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Development of *Euphausia pacifica* (krill) larvae is impaired under pCO_2 levels currently observed in the Northeast Pacific

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ABSTRACT: Despite the critical importance of euphausiids in marine food webs, little ocean acidification (OA) research has focused on them. *Euphausia pacifica* is a dominant and trophically important species of euphausiid throughout the North Pacific and the California Current Ecosystem, where low pH conditions are occurring in advance of those in the global ocean. We assessed the impact of reduced pH on the hatching and larval development of *E. pacifica* in the laboratory and characterized the pH to which *E. pacifica* eggs and larvae are currently exposed in Puget Sound, Washington (USA), a large estuary connected to the California Current. In 2 independent sets of laboratory experiments that lasted 6 to 22 d and which involved broods from 110 different females, we found that hatching is robust to a wide range of pH levels, but larval development and survival are reduced at pH levels that are currently observed within their habitat. Survival from 3 d post hatch to the calyptopis 2 stage was reduced by an average of 20% at pH 7.69 compared to pH 7.96. Even though this population experiences a range of pH conditions on seasonal and daily timescales, it may be living near the limits of its pH tolerance. Continued OA may push these organisms past their threshold, which could have cascading negative consequences for higher trophic levels.

KEY WORDS: Ocean acidification · Zooplankton · Euphausiids · pH · Survival · Exposure

INTRODUCTION

Ocean acidification (OA) is a reduction in the pH of the ocean over time that is primarily caused by the ocean's uptake of carbon dioxide from the atmosphere (IPCC 2011). Average surface ocean pH has declined 0.1 pH units (from 8.2 to 8.1) since the industrial revolution (Caldeira & Wickett 2003) and is predicted to decline an additional 0.3 pH units by 2100 (Feely et al. 2009). This predicted change represents a 150% increase in ocean acidity since the beginning of the industrial revolution. OA is happening at a faster rate than at any time during the past 50 million years (Hönisch et al. 2012) and has the potential to affect marine ecosystems throughout the world (Hofmann et al. 2010, Kroeker et al. 2013). Increased CO_2 affects both calcification and acid–base physiology in marine organisms; in some species, the increased metabolic costs of compensating for decreased pH can divert energy away from growth and reproduction (Melzner et al. 2011, Thomsen et al. 2013, Pedersen et al. 2014). In general, increased

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CO_2 decreases survival, calcification, growth, and development in marine invertebrates, but with highly variable responses among different groups (Kroeker et al. 2013).

Regional patterns of pH and aragonite saturation state vary; high-latitude waters and some coastal areas such as upwelling systems will experience harmful pH conditions sooner than the rest of the ocean due to a combination of natural and anthropogenic factors (Orr et al. 2005, Hauri et al. 2013). The California Current Ecosystem (CCE) is an ecologically productive and economically important upwelling system that has low and declining pH throughout the water column (Gruber et al. 2012, Hauri et al. 2013). During upwelling events in the CCE, high pCO_2/low pH deep water is driven up onto the continental shelf (Feely et al. 2008). Although this is a natural phenomenon, the CO_2 load of the upwelled waters is increasing over time as the oceans absorb anthropogenic CO_2, leading to increasingly acidified waters. The current anthropogenic carbon load has led to the shoaling of the aragonite saturation horizon (where dissolution and precipitation of the aragonite form of calcium carbonate are equally favored) by about 50 m depth across the shelf and to even reach the surface at times (Feely et al. 2008). Carbonate chemistry in the CCE has already shifted significantly from its pre-industrial range, and even greater changes are predicted in the near future (Hauri et al. 2013). In estuaries within the CCE, OA is exacerbated by runoff of low-pH freshwater and the respiration of organic matter, which increases CO_2 loading and drives the pH down even further (Feely et al. 2010). Organisms living in regions like the CCE, where ocean pH is naturally low and declining, could provide insight into species' responses to the chemical conditions expected elsewhere in the future. Are these species sensitive to the changes expected with OA, or do they display adaptation to the low and variable pH conditions currently experienced in their environment?

Despite their ecological importance, very few OA studies to date have focused on euphausiids. Experiments have shown that *Euphausia superba*, a keystone species in Antarctic ecosystems, has reduced hatching at 1250 µatm pCO_2 (pH 7.50) compared to 380 µatm and complete hatching failure at 2000 µatm pCO_2 (pH 7.36–7.40); however, at 3 d post hatch (dph) larval swimming activity is not affected at 1000 µatm pCO_2 (Kawaguchi et al. 2011, 2013). Even at moderate levels of 672 µatm pCO_2 (pH 7.84), adult *E. superba* increase feeding and nutrient excretion rates, which may indicate shifts in metabolism associated with acid–base regulation (Saba et al. 2012). Sub-adults of the north Atlantic krill *Nyctiphanes couchii* have reduced survival at pCO_2 concentrations between 1100 and 1700 µatm pCO_2 (pH 7.63 and 7.47), but other life stages have not been tested (Sperfeld et al. 2014). The few published studies on euphausiids are not sufficient to generalize the effects of increased CO_2 across species, especially without an evaluation of equivalent life stages. Early life stages generally have narrower physiological tolerances than other life stages and are predicted to be the most vulnerable to OA and other environmental changes (Kurihara 2008, Byrne 2011). The current literature characterizes crustaceans as generally tolerant to OA (Whiteley 2011, Kroeker et al. 2013, Wittmann & Pörtner 2013). However, very few holoplanktonic crustaceans have been studied to date, and those that have been tested are primarily copepods (Riebesell & Tortell 2011).

E. pacifica is a dominant zooplankton species throughout the North Pacific and the northern CCE (Brinton 1962), and an important prey item for fish, whales, and seabirds (Field & Francis 2006). The goals of this study were to characterize the pH conditions currently experienced *in situ* by *E. pacifica* eggs and larvae in Puget Sound, a large estuary adjoined to the northern CCE, and to test in the laboratory the influence of current and future pH levels on the development of this important species. During development from eggs to juveniles, *E. pacifica* pass through 2 nauplius stages and 1 metanauplius stage which are non-feeding, then 3 calyptopis stages (C1–C3) followed by 7 furcilia stages which are all feeding. *E. pacifica* have been successfully reared under laboratory conditions from egg to adulthood, but to our knowledge they have not been successfully mated in the lab. There are currently no published studies on the effects of increased pCO_2 on *E. pacifica*; however, sensitivity to pCO_2 can vary within and among species based on the organisms' exposure to pCO_2 in their environment (Maas et al. 2012, Kelly et al. 2013, Lewis et al. 2013). The population tested in this study inhabits an environment with large seasonal and geographic variation in pCO_2 and pH. Therefore, we hypothesized that the hatching and early development of Puget Sound *E. pacifica* populations would be robust to the intermediate pCO_2 levels that they currently encounter in the field, but would be reduced at higher pCO_2 levels that they may encounter in the future. We tested this hypothesis by sampling the pCO_2 conditions in the field and simulating those and projected future levels in the laboratory.

MATERIALS AND METHODS

Chemical conditions and euphausiid distribution

We characterized the pH environment currently experienced by *Euphausia pacifica* eggs and larvae at 2 stations in the northern end of Hood Canal in Puget Sound, Washington, USA (47.61° N, 122.94° W and 47.66° N, 122.86° W; Puget Sound Regional Synthesis Model [PRISM] stations P14 and P15; Fig. 1). The southwest station, P14, is deeper (180 m) than the northeastern station, P15 (130 m). Samples were collected during the day and night on 9–10 April and 15–16 June 2012. This design was chosen to sample during the spawning season of *E. pacifica* (approximately February to July) in an area where low pH waters occur (Feely et al. 2010). We collected physical and chemical data using a CTD profiler equipped with a pH sensor (SBE 18, Sea-Bird Electronics) and Niskin bottles, which were used to collect water at 6 depths for spectrophotometric pH, total dissolved inorganic carbon (DIC), and total alkalinity (TA) analyses. The CTD's pH sensor was not accurate, so it was only used to determine the shape of the pH profile relative to discrete measurements. Spectrophotometric pH was measured shipboard immediately after water sampling (Ocean Optics USB 2000+ Fiber Optic Spectrometer; m-cresol purple dye from Sigma Aldrich). TA was measured by open-cell potentiometric titration (according to SOP 3b in Dickson et al. 2007), and DIC was measured by acidification and quantification either using a Licor-700 CO_2 detector at the University of Washington's Friday Harbor Laboratory, or a CO_2 coulometer (UIC model CM5015) at the University of Washington's School of Oceanography. All chemistry samples from the field and experiments were analyzed according to Dickson et al. (2007) and Riebesell et al. (2010). We calculated full carbonate system parameters using CO2sys version 2.1 (Lewis & Wallace 2012) with $K1$ and $K2$ constants from Lueker et al. (2000), $KHSO_4$ constant from Dickson (1990), $[B]_T$ from Uppström (1974), and the total pH scale.

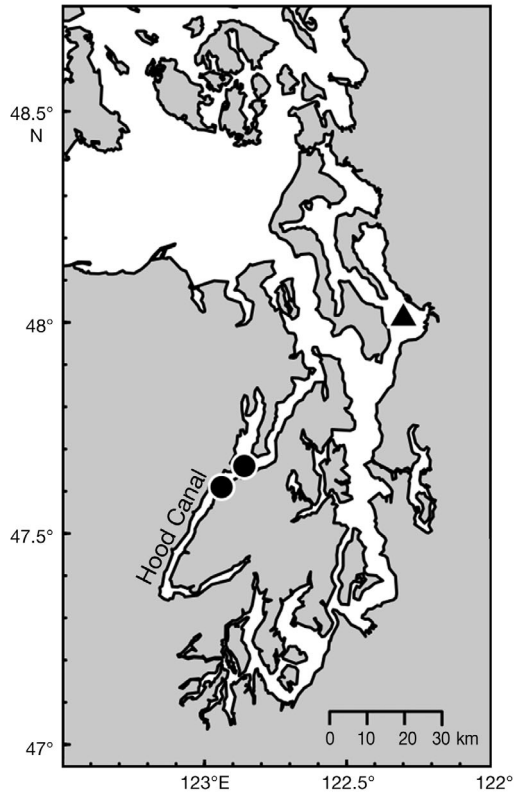

Fig. 1. Location of study area in Puget Sound, Washington, USA. Circles indicate field sampling stations P14 (southwest) and P15 (northeast); the triangle marks Possession Sound, where experimental organisms were collected

We collected zooplankton samples at the same locations where carbon chemistry was measured through a series of depth-stratified net tows. Larger life stages were quantified from oblique net tows using a 5-net, 335 µm mesh, 0.25 m^2 Hydro-Bios Multinet sampler; smaller life stages were sampled with a 60 cm diameter, 75 µm mesh, closing ring net lifted vertically over discrete depth ranges. For all collections, a flowmeter was used to monitor the volume of water filtered. Samples were preserved in a 5% buffered formalin and seawater solution. In the laboratory, samples were diluted to 4 to 8 times the settled volume and subsampled with a 10 ml Stempel pipette. We counted 4 to 9 subsamples, in which *E. pacifica* larvae were speciated and identified to the developmental stage.

Laboratory tests

We report findings from 2 sets of experiments. The first set of experiments (hereafter, Expt 1) characterized the sensitivity of *E. pacifica* hatching and development to the first feeding stage (C1) across a wide range of pH conditions. The second set of experiments (Expt 2) targeted a refined range of pH conditions based on Expt 1, and the development tests were extended to the second feeding stage (C2).

Organism collections

Ovigerous female *E. pacifica* were collected at night in May and June 2013 (Expt 1) and 2014 (Expt 2) from Possession Sound, WA (Fig. 1), between

22:00 and 01:00 h using a 1 m diameter, 2000 µm mesh ring net with a non-filtering cod end towed obliquely through the upper 50 m of the water column at 2 to 3 knots. Krill were kept in a cooler and transported back to the lab within 4 h. There, we distributed 1 healthy female per 500 ml jar of pre-equilibrated treatment water (described below), which was capped and held at 12°C. The following morning, all females were removed and any eggs were counted and left undisturbed in the 500 ml hatching jars.

Expt 1

Expt 1 was conducted at the National Oceanic and Atmospheric Administration Northwest Fisheries Science Center (NOAA NWFSC) in Seattle, WA. Treatment conditions were chosen to cover the broad range of pH conditions that *E. pacifica* currently experiences in Puget Sound (pH 8.0, 7.7, 7.4; pCO_2 400, 800, 1600 µatm, based on our field measurements described below) and may experience in the future (pH 7.3, 7.2; pCO_2 2400, 3200 µatm). The difficulty of collecting large numbers of ovigerous females from the field prevented us from populating all treatment conditions simultaneously. Instead, we conducted 5 separate trials to complete Expt 1, each with 2 pH treatments, using broods from a total of 57 different females. Hatching jars containing incubating krill eggs (or after hatching, larvae) were kept sealed in a 12°C water bath, with partial water changes every 2 d (described below) for 7 d, until approximately half of the larvae had reached the first feeding stage (C1). The eggs take approximately 36 h to hatch, so after 7 d, the larvae are approximately 5 d post hatch (dph). At the end of the experiment, living and dead larvae were sorted into separate groups and preserved in 5% buffered formalin for counting and staging. Hatching success was calculated from the initial egg counts and the number of hatched larvae (live + dead) found at the end of the experiment; survival was calculated over the duration of the larval phase (excluding egg mortality) as the proportion of hatched larvae that were alive at the end of the experiment; development was calculated as the proportion of the hatched larvae that had reached C1.

Seawater for each treatment was pH-conditioned by bubbling CO_2 gas and CO_2-free air generated with CO_2 adsorbers (Twin Towers Engineering) in 20 µm filtered natural seawater maintained at 12°C. Durafet pH electrodes (Honeywell Process Solutions) calibrated with a pH-certified Tris buffer (Dickson Laboratory, Scripps Institution of Oceanography) were used to monitor treatment water for the target values. Seawater for all treatments in a trial was conditioned in a single equilibration container, from which jars were filled when target pH levels were reached. Every 2 d, each jar was sampled for pH using a spectrophotometer (Ocean Optics USB 2000+ Fiber Optic Spectrometer) and m-cresol purple dye (Sigma Aldrich), after which 80% of the water was removed and replaced with freshly pH-conditioned treatment water. This resulted in 4 measurements of pH from each jar over the course of each 7 d trial. Discrete samples for TA of the treatment water were collected at the beginning and end of each experimental trial and measured by open-cell potentiometric titration according to SOP 3b in Dickson et al. (2007) at the NOAA NWFSC.

Expt 2

Expt 2 was conducted at the Shannon Point Marine Center in Anacortes, Washington, in a system of air-tight Plexiglas boxes supplied with atmospheric gas of pCO_2 treatment concentrations. This system can control pCO_2 in many individual containers through air–seawater diffusion, which allows tracking of individual larval development with minimal disturbance. Expt 2 consisted of 3 trials with 3 treatment levels; treatment conditions targeted pH 8.0, 7.7, and 7.6 (~400, 800, and 1200 µatm pCO_2). Broods from 53 different females were used in Expt 2 and eggs were spawned and counted as in Expt 1. On Day 5 of the experiment (3 dph), a portion of the larvae were gently pipetted from the hatching jars into glass petri dishes of pre-equilibrated seawater at a concentration of 1 larva per 20 ml. Half of the water in the hatching jars was then exchanged with seawater that had been pre-equilibrated to the treatment condition, and the remaining, undistributed larvae were left in these jars until Day 7, when they were sorted, preserved, and analyzed as in Expt 1.

On Day 6 (4 dph), larvae in petri dishes were checked for life stage and mortalities, 50% of the water in their dish was exchanged with pre-equilibrated seawater, and larvae were fed the alga *Heterocapsa triquetra* at 150 µg C l^{-1}. The krill larvae grown on this food source had visibly full guts, indicating that they fed well on it. Feeding and a 50% water change continued every second day for the remainder of the experiment. Survival and molting were tracked approximately daily for each individ-

ual. When larvae either died or molted to the C2 stage, they were removed from the experiment; thus survival was tracked to the C2 stage, but not beyond. Expt 2 consisted of 3 trials of 18, 23, and 19 d duration, by which time 85–100% of the larvae had either molted to C2 or died.

Treatment pCO_2-enriched seawater for experiment set-up and water changes was generated by stripping compressed (oil-less rotary compressor, Powerex) ambient air of CO_2 using CO_2 adsorbers (Twin Towers Engineering), and then mixing this CO_2-free air with pure CO_2 in precise concentrations using mass flow controllers (Sierra International). Ambient and CO_2 enriched air were bubbled into large containers of 0.2 µm filtered natural seawater for 24 h, or until the pCO_2 of headspace gas equaled the desired pCO_2 concentration. Treatment water was generated in a single equilibration container for each treatment level and then used to fill all jars in that treatment. CO_2 concentrations of the headspace gasses and gas streams into the headspace were measured using a Li-COR Li-820 CO_2 sensor. The hatching jars and petri dishes of larvae were left open inside airtight Plexiglas boxes (1 per pCO_2 level) supplied with atmospheric gas of the equivalent pCO_2 treatment concentrations in a 12°C temperature-controlled room. By supplying the airtight boxes with treatment gas, air–water gas exchange controlled the pCO_2 and bubbling was not needed. Carbonate chemistry of treatment conditions was verified with discrete DIC and pH samples taken in duplicate from the pre-equilibrated water each time it was dispensed, from the hatching jars on Days 1 and 5, and in the petri dishes prior to all feedings and water changes. Total DIC was measured using a Model AS-C3 total DIC analyzer, and spectrophotometric pH was measured using an Agilent 8453A UV-VIS diode array spectrophotometer.

Statistical analyses

In Expt 1, for which we have data only from the start and end of each trial, we assessed whether pH affected (1) the proportion of eggs that hatched, (2) the proportion of larvae that survived, and (3) the proportion of larvae that developed to the C1 stage. A mixed-effects logistic regression on a logit scale was used with the lme4 package (Bates et al. 2014) in R statistical software (R Core Development Team 2014). The 4 measurements of pH for each jar over the course of an experiment were averaged to define each jar's treatment conditions, and pH was converted to $[H^+]$ to define the model's fixed effect. Experimental trial and brood were considered as random effects in the model. Because *E. pacifica* eggs are sensitive to handling (Ross 1981), we did not split any broods and therefore brood and jar effects were confounded. Best-fit models were selected based on values of Akaike's information criterion corrected for small sample sizes (AICc; Burnham & Anderson 2002), and Hosmer-Lemeshow tests were used to verify the fit of the models.

In Expt 2, for which we have nearly continuous data on development and survival through each trial, survival and molting rates were analyzed using the survival package (Therneau 2014) in R statistical software (R Core Development Team 2014). Survival probabilities represent survival from 3 dph (Day 5), when live nauplii were initially moved to petri dishes for tracking to the C2 stage. We tested for differences between discrete treatments with a Cox proportional hazards model using robust variance estimates, which allowed the data to be clustered by brood. As in Expt 1, broods were not split, so brood and jar effects were confounded until Day 5, when larvae were distributed into petri dishes. This model does not allow for comparison of clustering by different factors, but preliminary data exploration indicated that petri dish alone, or together with brood, was not as predictive as brood alone. The Cox model gives the hazard ratio for 2 treatments, which describes the relative risk due to a treatment at any particular point in time. We also tested for pairwise correlations between hatching, survival, and development proportions of different broods with a logistic regression using the lme4 package (Bates et al. 2014) and Bonferroni correction in R statistical software (R Core Development Team 2014).

RESULTS

Field chemistry and krill distribution

In June, surface waters were warmer and less saline than in April, while deep waters had greater DIC and pCO_2, and lower pH. There was also a larger phytoplankton bloom in June than in April (chlorophyll maximum 10.9–22.7 mg m^{-3} and 1–2.2 mg m^{-3}, respectively), which corresponded with higher pH and lower pCO_2 in the surface waters in June. In April, pH measured by spectrophotometry was 8.11 in the surface waters, and pCO_2 (calculated from DIC and pH) was 320 µatm; in June, pH was 8.24 and pCO_2 was 209 µatm. Water chemistry was similar

between the 2 stations except in June when bottom waters at the deeper station (P14, 180 m) had higher DIC, pCO_2, and lower pH than at the shallower station (P15, 130 m; Fig. 2). Deep waters in April reached pH 7.61 (1101 µatm pCO_2) while in June, bottom waters were pH 7.56 (1241 µatm pCO_2) at Stn P15, and pH 7.48 (1516 µatm pCO_2) at Stn P14. When pH was calculated from TA and DIC, the profile was similar to that measured by spectrophotometry, but the absolute values were consistently lower (Fig. 3). Likewise, pCO_2 calculated from TA and DIC (not shown) was consistently higher than pCO_2 calculated from DIC and spectrophotometric pH (shown in Fig. 2). Water chemistry profiles were similar between day and night samplings; all profiles shown in Fig. 2 are from day samplings.

We observed much lower overall abundances of *Euphausia pacifica* eggs and larvae in April compared to June. The majority of larvae in April were C1 and C2 stages, which were concentrated in the surface waters (Fig. 3). In June, all early life stages were abundant. Again, the highest concentrations were observed towards the surface, but a large proportion of the population was also found below the pycnocline; approximately 37 to 42% of the eggs and nauplius 1 (first larval stage) were found below 20 m depth where the pH was <7.7, while 89 to 95% of nauplius 2 and metanauplius (second and third stages), 30 to 73% of C1 (fourth stage), and 68 to 89% of C2 (fifth stage) were below 20 m (Fig. 3). The nauplius 2 and metanauplius stages were distributed deepest, with 38 to 68% of nauplius 2 and 36 to 52% of metanauplii found below 50 m. Distributions of these early stages did not show differences between day and night samplings; all distributions shown in Fig. 3 are from day samplings.

Expt 1

Temperature was relatively stable within and between trials, with a mean ± SD of 12.4 ± 0.31°C (Table 1 and Table S1 in the Supplement at www.int-res.com/articles/suppl/m555p065_supp.pdf). Salinity was constant over the course of a trial (±0.26) and similar among trials, ranging from 29.1 to 30.0. The TA of the source water behaved similarly to salinity, staying relatively stable over the course of a trial (~14 µmol kg^{-1}) and ranging from 2002 to 2041 µmol kg^{-1} among trials. In both Expts 1 and 2, treatment conditions did not achieve our experimental targets with high accuracy, but were generally close and similar within treatments. pH conditions in Expt 1 varied among jars within a treatment as well as between water changes in the same jar (0.02–0.25 pH units); however, pH of the different treatments were distinct from each other (Table 1; Table S1). Average pH measured in the jars for the 5 target treatments of 400, 800, 1600, 2400, and 3200 µatm pC_2 was 7.88, 7.65, 7.40, 7.27, and 7.09; pCO_2 calculated from pH and TA was 565, 1004, 1833, 2509, and 3814 µatm, respectively, when pooled across trials.

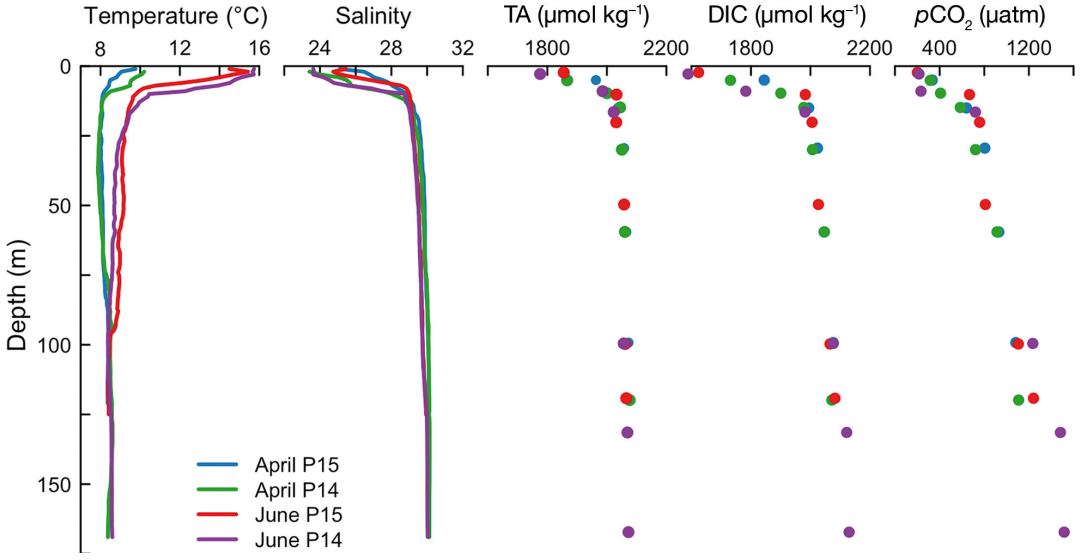

Fig. 2. Seawater chemistry in Puget Sound. Depth profiles of temperature, salinity, total alkalinity (TA), total dissolved inorganic carbon (DIC) and pCO_2 from stations P14 and P15 during April and June. Temperature and salinity were measured continuously with a CTD while TA and DIC were measured from discrete water samples. pCO_2 is calculated from discrete DIC and spectrophotometric pH measurements

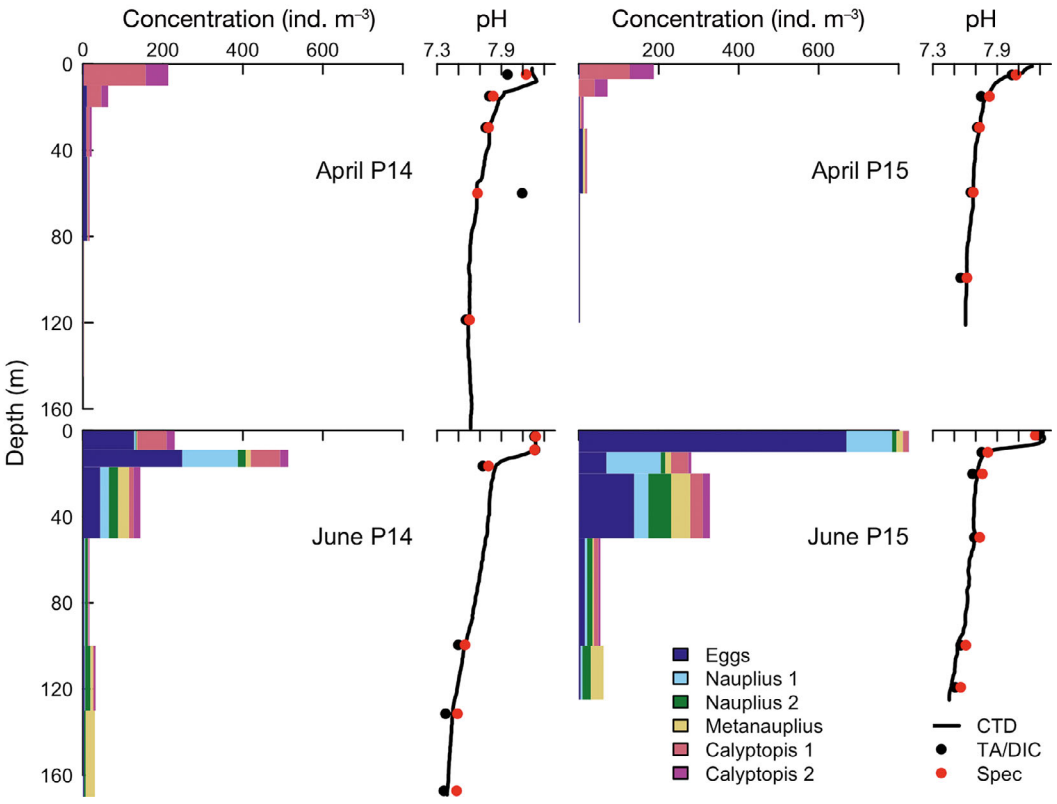

Fig. 3. Vertical distribution of *Euphausia pacifica* early life stages and their pH exposure in Puget Sound. Life stages listed youngest to oldest, with eggs closest to the *y*-axis and increasingly older stages to the right. Depth profiles of pH were calculated from discrete total alkalinity (TA) and dissolved inorganic carbon (DIC) samples (TA/DIC), and measured by spectrophotometry from discrete samples (spec). Continuous measurements from the CTD pH probe (black line) show the shape of the curve but have been shifted to approximately fit the discrete samples. The CTD probe was not calibrated for absolute measurements

Table 1. Treatment conditions for *Euphausia pacifica* during Expt 1. Average conditions grouped by experimental trial and target pCO_2 treatment, with the standard deviation of (n) measurements. Temperature, salinity, total alkalinity (TA), and pH were measured; pCO_2 and dissolved inorganic carbon (DIC) were calculated

Trial	Target pCO_2	No. of broods	Temperature (°C)	Salinity	TA (µmol kg^{-1})	pH (total scale)	pCO_2 (µatm)	DIC (µmol kg^{-1})
1	400	5	12.2 ± 0.1 (20)	29.1	2030	7.87 ± 0.03 (19)	585 ± 47 (19)	1938 ± 10 (19)
	3200	1	12.2 ± 0.1 (4)			7.08 ± 0.04 (4)	3890 ± 358 (4)	2173 ± 17 (4)
2	800	4	12.2 ± 0.1 (11)	29.2 ± 0.1 (2)	2022 ± 28 (2)	7.68 ± 0.06 (12)	942 ± 149 (12)	1989 ± 30 (12)
	2400	5	12.2 ± 0.1 (14)			7.29 ± 0.03 (15)	2397 ± 188 (15)	2096 ± 24 (15)
3	800	10	12.7 ± 0.9 (40)	29.2 ± 0.1 (2)	2022 ± 28 (2)	7.64 ± 0.03 (40)	1037 ± 77 (40)	2001 ± 20 (40)
	2400	5	13.1 ± 1.0 (20)			7.26 ± 0.07 (20)	2621 ± 484 (20)	2108 ± 29 (20)
4	400	4	12.3 ± 0.1 (16)	30	2021	7.89 ± 0.04 (16)	547 ± 55 (16)	1918 ± 13 (16)
	3200	4	12.3 ± 0.1 (16)			7.09 ± 0.04 (16)	3796 ± 402 (16)	2158 ± 19 (16)
5	400	10	12.2 ± 0.1 (40)	29.5 ± 0.2 (2)	2029 ± 6 (2)	7.88 ± 0.03 (38)	560 ± 37 (38)	1931 ± 7 (38)
	1600	9	12.3 ± 0.1 (36)			7.40 ± 0.03 (35)	1837 ± 114 (35)	2063 ± 8 (35)

All 3 biological responses varied significantly among broods. Model results indicated that survival and development, but not hatching, were also significantly affected by pH (Fig. 4; Table S2 in the Supplement). Average hatching in the 400, 800, 1600, 2400, and 3200 µatm pCO_2 target treatments was 31, 42, 34, 33, and 21%, respectively. The proportion of larvae that survived decreased at pH levels <7.4 (Fig. 4b); average survival in the 400, 800, 1600, 2400, and 3200 µatm pCO_2 target treatments was 90, 94, 58, 83, and 86%, respectively. The proportion of larvae to reach the C1 stage strongly decreased as pH

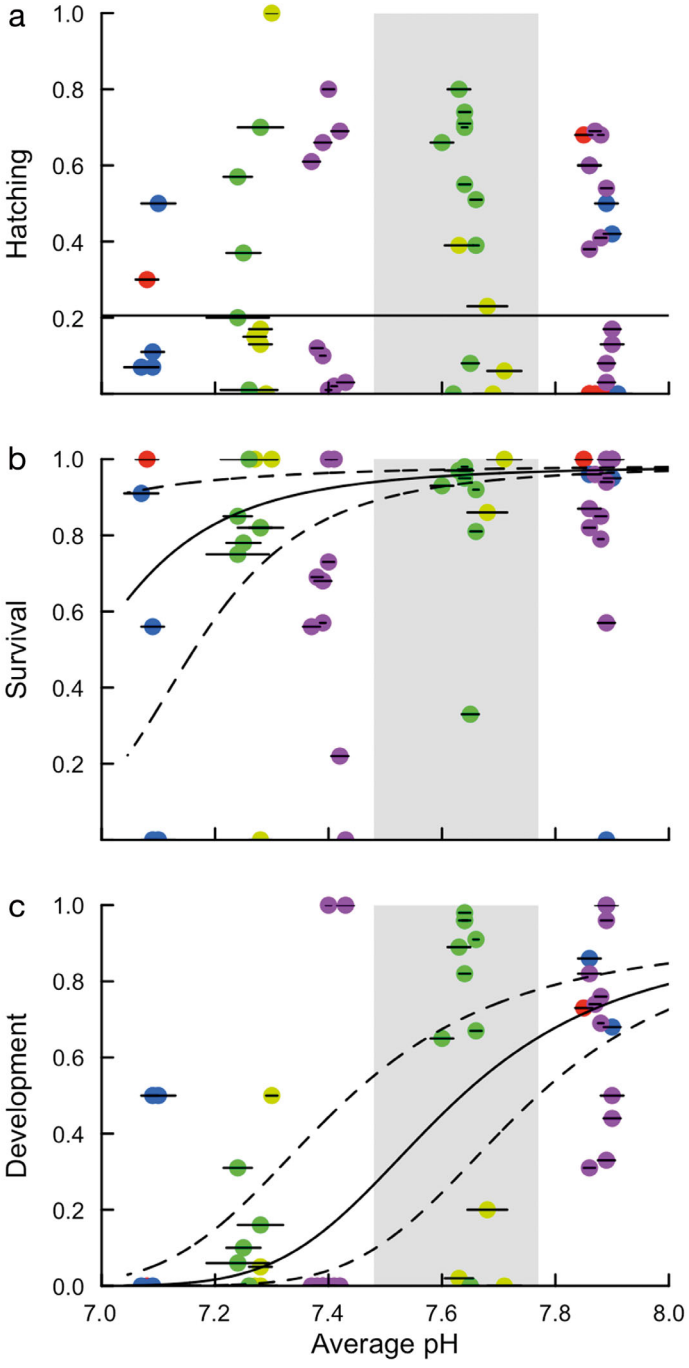

Fig. 4. *Euphausia pacifica* hatching, survival, and development to the calyptopis 1 (C1) stage as a function of pH in Expt 1. Each point represents the final proportion of a single female's brood that (a) hatched, (b) survived, and (c) developed to the C1 stage. Broods that were tested during the same trial share the same color. Horizontal error bars show the standard deviation from 4 pH measurements taken in each jar over the trial. Solid lines show the best-fit model, and dashed lines show the 95% CI for the effect of $[H^+]$ (no effect of $[H^+]$ detected on hatching). Shaded area shows the range of pH values observed in Hood Canal below the pycnocline in June

declined, starting at pH <7.8 and declining rapidly below pH 7.6 (Fig. 4c). Results from Hosmer-Lemeshow tests indicate that all 3 best-fit models fit the data (Hatching χ^2 = 0.1046, Survival χ^2 = 3.1262, Development χ^2 =1.2078; number of groups = 10).

Expt 2

Salinity and TA in Expt 2 varied among source water, hatching jars, and larvae dishes of the same treatment due to evaporation in the petri dishes (Table 2). Measured pH and calculated pCO_2 conditions also varied among different water types of the same treatment, but were generally consistent among broods and trials (Table 2; Table S3 in the Supplement). Average pH measured in the source water was higher than that measured in the hatching jars and petri dishes. Most of the larval development (3 dph to end of experiment) occurred in the petri dishes, where average pH was 7.96, 7.69, and 7.58 and calculated pCO_2 was 492, 956, and 1279 µatm, respectively; hereafter, these values are used to describe the treatment conditions, recognizing that the conditions the larvae experienced varied over time.

Hatching in Expt 2 was, again, variable among broods and not significantly different among treatments (Fig. 5a; Table S4 in the Supplement); average hatching in 400, 800, and 1200 µatm pCO_2 target treatments was 60, 72, and 68%, respectively. The larvae that were tested for survival and development through the C1 stage (as done in Expt 1 under a wider range of pH treatments) had high survival to 5 dph across all treatments and no differences among treatments (Fig. 5b; Table S4); average survival in the 400, 800, and 1200 µatm pCO_2 target treatments was 95, 97, and 97%, respectively. The proportion of larvae that had reached the C1 stage by 5 dph decreased as pH declined (Fig. 5c; Table S4). Results from Hosmer-Lemeshow tests indicate that the models fit the data (Hatching χ^2 = 0.01, Survival χ^2 = 0.255, Development χ^2 = 0.079, number of groups = 10).

The probability of larvae surviving to the C2 stage decreased at pH 7.69 (956 µatm pCO_2) and pH 7.58 (1279 µatm pCO_2), compared to pH 7.96 (492 µatm pCO_2). Average mortality before the C2 stage in 400, 800, and 1200 µatm pCO_2 target treatments was 26.5, 50, and 45.7%, respectively. Survival probability in pH 7.69 and pH 7.58 treatments was similar (Fig. 6a; log rank test: pH 7.96–7.69 contrast: χ^2 = 44.4, df = 1, p < 0.0001; pH 7.96–7.58 contrast: χ^2 = 38.1, df = 1, p < 0.0001; 7.69–7.58 contrast: χ^2 = 0.3, df = 1, p = 0.59). The Kaplan-Meier survival esti-

Table 2. Treatment conditions for *Euphausia pacifica* during Expt 2. Average conditions grouped by water type and target pCO_2 treatment with the standard deviation of (n) measurements taken over 3 trials. Temperature, salinity, dissolved inorganic carbon (DIC), and pH were measured; total alkalinity (TA) and pCO_2 were calculated

Target pCO_2	Temperature (°C)	Salinity	TA (µmol kg^{-1})	DIC (µmol kg^{-1})	pH (total scale)	pCO_2 (µatm)
Source water						
400		31 ± 1 (87)	2060 ± 46 (87)	1931 ± 50 (29)	7.98 ± 0.01 (29)	435 ± 3 (29)
800				2005 ± 38 (29)	7.72 ± 0.03 (29)	846 ± 11 (29)
1200				2030 ± 35 (29)	7.59 ± 0.03 (29)	1144 ± 15 (29)
Egg jars						
400	12.2 ± 0.4 (121)	31 ± 1 (33)	2110 ± 77 (33)	1991 ± 50 (10)	7.90 ± 0.04 (10)	553 ± 14 (10)
800				2058 ± 74 (12)	7.69 ± 0.04 (12)	922 ± 21 (12)
1200				2113 ± 86 (11)	7.55 ± 0.04 (11)	1318 ± 34 (11)
Larvae dishes						
400	12.3 ± 0.6 (1235)	33 ± 2 (110)	2239 ± 109 (109)	2092 ± 83 (37)	7.96 ± 0.05 (37)	492 ± 10 (37)
800				2161 ± 81 (36)	7.69 ± 0.03 (36)	956 ± 9 (36)
1200				2228 ± 120 (36)	7.58 ± 0.05 (36)	1279 ± 23 (36)

mates shows the point-wise 95% CI for the pH 7.96 treatment does not overlap with either the pH 7.69 or the pH 7.58 treatments until after Day 17 when the number of remaining larvae (n) became very small, indicating that survival was higher in the pH 7.96 treatment from Day 6 through the duration of the experiment (Fig. 6a). Survival data (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m555p065_supp.pdf) supports the proportional hazards assumption and use of the Cox Proportional Hazards model because the estimated −ln(−ln) survivor curves are nearly parallel (Kleinbaum & Klein 2005). The model yielded a survival hazard ratio of 2.29 (95% CI: 1.66–3.16) for the pH 7.69 treatment compared to pH 7.96, and 2.08 (95% CI: 1.42–3.04) for the pH 7.58 treatment compared to pH 7.96. In this application, hazard ratios describe the relative risk of dying at any particular point in time, with a higher ratio describing lower survival in the lower pH treatment compared to pH 7.96.

The probability curves for molting to C2 are statistically distinguishable among treatments (log rank test: $\chi^2 = 5.99$, df = 2, p = 0.05), but visual examination of the curves indicates that there was little difference until the end of the experiment, when the number of

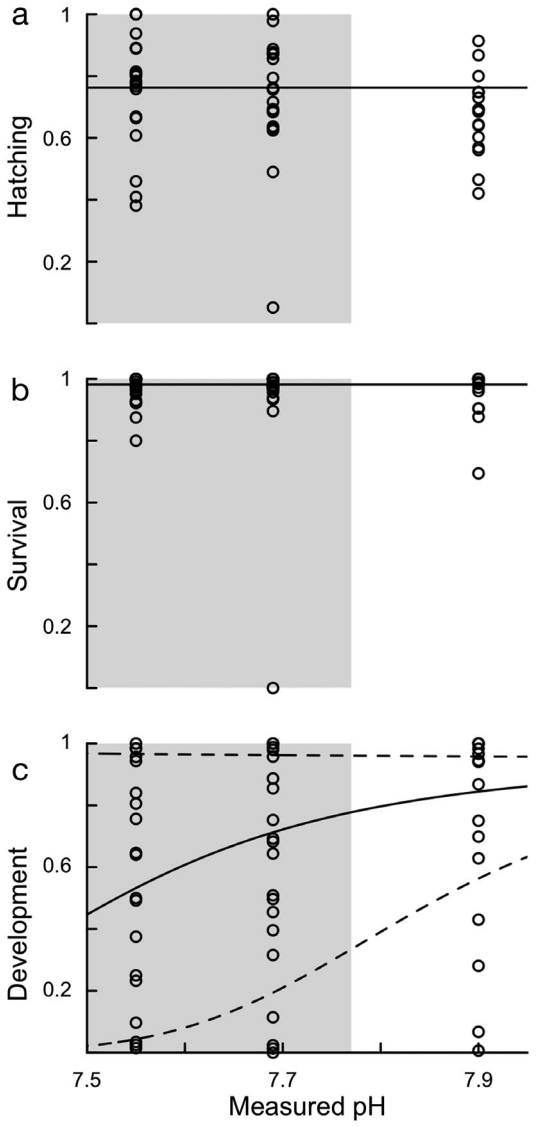

Fig. 5. *Euphausia pacifica* hatching, survival, and development to the calyptopis 1 (C1) stage as a function of pH in Expt 2. Each point represents the proportion of a single female's brood that (a) hatched, (b) survived, and (c) developed to the C1 stage at 5 d post hatch. Solid lines show the best-fit model and dashed lines show the 95% CI for the effect of $[H^+]$ (no effect of $[H^+]$ detected on hatching or survival). Shaded area shows the range of pH values observed in Hood Canal below the pycnocline in June

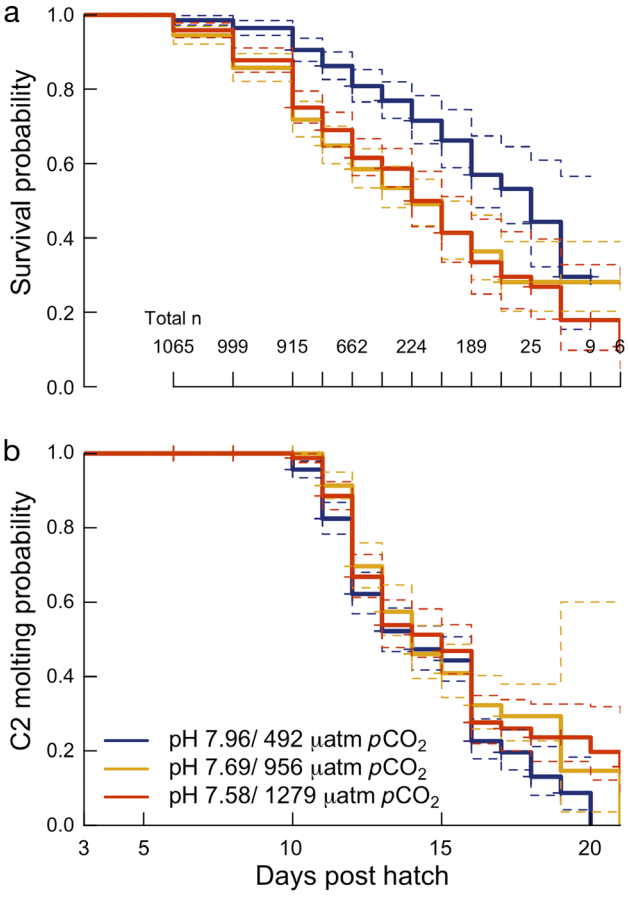

Fig. 6. *Euphausia pacifica* survival probability and probability of molting to the calyptopis 2 (C2) stage in Expt 2. (a) Survival probability (thick lines) of larvae from 3 d post hatch to the C2 stage raised at pH 7.96, pH 7.69, and pH 7.58. (b) Probability of larvae molting to the C2 stage (thick lines). Dashed lines show 95% CI. Total number of larvae remaining in all treatments (n) shown above *x*-axis in panel (a)

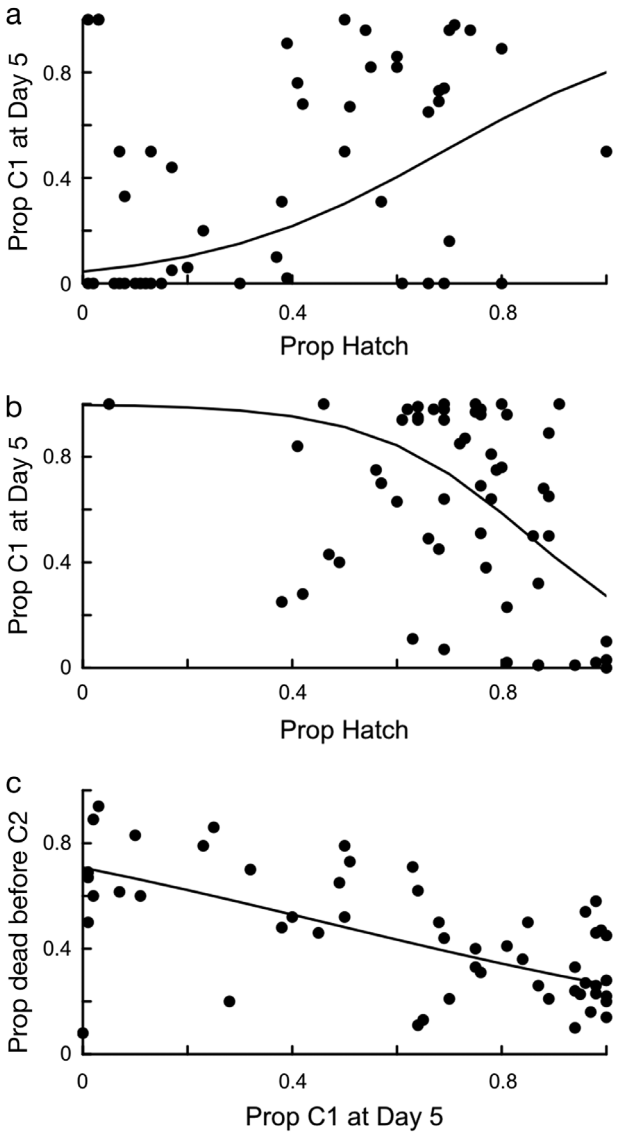

Fig. 7. Relationships among measured parameters of different broods of *Euphausia pacifica*. Statistically significant relationships were found between (a) the proportion (Prop) of larvae that reached the calyptopis 1 (C1) stage by 5 d post hatch (dph) and the proportion that hatched in Expt 1 (adjusted p = 0.04), (b) the proportion of larvae that reached C1 by 5 dph and the proportion that hatched in Expt 2 (adjusted p = 0.02), and (c) the proportion of larvae that died before reaching C2 and the proportion that reached C1 by 5 dph in Expt 2 (adjusted p < 0.0001)

animals in each treatment group was very small (Fig. 6b). Log rank tests put more weight on differences towards the end of the data set; to test for the influence of this weighting on our results, we truncated the dataset to Day 17 and re-ran the log rank test. With the truncated dataset, differences among treatment groups were no longer significant ($\chi^2 = 4.5$, df = 2, p = 0.11). The Wald and likelihood ratio tests were both non-significant on the entire data set (5.98 and 5.93, df = 2), indicating that molting probabilities were similar among treatments.

There was a significant negative relationship between the proportion of a female's brood that reached the C1 stage at 5 dph and the proportion that died before reaching the C2 stage (p < 0.0001; Fig. 7c). There were also statistically significant relationships between hatching success of broods and their development to the C1 stage at 5 dph in both experiments, but the direction of the correlation was not consistent (Expt 1: positive relationship, p = 0.041; Expt 2: negative relationship, p = 0.022; Fig. 7a,b). There were no significant relationships between hatching success and the proportion that died before reaching the C2 stage or the proportion alive at 5 dph.

DISCUSSION

We found that the early life stages of *Euphausia pacifica* are naturally exposed to a wide range of carbonate chemistry conditions in Puget Sound, ranging from pH 8.3 to 7.5 during their egg to C2 stages. Two sets of experiments in the laboratory indicated that the pH conditions currently experienced by this species in sub-surface waters of Puget Sound can lead to developmental delays and decreased larval survival.

Our field surveys showed that in June, significant proportions of *E. pacifica* early life stages are found between 20 and 50 m water depth, where they are exposed to low pH/high CO_2 waters. Vertical distributions of *E. pacifica* early life stages are not commonly reported in the literature, but the depth distributions we observed are similar to a published distribution in the southern Yellow Sea, where *E. pacifica* eggs are distributed below 20 m in the high chlorophyll layer, nauplius 1 are distributed below 10 m, nauplius 2 and metanauplius extend nearly the whole water column, and most C1 and C2 are restricted to the upper 30 m (Liu & Sun 2010). We observed pH values of 7.7 to 7.5 below the pycnocline in Hood Canal, which are similar to those observed by Feely et al. (2010) in the same region in 2008, indicating that such low pH conditions frequently occur in this area. Seawater pH values below 7.7 are also observed below the pycnocline in the Strait of Juan de Fuca (Feely et al. 2010) and on the continental shelf of the US west coast (Feely et al. 2008), where *E. pacifica* is abundant. Salinity and TA profiles were similar among months, whereas DIC concentrations in mid and deep waters were higher in June than in April — likely due to increased respiration — and contributed to lower pH and higher pCO_2 at depth at the time when *E. pacifica* was also more abundant.

E. pacifica hatching in the laboratory was similar across a wide range of pH conditions, but exposure to low pH slowed development and decreased survival of the larvae. In contrast, *E. superba* hatching success decreased at pH 7.5/pCO_2 1250 µatm in laboratory studies (Kawaguchi et al. 2011, 2013). Our study is the first to look at larval development of krill under increased pCO_2 through several life stages; *E. superba* larvae have only been observed under elevated CO_2 (1000 µatm) for up to 3 d post hatch, with no observed effects on larval swimming activity (Kawaguchi et al. 2011). We observed developmental delays in non-feeding stages (development to the C1 stage) and increased mortality in feeding stages (mortality before the C2 stage). In this study, 50% of the larvae reared at pH 7.69 died before the C2 stage, compared to 27% of larvae reared at pH 7.96. This decline occurred over just the first 20 d of life; it takes *E. pacifica* an average of 45 d to reach the juvenile stage (Ross 1981). Both developmental delays and increased mortality are ecologically important for *E. pacifica* populations. Although some general trends in pH sensitivity across taxa are emerging (Dupont et al. 2010, Hofmann et al. 2010, Kroeker et al. 2013), the idea that crustaceans are generally tolerant (Whiteley 2011, Kroeker et al. 2013) should be carefully considered and re-examined after more species and life stages have been studied.

Our results indicate that *E. pacifica* developmental stages are differently affected by low pH conditions. Larvae were slower to molt into the C1 stage at lower pH levels in both experiments; the highest pH at which this occurred was pH 7.69 (956 µatm pCO_2) in Expt 2. However, we did not find a difference in time to molt to the C2 stage at this pH level (as measured by molting probability), an apparent conundrum that is discussed below. Larval survival decreased with pH in both experiments, but again with differences between stages. Survival to 5 dph was not affected by pH 7.69 or 7.58 (956 and 1279 µatm pCO_2), but survival to the C2 stage was decreased at those pH levels (as indicated by hazard ratios >1). The C1 stage is one of the longest developmental stages in the early life cycle of *E. pacifica*; as the first feeding stage, it involves a large change in physiology and behavior, and may represent a bottleneck for their larval development (Feinberg et al. 2006). The transition to C1 is also the first true molt; although we did not determine whether the observed mortalities occurred during the molting process, increased pCO_2 has been reported to increase the frequency of molting-related deaths in sub-adults of Atlantic krill (Sperfeld et al. 2014), so the differences in mortality among treatments we observed after transition to C1 may have been due to the energetic requirements associated with molting.

Increased energy expenditures early in life can lead to latent or carryover effects that influence later performance (Pechenik 2006). The mono-algal diet supplied in this laboratory study was potentially less nutritious than food available in the field, which could increase the importance of carry-over effects from non-feeding stages; however, we observed slowed development to the C1 stage during life stages when larvae are completely dependent on endogenous energy sources, indicating that the larvae were stressed by reduced pH even before they began feeding. The mortality and development rates we observed in our control conditions are similar to

those reported in previous laboratory studies, giving no clear indication of extra stress induced by the rearing methods (Ross 1981, Feinberg et al. 2006).

Females' broods that developed faster over the first 5 d after hatching had higher survival to the C2 stage, as indicated by the negative correlation between the proportion of a female's brood that reached C1 by 5 dph and the proportion that died before reaching C2. This may indicate that there are different inherent susceptibilities to pH in the population, which could explain the differences observed between the effects of pH on development rate to C1 and C2 stages. If individuals which had slower growth to the C1 stage were also more likely to die before reaching the C2 stage, our measure of time to molt to C1 would include both susceptible and robust individuals, whereas the measure of time to molt to C2 would have a larger proportion of robust individuals because of the higher mortality among susceptible individuals. There were also statistically significant relationships between the proportion of larvae that reached the C1 stage by 5 dph and the proportion of eggs that hatched in Expts 1 and 2. However, the sign of this relationship differed between experiments, and visual examination of those data shows that although statistically significant, this relationship is unlikely to be biologically relevant (Fig. 7).

We observed high variability in all measured parameters (hatching, survival, development) among broods, which may indicate heritable differences among individuals. We left each brood intact and undisturbed before hatching, so experimentally, this variability combines potential differences among individual jars and parental effects, which in turn includes both heritable genetic factors and physical condition based on environmental experience, diet, etc. However, we expect that parental effects primarily drove the variability we observed. This work and other studies of euphausiids (Ross 1981, Kawaguchi et al. 2013) have shown high inter-brood variability in hatching, and we also observed inter-brood variability in later development after larvae from each brood were distributed from their individual jars into multiple petri dishes. Furthermore, similar experiments on copepods in which each female's brood was split into multiple jars showed that the variability among broods was larger than that within broods split across different jars (unpublished data). We cannot determine from our data whether this inter-brood variability is due to heritable factors or environmental experience. If there is a subset of the population that is more resilient to low pH, and this resilience is due to genetic factors, those individuals may represent the potential for this population to adapt to low pH, as has been observed in other marine invertebrates (Parker et al. 2012, Kelly et al. 2013).

E. pacifica showed negative responses to low pH in the laboratory at levels that occur within their current field distribution — pH levels that are expected to become more common in the future, which may threaten this species through a decline in suitable habitat. These results highlight the need to conduct OA experiments within the context of an organism's habitat to understand responses to both current and future conditions (McElhany & Busch 2013). Although atmospheric CO_2 levels of 950 ppm are not predicted to occur until after 2100, seawater pCO_2 at these and higher levels are currently observed *in situ* within areas of *E. pacifica* habitat. *E. pacifica* free spawn their eggs at night near the surface where the pH is most often high, but as the eggs and early larvae sink through the water column, they experience decreased pH at intermediate depths until they are strong enough to swim back towards the surface.

Anthropogenic carbon emissions since the preindustrial era have resulted in pH declines in Puget Sound of up to 0.11 in the surface waters and 0.06 in deep waters (Feely et al. 2010); these changes are small compared the values we observed (pH 7.56–7.48 in deep waters) indicating that low pH habitats existed in Puget Sound before the industrial revolution. Despite this, the *E. pacifica* larvae we reared in the laboratory were sensitive to these pH conditions. Because time series data on euphausiids in Puget Sound are scarce, we are unable to conjecture about how their populations may be currently shaped by pH. In the CCE, krill are concentrated near highly productive upwelling zones that are also places of low pH due to the upwelling of high-pCO_2 deep waters to the surface (Santora et al. 2012). This co-occurrence of low pH with high food levels could lead to tradeoffs between food availability and suitable pH conditions, which may become rarer as the pH of upwelled waters declines. In some organisms, abundant food has been shown to offset the energetic costs of compensating for low pH (Melzner et al. 2011, Thomsen et al. 2013); this has not yet been demonstrated in euphausiids, but could confer some resilience if food levels increase with pCO_2 in the field.

In both our study and the *E. superba* experiments of Kawaguchi et al. (2011, 2013), the females extruded eggs directly into pH-manipulated seawater, but effects of pH on oogenesis and fertilization were not tested. We are unaware of any work that has tested for these effects in krill; however, evidence from copepods suggests that male exposure has fur-

ther negative effects on offspring compared to when females alone are exposed (Cripps et al. 2014). The effects of parental exposure and acclimation may be important in krill, but cannot easily be tested for in this species because they have not been successfully mated in the laboratory.

In both experiments, the CO_2-equilibrated water for each treatment was generated in a single equilibration container, a pseudoreplication issue recently identified by Cornwall & Hurd (2016) as common among CO_2 manipulation experiments. In this study we have partially accounted for this through replication in time and through statistical techniques. Expt 1 was partially replicated in time: the 400 vs. 3200 µatm pCO_2 and 800 vs. 2400 µatm pCO_2 trials were each repeated, allowing for trial to be included in the mixed model. Three separate trials that overlapped in time were conducted in Expt 2, and trial was included as a random factor in the statistical model. Logistical constraints always influence experimental design, and we attempted to maximize the number of different females we tested because of the known variability among broods from different females. We conclude that the weight of evidence from 2 completely separate experimental systems is compelling despite the less-than-ideal experimental design.

E. pacifica early life stages show developmental delays and significantly decreased survival in the laboratory under pH conditions to which they are currently exposed in the field. This and other studies continue to demonstrate the importance of OA experiments that are designed in the context of the organisms' habitat, target the most vulnerable life stages, and are long enough to detect carryover effects. This study, along with the other limited OA work on krill, indicates that krill, particularly the early stages, may be more sensitive to OA than other crustaceans, which could have profound implications for the future of their populations and the organisms that prey on them. The negative response of *E. pacifica* larvae to reduced pH in this laboratory study indicates that they may be living at the edge of their current tolerance.

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