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Effects of oxygen depletion on field distributions and laboratory survival of the marine copepod *Calanus pacificus*

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Copepods dominate the zooplankton, but surprisingly little is known of their tolerance to the increasing threat of hypoxia. We measured abundances of the calanoid copepod *Calanus pacificus* in relation to oxygen concentrations in the field and established its tolerance to low dissolved oxygen (DO) in the laboratory. *In situ* distributions of female *C. pacificus* were assessed with depth-stratified net sampling in Hood Canal, a seasonally hypoxic sub-estuary of Puget Sound, Washington. No clear avoidance of DO levels from supersaturated down to 2.0 mg DO L^{-1} was observed; DO levels <2 mg L^{-1} were rare in our sampling. In the lab, at 13°C female *C. pacificus* exhibited 100% 24-h survival at DO levels down to 1.7 mg DO L^{-1}; below 1.5 mg DO L^{-1}, survival sharply declined, with 25% survival at 1.2 mg L^{-1} and complete mortality within 1 h at 0.9 mg L^{-1}. *Calanus pacificus* is one of the dominant copepods throughout much of the North Pacific and an important trophic link in the ecosystem. Our measurements indicate that they have a steep threshold in oxygen tolerance that is similar to those measured for other calanoid copepods and which could limit their habitat in years of severe oxygen depletion.

KEYWORDS: *Calanus pacificus*; hypoxia; survival; oxygen depletion; vertical distribution

INTRODUCTION

Globally, coastal hypoxia is increasing in geographic distribution, duration and frequency of occurrence (Doney, 2010). Hypoxia occurs naturally in some regions, while in many others anthropogenic influences including fertilizer and sewage run-off lead to eutrophication, which through biological processes, intensifies oxygen drawdown (Howarth, 2008). As the world population has grown and temperatures have increased through global warming, the incidence of coastal hypoxia has too: nine oxygen-depleted systems were reported prior

to 1960, with that number roughly doubling every 10 years (Diaz et al., 2003). As of 2002, 146 regions were reported to experience coastal hypoxia.

Increased hypoxia and anoxia are adversely changing the environment as shown by habitat degradation, changes in trophic-web structure, and decreased biodiversity (Howarth, 2008; Vaquer-Sunyer and Duarte, 2008). In many cases, high-value species of demersal fish and shellfish have been displaced (Diaz, 2001; Rabalais et al., 2001; Diaz et al., 2003) leaving instead less valuable pelagic species. Generally, motile fish and benthic crustaceans are absent from bottom habitats when dissolved oxygen (DO) falls below 1.5–2 mg DO L^{-1}; many less motile invertebrates die at oxygen concentrations below 1.5 mg DO L^{-1} (Rabalais et al., 2001). While there is substantial literature on the effects of hypoxia on fisheries and benthic organisms, less is known about its effects on the underlying pelagic trophic web that supports high-value species.

In complex marine food webs, often a key trophic position is filled by one or a few species of calanoid copepod (Bollens and Frost, 1989; Schukat et al., 2013). In particular, species in the genus *Calanus* tend to play important trophic roles because of their relatively large size, lipid stores and abundance, which make them preferred diet items of many zooplanktivores (Economou, 1991; Falk-Petersen et al., 2007). As one of the most important species in marine food webs, *Calanus* have been more intensively studied than any other group of zooplankton (Marshall and Orr 1972; Vidal, 1980). Yet literature on the oxygen tolerance of *Calanus*, and indeed of any copepod, that inhabits seasonally hypoxic regions is extremely limited. Studies of oxygen tolerance have been conducted on *Acartia tonsa*, *Oithona colcarva*, *Labidocera aestiva*, *Calanoides carinatus* and *Centropages hamatus* (Roman et al., 1993; Stalder and Marcus, 1997; Auel and Verheye, 2007) and a few studies have assessed behavioral avoidance in the laboratory (e.g. Decker et al., 2003) or inferred it from field distributions (e.g. Keister et al., 2000; Keister and Tuttle, 2013). Together, the studies indicate species-specific differences in tolerances with individuals of some species surviving DO as low as 0.6 mg L^{-1} but 100% mortality of others at 1.4 mg DO L^{-1}. Only one previous study that we are aware of has measured the oxygen tolerance of a species of *Calanus* (Ruz et al., 2015), and that was from a system with a permanent Oxygen Mimimum Zone (OMZ). Overall, surprisingly little information exists on the physiological tolerances of copepods to low DO.

This study focused on female *Calanus pacificus*, the dominant *Calanus* in the North Pacific, to better understand their oxygen tolerance and behavioral responses to low oxygen concentrations in the field. We measured vertical distributions of zooplankton and oxygen as a component of a project designed to assess the effects of seasonal hypoxia on predator–prey interactions between mesozooplankton and fish. To help explain the observed *in situ* patterns, the 24-h survival of *C. pacificus* was measured in the laboratory under controlled conditions. We hypothesized that abundances of *C. pacificus* would be positively related to oxygen concentrations in the field, and that their distributions would reflect complete avoidance of regions where oxygen was below their lethal limit.

METHOD

Study site—Sampling was conducted in Hood Canal (Fig. 1), a seasonally hypoxic sub-estuary of Puget Sound, Washington, USA. Hood Canal's deep, narrow basin is fronted by a glacial sill that inhibits circulation, which combined with high primary production, regularly leads to oxygen depletion in late summer and autumn (Gregg and Pratt, 2010). DO is routinely lowest in the southern-most reaches of Hood Canal and increases northward towards the ocean. Strong winds or tidal events can mix low oxygen waters up into the water column and may lead to displacement of the oxygen minimum layer into near-surface water (Cannon, 1975).

Field sampling—Sampling was conducted from the *R/V Clifford A. Barnes* on monthly cruises June to October in both 2012 and 2013 (10 cruises in total). Four stations were sampled on each cruise with approximate depths of (from north to south): 80 m (Dabob), 170 m (Duckabush), 120 m (Hoodsport) and 70 m (Union) (Fig. 1). Data used in this study were from the northern-most and southernmost stations (Dabob and Union). Conductivity, temperature, depth and DO were recorded using a Sea-Bird Electronics (SBE) 9 CTD array equipped with an annually-calibrated SBE 43 oxygen sensor and a WET Labs ECO-AFL/FL fluorometer. A Niskin rosette was used to collect water at 3–5 targeted water depths above and below the oxycline at each station to calibrate the CTD DO probe; oxygen was analyzed using the modified Winkler titration method (Carpenter, 1965a; Carpenter, 1965b).

Zooplankton were collected using a 0.25 m^2 HydroBios Multinet equipped with five 200-μm mesh plankton nets that were opened and closed remotely to sample 4–5 discrete depth layers at each station. Depth strata sampled in each tow were chosen based on DO profiles to sample the surface mixed layer, within the oxycline, in any regions of mid-water DO minima, and one or more deep strata. Paired day and night samples
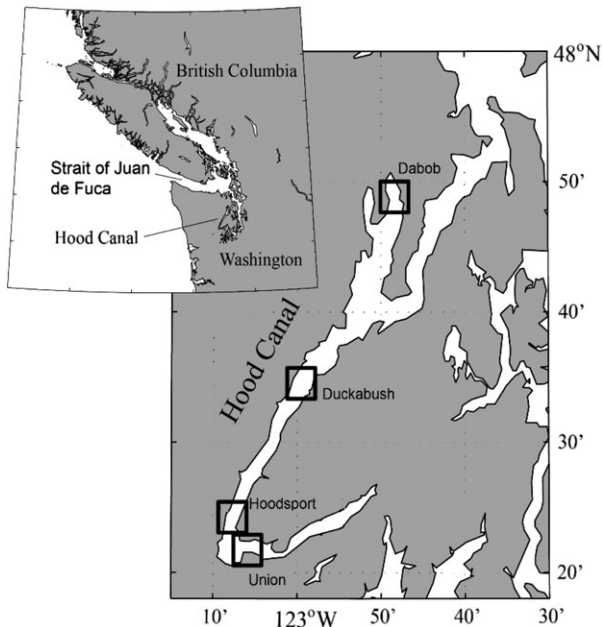
Fig. 1. Map of sampling stations in Hood Canal, Washington, USA. Data for this study were primarily analyzed from the northernmost (Dabob) and southernmost (Union) stations.

were collected at each station. The Multinet was equipped with inner and outer flow meters to measure the water volume filtered and monitor for clogging. Samples were preserved in 5% buffered formalin in seawater and returned to the lab for identification where 200–1300 (median = 445) individual zooplankton were identified, staged and counted per sample, of which the number of female *Calanus* counted ranged from 0 to 171 per sample. A total of 132 individual samples were analyzed for this study.

Laboratory Experiments—We collected zooplankton off of Shilshole Bay, Seattle by gentle vertical hauls (0.2 m s^{-1}) using a 1-m diameter ring net with 571-μm mesh equipped with a non-filtering cod end. Female *C. pacificus* were selected from the hauls and held for 24 h prior to experiments to allow recovery from capture stress. We completed three survival trials. Each trial consisted of an ambient DO control treatment (~7.0–8.0 mg DO L^{-1}) and one or more low-DO treatments (ranging 0.9–1.8 mg DO L^{-1}). Seawater for the treatments was collected from below the pycnocline in Puget Sound, held at 13°C, then filtered through a 20-μm sieve and diluted to 30.0 (typical sub-surface conditions in Puget Sound) with deionized water 24 h before experiments.

We attained target DO levels by vigorously bubbling 20-L carboys of the filtered seawater with N$_2$ gas. We estimated the DO level during N$_2$ bubbling using a Pinpoint II Oxygen Monitor (American Marine Inc.)

which had been calibrated by oxygen titrations using the modified Winkler titration method (Carpenter, 1965a; Carpenter, 1965b) on a Metrohm 765 Dosimat burette. Because the benchtop meter is not highly accurate, after bubbling each 20-L carboy to a target DO concentration, a minimum of three 125-ml biological oxygen demand (BOD) bottles were filled, then immediately fixed and sealed for Winkler titration to confirm the starting (T_0) oxygen concentration; as performed, the modified Winkler method has a total estimated error of <0.01 mL L^{-1} (Murray and Riley, 1969). Fifteen to twenty 300-mL BOD bottles per DO level were then immediately filled from the same treatment water, and one healthy female *C. pacificus* was placed in each bottle. These 300-ml bottles were placed in the dark at 13°C for 24 h. Within 1 h after introducing females and after 24 h (T_{24}), the bottles were examined to determine whether the females were alive or dead. At T_{24}, after examining for mortality, we randomly selected a minimum of three (up to six) of the 300-mL bottles and immediately fixed them for oxygen titration, which was conducted within 48 h of completing the trial.

Survival at each oxygen concentration was calculated as the percent of females in each treatment that were alive at T_{24}. The oxygen concentration estimated to be lethal to 50% of the experimental females after 24 h (the LC50) was estimated from the survival curve by fitting a linear regression using MatLab software version R2016a (The MathWorks Inc., Natick, MA).

RESULTS

Field results—As is typical in Hood Canal, oxygen was lower at Union than at Dabob, and persistently declined over the summer (Fig. 3). We sampled across a wide range of DO concentrations, from hypoxic sub-pycnocline waters at Union in autumn (minimum DO measured = 1.3 mg L^{-1}) to supersaturated surface phytoplankton blooms in early summer at Hoodsport (maximum of 15.7 mg DO L^{-1}). Upper 3-m temperatures ranged from 10 to 22°C; sub-pycnocline temperatures ranged from ~8 to 12°C. In summer when oxygen levels were lowest, oxygen profiles showed deep water oxygen depletion typical of many estuaries (Fig. 2 left panel) which were displaced upward into the water column when dense ocean water intruded into the fjord in late summer to create mid-water oxygen minimum layers (Fig. 2 right panel).

Female *C. pacificus* were collected in 63 of the 132 plankton nets analyzed. Densities (excluding 0 values and two very high outliers) ranged from 0.2 to 157 female *C. pacificus* m^{-3} (Fig. 3). We did not find a strong relationship between female abundance and oxygen
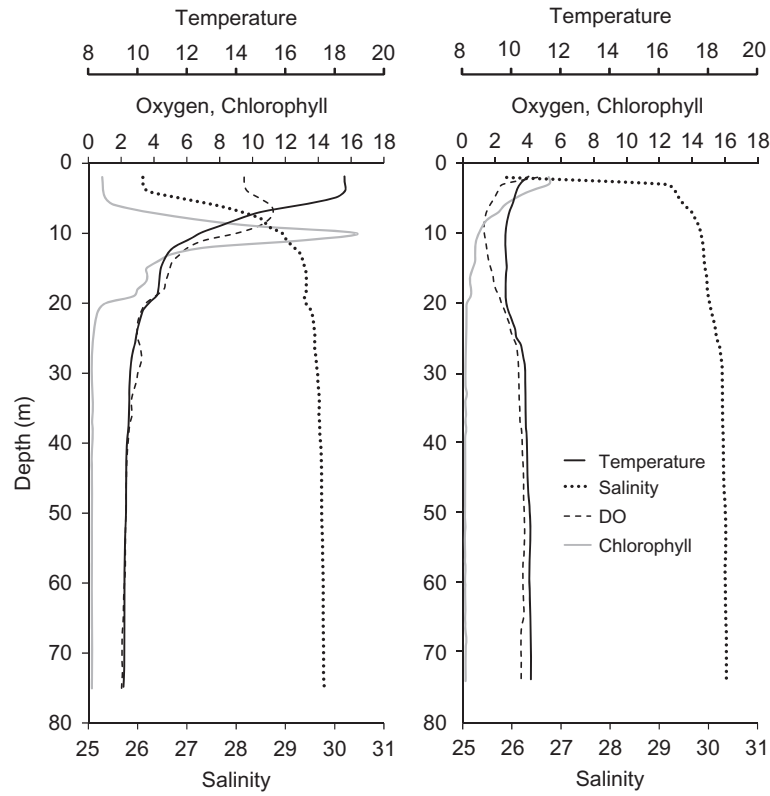
Fig. 2. CTD profiles from Union in (left panel) August 2013 and (right panel) October 2013 as examples of conditions present during the field study. Temperature (°C), salinity, DO (mg L^{-1}) and fluorescence as chlorophyll (µg L^{-1}) from profiles are shown. Summary data from all net tows are given online in the Supplementary Information.
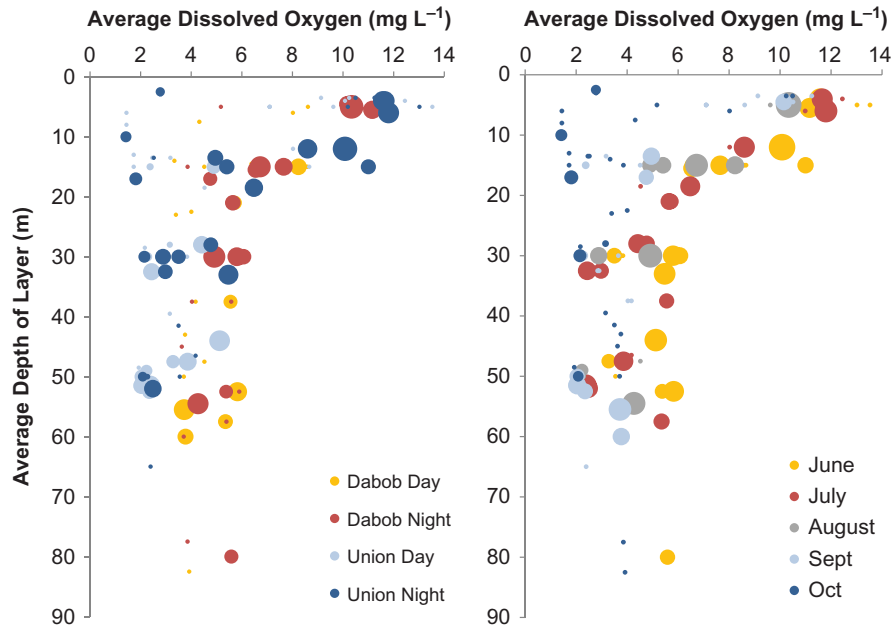

Fig. 3. Abundances of *C. pacificus* females as Log$_{10}$ (ind. m^{-3} +1), relative to average depth of the layer sampled (*y*-axis) and average DO of that layer (*x*-axis). Abundances are shown as circles colored by (left panel) station and time or (right panel) month sampled, with the largest circle scaled to the maximum abundance (960 ind. m^{-3}) and the smallest to zero. All individual samples shown regardless of time of day collected.

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over the range of DO concentrations we sampled in the field when including zero values (Pearson $R^2 = 0.04$). *Calanus pacificus* females did show a tendency towards lower abundances with lower oxygen, and were rare in nets that sampled in depth strata where DO values were <1.9 mg L^{-1}, regardless of where in the water column (deep or near surface) the low-DO layers occurred or whether the sampling was conducted during day or night. Female abundances declined from early to late summer, but did not significantly differ between stations. Day–night differences in depth distributions reflected diel migration from deeper waters during the day towards the surface and night.

Laboratory results—Over the three 24-h experimental survival trials, three ambient control DO concentrations of ~7.7–8.0 mg L^{-1} were tested against seven low-DO treatments that averaged 0.92–1.84 mg DO L^{-1} at T_0 (Table I). Because of the difficulty in homogenizing the treatment water and maintaining constant oxygen concentrations during experimental setup, jars titrated from the five moderately low-DO treatments showed some overlap among initial and final DO concentrations. That is, the treatments that averaged 1.36–1.56 mg DO L^{-1} at T_0 were not discrete from each other, whereas the lowest (0.92 mg L^{-1}) and highest (1.84 mg L^{-1}) of

the low-DO treatments were discrete from the rest. DO concentrations in the experimental jars were higher at T_0 than at T_{24} by an average of 0.26 mg DO L^{-1}, indicating that respiration occurred in the sealed jars during the trials. Since the oxygen concentration experienced by the females was therefore not constant, the DO concentrations shown in Fig. 4 and used in the text below as treatment levels are the average DO from all jars titrated at T_0 and T_{24} in each treatment.

All female *C. pacificus* survived 24 h in the three control treatments at 13°C, most (13 of 15; 87%) survived in the highest of the low-DO treatments (1.70 mg DO L^{-1}), and all survived in the next highest treatment level in Trial 2 (1.44 mg DO L^{-1}), whereas only 70% survived the same average level in Trial 3. All females died within 1 h of T_0 in the lowest oxygen concentration (0.92 mg L^{-1}). Overall, 24-h survival showed a sharp decline with decreasing oxygen concentrations between the range of ~1.4–0.9 mg DO L^{-1} with 100% survival at higher concentrations and 0% survival at the lowest concentration tested. Fitting a linear regression over the range of 0–100% survival between 0.9 and 1.7 mg DO L^{-1} resulted in an R^2 of 0.75 and an estimated LC_{50} of 1.3 ± 0.1 mg L^{-1}.

Table I: DO and female C. pacificus *survival data from survival experiments*

	Trial 1			Trial 2			Trial 3			
	Control	LDO-1	LDO-2	Control	LDO-1	LDO-2	Control	LDO-1	LDO-2	LDO-3
Titrated DO at T_0 (mg L^{-1})	8.08	**0.91**	*1.34[b]*	7.80	1.89	1.58	7.67	1.36	1.48	1.57
	7.89	**1.04**	*1.35[b]*	7.72	1.86	1.57	7.68	1.50	1.45	1.54
	7.92	**0.80**	*1.37[b]*	7.70	1.78	1.54	7.73	1.44	1.52	1.61
					1.83					1.54
										1.47
										1.39
Average at T_0[c]	7.97	0.92[a]	1.36[b]	7.74	1.84[c]	1.56[d]	7.70	1.43[b,e]	1.48[e]	1.52[b,d,e]
Titrated DO at T_{24} (mg L^{-1})	7.66	N/A[a]	**1.07**	7.31	1.56	1.29	7.44	1.23	1.26	1.42
	7.55		**1.07**	7.25	1.56	1.35	7.51	**1.10**	**1.19**	**1.27**
	7.70		**1.10**	7.26	1.54	1.33	7.48	**1.10**	1.27	1.44
								1.25		**1.30**
								1.07		
								1.23		
Average at T_{24}	7.64	N/A[a]	1.08	7.27	1.55	1.32	7.48	1.16	1.24	1.36
Average DO (used in Fig. 4) ± 1 Std. Dev.	7.80±0.20	0.92±0.12	1.22±0.15	7.51±0.26	1.70±0.16	1.44±0.14	7.59±0.12	1.30±0.15	1.36±0.14	1.44±0.11
C. pacificus: # Survived / # tested	18/18	0/18	5/18	18/18	13/15	15/15	14/14	5/20	11/20	14/20
C. pacificus: % Survival	100%	0%	28%	100%	87%	100%	100%	25%	55%	70%

Average DO values used as treatment levels in Fig. 4 and the text was calculated as the mean of average T_0 and average T_{24} concentrations; Std. Dev. was calculated from all individual T_0 and T_{24} titration values. LDO-1, LDO-2 and LDO-3 represent the low-DO (LDO) treatment levels in each trial. Bold numbers represent samples in which the female had died by T_{24}. Superscripts on Average at T_0 LDO values indicate overlapping ranges of oxygen concentration between treatments at T_0.
[a]100% mortality of all females within 1 h of setup eliminated the need for T_{24} titrations.
[b]Titrations were not conducted at T_0, so T_0 data (*italicized*) were estimated from T_{24} values + the average of ΔDO (T_0–T_{24}) from all other titrations.
[c]Similar superscripted numbers indicate treatments with overlapping range of oxygen concentrations at T_0.
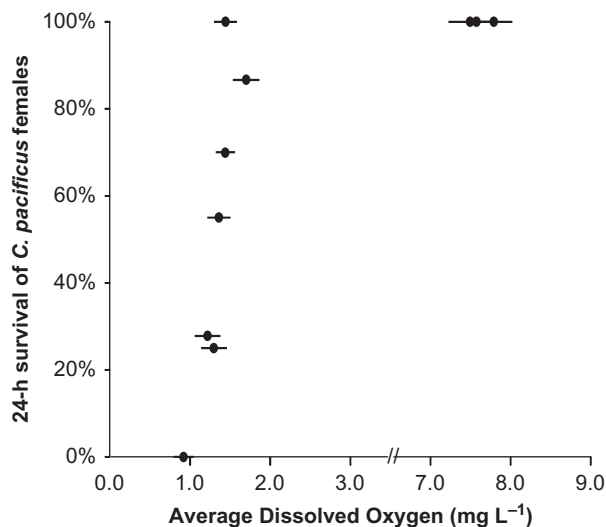
Fig. 4. The relationship between percent 24-h survival of *C. pacificus* females and DO (mg L^{-1}). See Table I for oxygen concentration calculations; error bars were calculated as one Std. Dev. of all T_0 and T_{24} oxygen titration values within each treatment.

DISCUSSION

We did not find evidence of avoidance of hypoxic layers, or decreased abundances of *C. pacificus* females where oxygen concentrations were low in the field. Female abundances were lowest in late summer when oxygen was lowest, but did not significantly differ between stations despite large differences in oxygen. At Union, females were abundant at oxygen levels down to 1.9 mg L^{-1} and were present in low numbers in water column layers where oxygen concentration (averaged across the depth of the net tow) was as low as 1.4 mg L^{-1}. Although *C. pacificus* females were absent or in very low numbers at the lowest DO levels we sampled (<1.9 mg L^{-1}), a scarcity of samples at low DO prohibits drawing conclusions about whether they actively avoided those layers. Notably, some of the lowest oxygen concentrations occurred in the upper 20 m of the water column (Fig. 3), so low female abundance in those layers was not simply due to avoidance of deep water where oxygen depletion typically occurs in estuaries.

To test whether the observed field distributions reflected physiological tolerance for low oxygen, as opposed to an inability to sense and move to avoid stressful oxygen levels, we tested the survival of female *C. pacificus* in response to DO in the lab. Our results suggest there is a tight range in DO concentration that defines a rapid decline in 24-h survival of *C. pacificus* females; from 100% survival at ~1.5 mg DO L^{-1} to 0% survival at 0.9 mg DO L^{-1} at 13°C. Thus, the laboratory results suggest that at ≤13°C, females would not need to avoid concentrations of 1.5 mg L^{-1} to survive periods shorter than 24 h. Based on the laboratory results, females would be expected to avoid concentrations <1.5 mg L^{-1}, particularly in regions with warmer temperatures and hence higher BOD (Brown et al., 2004), but we did not observe oxygen concentrations in the field lower than 6 mg L^{-1} where temperature was ≥13°C. We were not able to assess whether females collected from low oxygen layers were alive at the time of collection, so there is the possibility that some collected from deep hypoxic water may have been sinking carcasses.

The oxygen survival thresholds we found for Hood Canal *C. pacificus* were similar to those reported for the copepods *A. tonsa* and *O. colcarva* which also occupy seasonally hypoxic regions. Stalder and Marcus (1997) reported a rapid decline in survival of Florida Gulf Coast *A. tonsa* between 1.4 (100% survival) and 0.9 mg DO L^{-1} (0–10% survival) at 20°C. Roman et al. (1993) reported that complete mortality of Chesapeake Bay *A. tonsa* occurred at ≤1.7 mg L^{-1} and significant mortality of *O. colcarva* at <2 mg L^{-1} at 20°C, but that ~15% of *O. colcarva* survived concentrations as low as 0.6 mg L^{-1}. It is clear that inter-specific and even inter-population differences in oxygen tolerance exist, but among species that inhabit seasonally hypoxic regions, thresholds at which decreased survival and complete mortality occur appear to be within the fairly small range of ~1–2 mg DO L^{-1}.

These values are in stark contrast with those reported for copepods that are adapted to life in the low oxygen environments of permanent OMZs. While the diversity of copepods that can inhabit the core of OMZs is low compared to oxygenated surface waters (Wishner et al., 2008), some copepods such as *Lucicutia grandis*, *Eucalanus elongatusof* and *Spinocalanus antarcticus* can be abundant in <0.05 mL L^{-1} (<0.07 mg L^{-1}) deep in the Arabian Sea OMZ (Wishner et al. 2000, 2008). On the other hand, copepods such as *Subeucalanus subtenuis* suffer high mortality in 5% O$_2$ saturation (0.6 mg L^{-1} at 10°C) despite inhabiting the Eastern Tropical Pacific, so are restricted to near surface waters above the OMZ (Cass and Daly, 2014).

The measured survival of *C. pacificus* females in response to oxygen was similar to that found for the closely related *C. carinatus* in the Benguela upwelling system (Auel and Verheye, 2007). These authors reported 100% survival of female and stage CV *C. carinatus* at 1.6 mg DO L^{-1}, a sharp decline in survival between ~1.0 and 0.5 mg L^{-1} and complete mortality at 0.2 mg L^{-1}. The lower oxygen concentration at which complete mortality occurred may again reflect the difference between copepods that inhabit regions with permanent OMZs versus seasonally hypoxic regions.

Ruz et al. (2015; in review) reported that >85% of *Calanus chilensis* females collected from the Humboldt OMZ region survive 24-h at DO concentrations between 0.6 and 2.0 mg L^{-1}, but that females collected within low DO water have lower respiration and other measures of metabolism than those collected from above the OMZ. Despite occupying an OMZ region, *C. carinatus* seems not to have evolved tolerance to oxygen levels found in the core of the OMZ (<0.8 mg L^{-1}), as some other copepods (e.g. *Pleuromamma robusta* and *Aetideopsis carinata*) in the Benguela System have (Auel and Verheye 2007).

Calanus, like other large zooplankton, migrate vertically to avoid visual predation near the surface during the day (Frost, 1988; Dagg et al., 1998) or during diapause (Dahms, 1995). Fish are generally more sensitive to low DO than zooplankton (Miller *et al.*, 2002) and may exhibit physiological responses or avoidance of DO levels <3–5 mg L^{-1} (Breitburg et al., 2001; Vaquer-Sunyer and Duarte, 2008; Ekau et al., 2010). Since *C. pacificus* are able to tolerate much lower oxygen levels than many fish, depths where DO is <3 mg L^{-1}, but above their tolerance threshold of ~1.5 mg DO L^{-1}, may serve as additional predation refuges. In coastal upwelling areas, some species of *Calanus* have been observed to migrate into OMZs. For example, *C. chilensis* was observed in high densities (60% of the population) in DO ranging from 0.92 to 1.38 mg L^{-1}, likely to avoid predation by a local anchovy population in the Humboldt upwelling system (Hirche et al., 2014). Off the Santa Barbara Basin, *C. pacificus* was found to migrate to just above the OMZ for increased protection from predators while in the fifth copepodid (CV) stage, then enter a diapause state for the winter (Osgood and Checkley, 1997). Alldredge et al. (1984) observed high abundance of diapausing stage CV *C. pacificus* in very low oxygen (0.2 ml L^{-1}) deep waters of the Santa Barbara Basin. The ability to tolerate more severe conditions than predators may be an important trait that permits survival of populations.

CONCLUSIONS

We examined distributions of adult female *C. pacificus* in relation to oxygen in a seasonally hypoxic sub-estuary of Puget Sound, Washington, and conducted laboratory experiments to quantitatively define their physiological tolerance to DO in an effort to contribute to the understanding of the effects of hypoxia on marine trophic interactions. We found little evidence of avoidance of hypoxia in the field at oxygen concentrations ≥1.9 mg L^{-1}. Laboratory results confirm that the copepods are

tolerant of oxygen levels as low as ~1.5 mg L^{-1} over a 24-h period at 13°C, but suffer complete mortality at 0.9 mg L^{-1}. *Calanus pacificus* is one of the dominant *Calanus* species of the Northeast Pacific (Star and Mullin, 1981). By gaining a thorough understanding of the physiological limits and behavioral responses of this important species to environmental stressors, we will be better able to predict changes in marine ecosystems in the face of climate change.

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