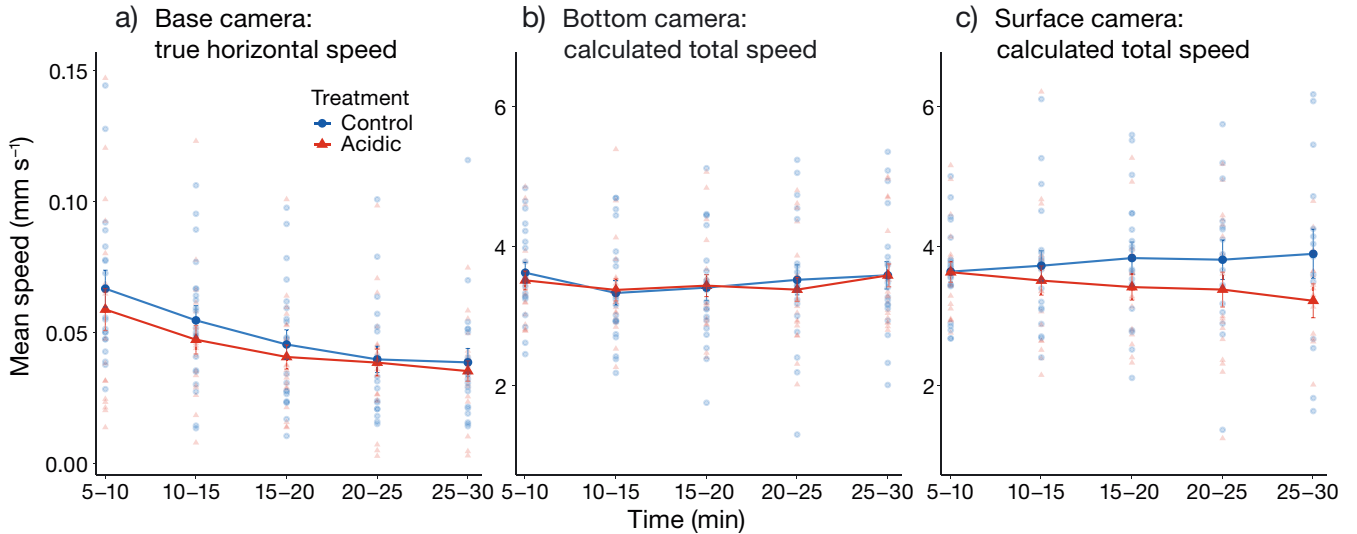


Fig. 6. Mean ( $\pm$ SE) copepod swimming speeds from different camera views during the acidification experiments. (a) True horizontal speed observed by the base cameras. Treatment and control tanks differed significantly ( $p = 0.007$ ). (b) Calculated total speed from front-facing bottom camera (below the halocline and above the base) and (c) surface camera (above the halocline) during pH experiments. There was a significant treatment  $\times$  collection site interaction for the surface camera. Blue lines: mean speed in control tanks; red lines: mean speed in treatment tanks. Note the difference of scale in (a)

the bottom water (but above the base), *TS* varied with time, with no significant difference between treatments (time:  $p = 0.003$ ; time<sup>2</sup>:  $p = 0.002$ ) (Fig. 6b). Within surface waters, *TS* significantly varied with treatment (but not time), with a significant treatment by collection site interaction (treatment:  $p = 0.0003$ ; treatment  $\times$  site:  $p = 0.02$ ) (Fig. 6c).

### 3.5. Differences among collection sites

Changes in swimming speed in both bottom and surface waters in response to hypoxia, and in surface waters in response to acidification, varied depending on collection site (Fig. 7). In hypoxia experiments, mean *TS* within the bottom water was significantly

slower in hypoxic tanks relative to controls for copepods collected from the Main Basin ( $p = 0.008$ ) and South Sound ( $p = 0.004$ ) but not from Hood Canal (Fig. 7a). Mean *TS* within surface waters was significantly faster in hypoxic tanks relative to controls for copepods from Hood Canal ( $p < 0.0001$ ) and to a lesser extent South Sound ( $p = 0.054$ ) but not Main Basin (Fig. 7b).

In acidification experiments, there were no differences in swimming speeds among copepods from different collection sites within the bottom waters (Fig. 7c). However, in surface waters, the mean speed of copepods from Hood Canal was  $0.51 \pm 0.14 \text{ mm s}^{-1}$  slower in acidic tanks than in control tanks ( $p = 0.002$ ), but copepods collected from Main Basin did not exhibit differences (Fig. 7d).

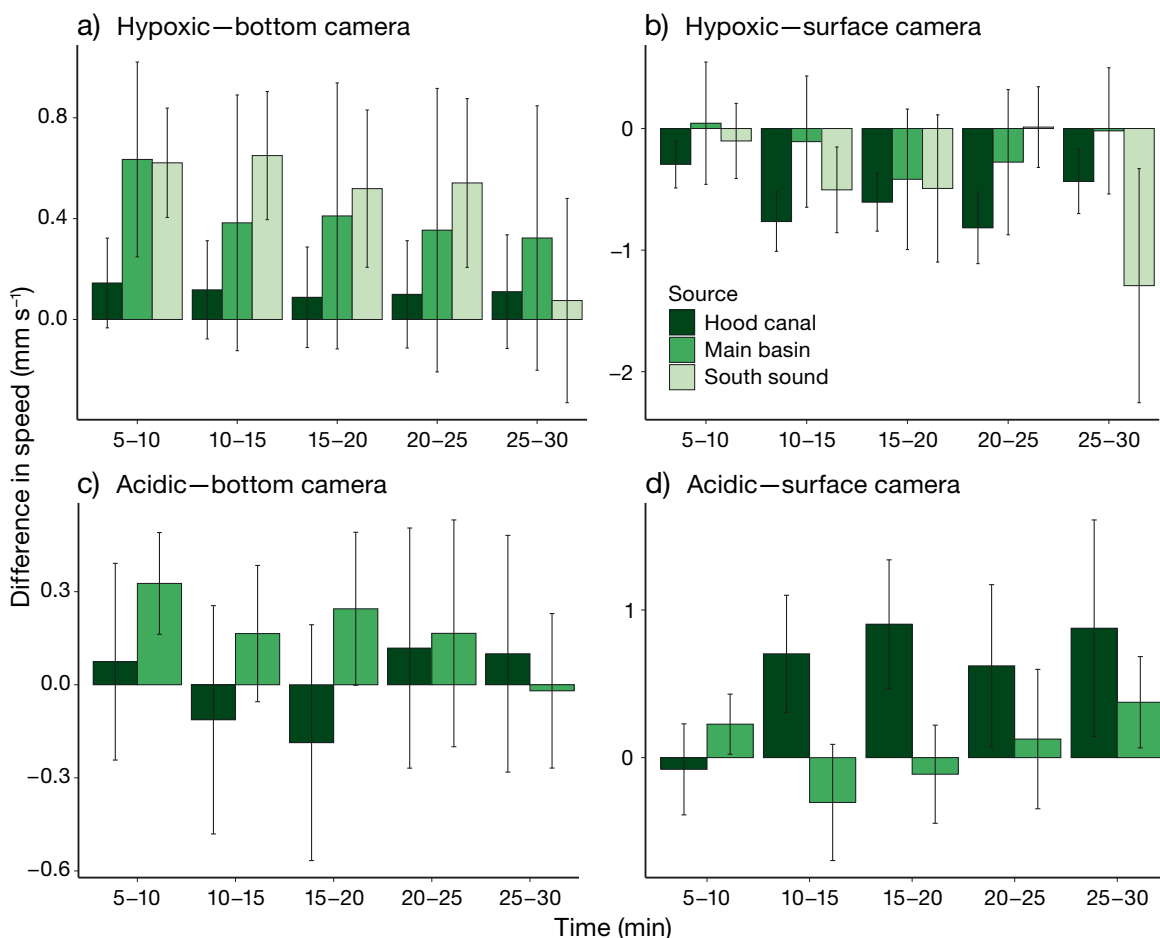


Fig. 7. Difference (control – treatment) in mean ( $\pm$  pooled SE) swimming speeds between control and treatment tanks among copepods collected from Hood Canal, Main Basin, and South Sound. (a) In hypoxia experiments, changes in mean total swimming speeds observed by the bottom camera (but not including the base) were significantly slower in hypoxic tanks relative to control among copepods collected from the Main Basin ( $p = 0.008$ ) and South Sound ( $p = 0.004$ ) but not Hood Canal. (b) In surface waters, the largest swimming speed differences were observed among copepods from Hood Canal ( $p < 0.0001$ ). (c) In acidification experiments, there was no significant difference in swimming speed responses among copepods from different collection sites in bottom waters. (d) In surface waters, only copepods from Hood Canal swam significantly slower in acidic tanks relative to control tanks ( $p = 0.002$ )

Changes in moribundity, mean height, and swimming speed at the base of the tanks (observed by the Base Cameras) did not vary among collection sites in response to either hypoxia or acidification.

#### 4. DISCUSSION

Due to a combination of natural conditions, climate change, and increased development in coastal areas, the oceans are predicted to experience widespread oxygen deficiency and acidification by the end of the century (Doney 2010, Melzner et al. 2013, Breitburg et al. 2018). Our laboratory experiments demonstrated significant effects of bottom water hypoxia and acidification on behavioral avoidance, swimming statistics, and apparent mortality rates in the copepod *Calanus pacificus* (summarized in Table 3). Copepods showed strong responses to hypoxia and weaker but significant responses to acidification. When exposed to hypoxic ( $0.65 \text{ mg O}_2 \text{ l}^{-1}$ ) bottom waters, *C. pacificus* were higher in the water column, exhibited slower swimming speeds within bottom waters, faster swimming speeds within non-stressful surface waters, and had higher apparent mortality rates relative to controls. When exposed to acidic (7.48 pH) bottom waters, *C. pacificus* swam slower but did not show changes in apparent mortality or vertical position. Interestingly, for a subset of our swimming speed measurements, we observed a significant effect of collection site on the magnitude of differences between treatment and control tanks.

Table 3. Responses of copepods to hypoxic or acidic bottom waters. \*Significant treatment effect; † significant treatment  $\times$  collection site interaction

Stressor	Metric	Camera view	Change relative to control
Hypoxia	Moribundity	Base camera	Increase *
	Mean height	All cameras	Increase *
	Calculated total speed ( $TS$ )	Surface camera	Increase *,†
	Calculated $TS$	Bottom camera	Decrease †
	True horizontal speed ( $HS_{tr}$ )	Base camera	Decrease *
Acidification	Moribundity	Base camera	No change
	Mean height	All cameras	No change
	Calculated $TS$	Surface camera	Decrease *,†
	Calculated $TS$	Bottom camera	No change
	$HS_{tr}$	Base camera	Decrease *

These may suggest an evolved or learned difference between animals from different locations in response to the historical presence or absence of chemical stressors.

##### 4.1. Changes in moribundity

We interpret the moribundity metric to imply a high likelihood of impairment or impending mortality, because lying immobilized on the bottom of the tank is not normal behavior for *C. pacificus*, and because oxygen levels of  $0.65 \text{ mg l}^{-1}$  have been shown to cause 100% mortality in *C. pacificus* within 60 min (Grodzins et al. 2016). Increased moribundity in our experiments suggests potential increases in mortality under analogous natural conditions, which are increasingly encountered by copepods in eastern Pacific habitats. Oxygen conditions in our experiments resulted in 25% mortality after 90 min—a 118% increase relative to controls, but still low compared to Grodzins et al. (2016). A possible interpretation for the difference is that in our experiments, a subset of *C. pacificus* periodically sought refuge in normoxic waters above the halocline. In the absence of avoidance, hypoxia experienced *in situ* in present-day Hood Canal and other coastal areas is likely to result in even higher mortality than observed in our 90 min experiments.

Consistent with previous findings that copepods are generally robust to the low pH conditions they encounter *in situ* (Mayor et al. 2012, Weydmann et al. 2012), we found no significant difference in moribundity between control and treatment tanks during acidification experiments. In many coastal and upwelling systems, hypoxia and acidification occur in conjunction; our results imply that oxygen stress will have a larger impact on copepod mortality *in situ* relative to acidification. Overall moribundity was higher in acidification experiments, with an average of 1.2 more moribund copepods in the control tanks during acidification experiments than in control tanks during hypoxia experiments. However, we hypothesize that copepods collected later in the season for acidification experiments than for hypoxia experiments were in a different physiological state, possibly due to seasonal changes in food availability. We speculate that the later collection date may have resulted in marginally increased moribundity rates in both treatment and control tanks.

#### 4.2. Changes in depth distributions

Our laboratory observations suggest that avoidance may be an important mechanism contributing to observed *in situ* shifts in copepod vertical distributions when stressful bottom water conditions are present. A majority of copepods, whose normal downward swimming behavior would have kept them within quickly lethal concentrations of dissolved oxygen, survived the duration of the 90 min experiment. Our interpretation is that individual copepods periodically sought refuge in normoxic surface waters. The upwards shift in vertical position in hypoxic tanks, the ongoing presence of swimming paths within lethally stressful bottom waters, and low moribundity together suggest that copepods attempted to continue their normal downward swimming, but when hypoxic bottom waters were present, periodically reversed that behavior to move above the hypoxic layer.

There are important differences between laboratory and field conditions to consider when translating results to the field. Notably, for our experiments, downward migrations in the field occur over much larger water columns across broader chemical gradients (typically on the order of m versus mm). As a result, copepods in the laboratory may be more likely to sense changes in water chemistry as they experience steeper gradients and more rapid changes with depth. This difference suggests tank experiments might overestimate some behavioral responses relative to *in situ* water columns. Conversely, swimming across broader natural gradients would provide more time for physiological effects to induce behavioral responses, such as up-swimming to escape stressful bottom layers, before full impacts of stressors become incapacitating. In that case, tank experiments might underestimate *in situ* behavioral responses. Despite the unclear implications of these limitations, recording copepod swimming paths in the laboratory yielded new insights into specific mechanisms leading to the apparent upwards shift in population distributions above stressful waters observed in many natural systems.

Interference with DVM into deep waters and a resultant congregation of copepods near the oxycline could have profound influences on predator–prey interactions. Hypoxic bottom waters can compress available habitat and shift copepod distributions upwards (Keister et al. 2000, Pierson et al. 2009, Roman et al. 2012, Keister & Tuttle 2013). In Hood Canal, the oxycline separating oxygen-rich surface waters and hypoxic bottom waters can be as shallow as 15 m in a

120 m water column (ORCA Mooring Data 2022), apparently compressing available habitat by as much as 88%. Our laboratory results suggest that *in situ* congregations of copepods observed near the halocline could be due to copepods attempting their normal downward migration but periodically ascending to recover from exposure to hypoxic conditions (Ludsin et al. 2009, Zhang et al. 2009). Encounter rates and foraging efficiency of both visual and non-visual predators are likely to increase if copepods aggregate near the oxycline during the day (Craig 2012), with commensurate increases in copepod mortality.

Suppression of downward migration may also impact carbon transport to depth. In shallower systems, such as hypoxic estuaries, a large percentage of the carbon flux to depth is the direct sinking of organic matter from the surface which, in summer, is primarily composed of fixed carbon associated with phytoplankton blooms (Baker et al. 1985, Turner 2002, Svensen et al. 2007). Zooplankton mediate the flux of fixed carbon by grazing on phytoplankton (Lorenzen et al. 1981). When bottom waters are not stressful, zooplankton may transport carbon into deeper waters during DVM. However, when downward migration is suppressed, fecal pellets produced in surface waters will likely be recycled through consumption and bacterial decomposition before sinking to the seafloor (Kjørboe 1997, Turner 2002). This shallow remineralization may act to retain nutrients in the surface waters, reducing the carbon flux to depth and acting as a negative feedback on hypoxia.

Finally, shallower migrations could increase population losses from estuaries and upwelling regions, where net transport of surface water is typically oceanward (Peterson et al. 1979, Falkenhaus et al. 1997, Giddings & MacCready 2017). In the absence of hypoxic bottom waters, migrating copepods spend part of the day in deeper waters where net flow is landward, acting to retain them in coastal environments (Hill 1991, Falkenhaus et al. 1997, Batchelder et al. 2002, Tommasi et al. 2013). The extent to which shallower copepods are advected offshore is location- and time-specific, mediated by bathymetry and local climatic and oceanographic conditions such as freshwater runoff, tidal currents, and wind patterns (Falkenhaus et al. 1997).

#### 4.3. Changes in swimming speed

We observed significant changes in swimming speeds when *C. pacificus* were exposed to either hypoxic or acidic bottom waters. Copepods in hypo-

xic tanks swam slower below the halocline and faster above the halocline relative to controls. We hypothesize that this observed change in swimming speed indicates a behavioral avoidance mechanism used to regulate and ultimately survive exposure to hypoxic bottom waters. A reduction in swimming speed within the hypoxic bottom layer could prolong the time copepods can tolerate metabolic stress. Conversely, an increase in swimming speed may reflect behavior used to swim out of the stressful layer. In acidification experiments, copepods in tanks with acidic bottom waters swam significantly slower than those in control tanks both at the base of the tanks (observed by the Base Cameras) and above the halocline (observed by the Surface Camera). To our knowledge, this laboratory study is the first to report a significant behavioral response of a calanoid copepod to changes in pH, potentially indicating a sublethal stress response to acidic waters that may be observable in the field.

While potentially important in avoiding chemical stress, significant increases in swimming speed increase copepods' metabolic demands. The total metabolism of *C. euxinus* is 2–4 times higher at maximal locomotion compared to basal metabolism (Svetlichny et al. 2000). An organism's metabolism is also influenced by environmental variables such as temperature and dissolved oxygen. While temperature was constant in our experimental tanks, temperatures *in situ* are typically warmer in surface waters, exacerbating the metabolic costs of increased swimming above the halocline (Roman et al. 2019, Svetlichny et al. 2000). However, the potential for increased feeding on phytoplankton near the surface may be able to offset some of the increased metabolic costs associated with exposure to chemical stress.

Changes in swimming speed also affect trophic interactions by directly influencing encounter rates between predators and prey (Visser 2007). Many calanoid copepods are cruise feeders, using their mouth appendages to propel them through the water while also bringing food particles toward them (Van Duren & Videler 1996). The encounter rate of cruise feeders is determined by prey density and swimming speed, with encounter rates increasing with speed (Frost 1972). Additionally, rheotactic predators that consume copepods detect their prey through fluid signals, making faster-swimming prey easier to detect (Kjørboe et al. 2014). We hypothesize that stress-induced increases in swimming speed will increase predation on copepods because of increased encounters and increased detection by rheotactic predators (Lima & Dill 1990, Visser 2007).

With hypoxia and acidification typically occurring in conjunction in natural systems, it will be important to understand how encountering the 2 stressors simultaneously may affect swimming speed. Tomasetti et al. (2018) observed additive negative effects of low pH combined with moderately low DO on larval crustacean survival. In both our hypoxia and acidification experiments, *C. pacificus* showed significant changes in swimming speed above the halocline, but the direction and magnitude of change differed. In addition, *in situ* surface waters are typically warmer than deep waters, which may further impact copepod responses to stressful bottom waters. Although constraints on our experimental design prevented us from assessing interactions, understanding co-occurring stressor effects on swimming speed is a priority for future research.

#### 4.4. Differences among collection sites: Evidence for local adaptation?

In our experiments, copepods from Hood Canal (which regularly experiences both hypoxia and acidification) showed significantly different responses in swimming speed to both hypoxia and pH compared to copepods from Main Basin (and South Sound during hypoxia experiments). Copepods from the different sub-basins of Puget Sound are genetically distinct and differ in population structuring across basin-wide spatial scales (Nuwer 2008). We hypothesize that these behavioral differences reflect local adaptation of a subpopulation of *C. pacificus* from Hood Canal, driven by the site's historical chemical conditions.

Localized, potentially adaptive, behavioral variations have been previously observed in copepods. *Acartia tonsa* from areas historically exposed to low oxygen move vertically to avoid hypoxic bottom waters, while *A. tonsa* from high oxygen areas do not (Decker et al. 2003). Similarly, respiration rates after exposure to elevated partial pressures of CO<sub>2</sub> (*p*CO<sub>2</sub>) differed between populations of *Pseudocalanus acuspes* from areas differing in natural fluctuations of *p*CO<sub>2</sub> (Thor & Oliva 2015).

The specific mechanisms enabling local adaptation could include physiological adaptations such as enhanced ventilatory capability, large surfaces, short diffusion distances, and respiratory proteins with high oxygen affinity (Childress & Seibel 1998) or behavioral adaptations in responses to a sensory input (Dam 2013). In our experiments, increased swimming speeds above hypoxic waters paired with



maintained swimming speeds within hypoxic waters may reflect adaptive behaviors in a subpopulation of copepods to escape lethal bottom waters common in Hood Canal. If so, local adaptation merits further study as a potential indicator of long-term resilience of copepods to increasing chemical stress.

#### 4.5. Conclusions

With coastal systems continuing to change, our observations in stratified laboratory water columns are important for understanding ecosystems currently stressed by hypoxia and acidification and for predicting future ecosystem responses. Our experimental results indicated changes in depth and swimming speed when exposed to hypoxic bottom waters and changes in swimming speed when exposed to acidic bottom waters. These sub-lethal effects have implications for the distribution and abundance of copepods, predator–prey interactions, and biogeochemical cycling. These behavioral changes may have contributed to observed mortality estimates, which were much lower than expected under prolonged exposure to hypoxia. Sub-lethal effects of hypoxic and especially acidic conditions on copepods are only just starting to be explored in the literature. With advances in ocean technology and the improving ability to observe individual organisms in the field, our results suggest metrics for *in situ* swimming characteristics, such as changes in swimming speed, that could prove a useful tool for monitoring ecosystems impacted by climate and anthropogenic changes.

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