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Prior exposure to low salinity affects the vertical distribution of *Pisaster ochraceus* (Echinodermata: Asteroidea) larvae in haloclines

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ABSTRACT: Increased freshwater discharge during spring/summer from the Fraser River into Puget Sound makes this region ideal to study the effects of low salinity (~20‰) on the vertical distribution of marine invertebrate larvae. We investigated whether exposing early developmental stages of the sea star *Pisaster ochraceus* to low salinity for 3, 7, and >25 d affects larval morphology and the ability of later developmental stages (brachiolariae) to swim to the surface in the presence or absence of haloclines. We also determined the effect of food patches at the halocline on swimming behavior. Larvae reared in low salinity throughout development had 55–95% shorter posterolateral arms than those in other treatments. In the absence of a halocline, exposure to low salinity for 7 or >25 d reduced the percentage of brachiolariae that swam to the surface (3 and 30%, respectively) compared to brachiolariae held for 0 or 3 d at low salinity (64 and 84%, respectively). Brachiolariae with no exposure to low salinity remained around the halocline in the presence of food, while those reared in continuous low salinity swam directly to the surface. Brachiolariae acclimated to low salinities and then transferred to a stratified water column avoided the halocline even in the presence of food. We conclude that exposure of early developmental stages to low salinity affects the swimming ability of brachiolariae. Our results suggest that sea star populations in the Pacific Northwest could be threatened if the influence of the Fraser River during the spring/summer continues to intensify in the southern Puget Sound region.

KEY WORDS: Sea stars · Bipinnariae · Brachiolariae · Morphology · Posterolateral arm length · Vertical distribution · Halocline · Salinity

INTRODUCTION

Tremendous amounts of ice melt in recent decades have decreased the salinity of surface waters of the Pacific Ocean, including the Salish Sea in the Pacific Northwest, by 0.1–0.5‰ since 1950. Based on climate models, salinity in this region is likely to continue decreasing as a result of increased ice melt and precipitation (Stocker et al. 2013). The Salish Sea, which encompasses Washington State's (USA) Puget Sound, the Strait of Juan de Fuca, and the San Juan Islands and British Columbia's (Canada) Gulf Islands and the Strait of Georgia, also receives freshwater input from several rivers, including the Fraser River, the second largest in the region. The rivers move meltwater from several mountain ranges surrounding the Salish Sea, viz. the Olympic Mountains and the Vancouver Island range on the west, and the Cascade range on the east.

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Riche et al. (2014) recently noted that the number of days during the year when the temperature of the Fraser River is above 18°C has increased from roughly 10–50 to 50–100. The higher surface temperatures together with substantial regional runoff create stratified conditions in the Salish Sea. Mixed brackish water (20–30‰) is found in the surface layers to 5–20 m in depth, and saline waters (34‰) from the Pacific occupy the lower layers (Masson & Peña 2009, Khangaonkar et al. 2011, Sutherland et al. 2011; Fig. 1A). The effects of the Fraser River in the San Juan region are generally determined by wind direction and strong tidal currents. Data collected between 2011 and 2014 at 1.7 m depth revealed high amplitude (from 21 to 30‰) and frequency (3–6 events) of salinity fluctuations (Fig. 1B) during late spring and summer directly linked to increased freshwater discharge from the Fraser River. Unfortunately, these fluctuations coincide with the reproductive season of many marine invertebrates in the region.

Repeated fluctuations in salinity could have devastating effects on the population abundance of marine invertebrates, especially stenohaline osmoconformers with limited ability to regulate their internal ion content. Planktotrophic larval stages of these invertebrates could experience increased larval mortality (Stickle & Diehl 1987), decreased developmental rates (Pia et al. 2012, Bashevkin & Pechenik 2015), and decreased ability to swim through haloclines (Lance 1962, Metaxas & Young 1998a,b,c, Lougee et al. 2002).

The strength and duration of stratification are important in initiating and sustaining phytoplankton blooms in the Puget Sound region (Masson & Peña 2009, Durham & Stocker 2012, Benoit-Bird et al. 2013, Benoit-Bird & McManus 2014). Thin phytoplankton layers (a few cm to 5 m thick) are found at the base or within haloclines, pycnoclines, or thermoclines (Dekshenieks et al. 2001, Erga et al. 2003, McManus et al. 2012, Benoit-Bird et al. 2013, Sevadjian et al. 2014, Benoit-Bird & McManus 2014). These thin layers have the potential to attract predator aggregations (Durham & Stocker 2012, Sevadjian et al. 2014, Benoit-Bird & McManus 2014) as observed in several laboratory studies (Vázquez & Young 1996, Lougee et al. 2002, Menden-Deuer & Grünbaum 2006, Sameoto & Metaxas 2008, Arellano et al. 2012).

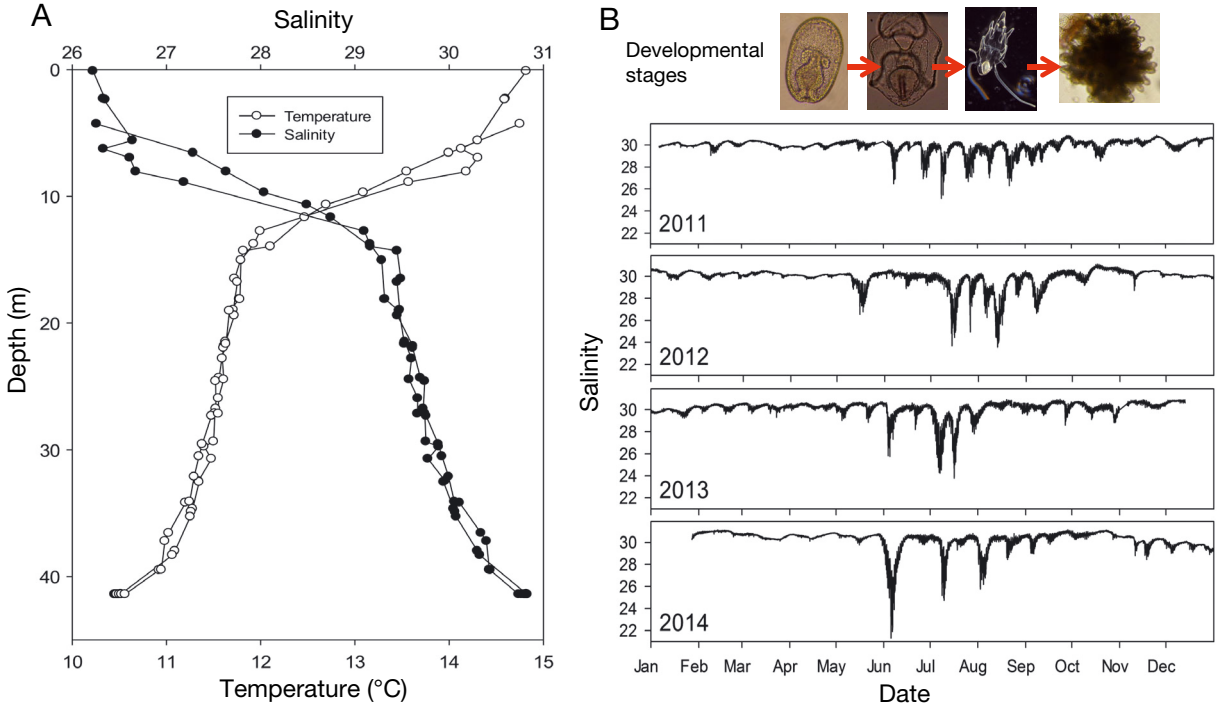

Fig. 1. (A) Depth profile of seawater salinity and temperature near Cantilever Point, Friday Harbor, WA, 2 August 2014. Samples were taken sequentially from 4 to 41 m and then back to the surface over approximately 15 min. These samples were taken during a freshening event at Friday Harbor Laboratory (see panel B); the thermohalocline is otherwise less well defined (data not shown). (B) Salinity at Cantilever Point, 2011–2014. Data recorded hourly at 1.7 m depth. Note salinity is typically ~30 psu in winter; freshening events occur in spring and summer, often at 2 wk intervals. Pictures indicate approximate times when the gastrula, bipinnaria, brachiolariae, and juvenile stages of *Pisaster ochraceus* are found in the water column in the San Juan Islands, WA. Pictures not to scale

Predators use specific adaptations to locate and exploit prey. For example, laboratory studies revealed that the swimming speeds and turning rates of the planktonic predator *Oxyrrhis marina* increased significantly in the presence of a 5 mm thin phytoplankton layer of *Isochrysis galbana* (Menden-Deuer & Grünbaum 2006). They noted that these behaviors increased effective prey availability to the predators.

A keystone marine invertebrate species in the Pacific Northwest is the sea star *Pisaster ochraceus* (Paine 1966, Menge & Sanford 2013). The spawning season of *P. ochraceus* is typically late March through June, though a few ripe individuals can be found as late as mid-July (Strathmann 1987, Sanford & Menge 2007, Menge & Sanford 2013), and depending on food availability and physical conditions, the larvae can remain in the water column for as little as 39 d (George 1999) or up to over 228 d (Strathmann 1987). Larvae (gastrula, bipinnaria, and brachiolaria stages) could thus be exposed to multiple low-salinity events in surface waters during their development (Fig. 1A,B).

In a recent laboratory study, Pia et al. (2012) observed salinity-induced morphological changes in *P. ochraceus* larvae. Brachiolariae reared at 32‰ throughout development were long and slender with long posterolateral arms, while those exposed to 20‰ for 3 or 14 d were short and wide with short posterolateral arms. These pronounced shape changes lasted for 37 d. Strathmann (1971) noted that the posterolateral arms of this sea star are mainly used for swimming. However, these studies did not address the effect of morphological changes on the swimming ability of brachiolariae in haloclines.

Although studies have shown that larvae are capable of avoiding low-salinity waters, with older larvae being more sensitive (Sameoto & Metaxas 2008, Arellano et al. 2012), the effects of prior exposure to low salinity on the vertical distribution of *Pisaster* larvae have not been addressed. Larvae reared in low salinity that develop shorter posterolateral arms might not be able to increase their swimming speeds and turning rates in the presence of food at the halocline. Furthermore, although haloclines are a regular feature in many places (Lougee et al. 2002, Garza & Robles 2010, Khangaonkar et al. 2011, Sutherland et al. 2011), only a few studies have observed the behavior of planktonic organisms in haloclines over a 24 h period (Pennington & Emlet 1986, Lougee et al. 2002).

The goals of the present study were to (1) demonstrate that exposing embryos and larvae of *P. ochraceus* to low salinity during development affects larval morphology including posterolateral arm length used for swimming and (2) determine the vertical distribution of later developmental stages (brachiolariae) in cylinders with and without food. The later developmental stages were used because their vertical distribution in cylinders with and without a halocline can be easily quantified. Furthermore, this stage is characterized by the development and elongation of 5 pairs of larval arms (anterodorsal, preoral, postoral, posterodorsal, and posterolateral arms) used specifically for swimming (Strathmann 1971, Lacalli 1996, George 1999, Pia et al. 2012).

MATERIALS AND METHODS

Collection site

To determine the effect of salinity fluctuations on the vertical distribution and behavior of larvae in haloclines, 2 separate sets of experiments were carried out in June 2011 and 2012. In each year, 7 adult *Pisaster ochraceus* were collected along the rocky intertidal coast at Cantilever Point, Friday Harbor Laboratories (FHL), Washington, USA (48° 32′ 46″ N, 123° 0′ 46″ W). Sea stars were collected from this site because freshening events (reduction from the typical salinity of 30‰ during spring and summer) are commonly observed at 1.7 m depth (SeaBird MicroCats; SBE37, Seabird Electronics; Fig. 1B). These events are due to runoff from the Fraser River and often occur at 2 wk intervals. The timing, frequency, and magnitude vary from year to year, due to variations in river flow, tidal exchange, and wind speed and direction. A second sensor was used to profile water conditions with depth to show that higher water temperatures were associated with these freshwater intrusions, which can extend down to 15 m depth (Fig. 1A).

Spawning, fertilization, and larval rearing

In 2011, 4 adult *P. ochraceus* were injected with 2 ml of 10^{-4} M 1-methyladenine to induce spawning. The eggs of 2 of the females were washed with 0.45 µm filtered seawater (FSW) to remove any debris, and a sample was photographed with the use of a video camera attached to a compound microscope. Egg diameter was measured using imageJ (mean ± SE, 174.5 ± 8.5 µm, n = 60). The rest of the eggs were fertilized with 8 to 10 drops of dilute sperm, and subsamples examined under the micro-

scope revealed 100% fertilization success. In 2012, 2 females and 1 male were injected with 1-methyladenine and spawned in a tank with running seawater. Fertilization success was 99%, and mean egg diameter was 156.5 ± 6.9 µm (n = 40).

In both years, embryos were placed in large 4 l jars in a sea table (78.7 × 129.5 × 16.5 cm) with continuously flowing seawater at 11–15°C. Embryos were left to develop for 2 d to the gastrula stage before distributing them among the various treatments.

Food and larvae in the jars were kept in suspension with the aid of a system of swinging paddles (Strathmann 1987). Water changes in each jar were made weekly to maintain a high water quality while minimizing damage to embryos and larvae. Approximately 85% of the seawater was siphoned from each jar and replaced with fresh 0.45 µm FSW. A 150 µm mesh at the end of the siphon tube prevented loss of larvae during siphoning. Once or twice a week extra FSW was added to each jar to maintain a high water quality.

Based on studies by Schiopu et al. (2006), 1800 to 2000 larvae jar^{-1} were fed a mixed algal diet of *Dunaliella tertiolecta*, *Isochrysis galbana*, and *Rhodomonas* sp. at initial concentrations of 1000, 6529, and 556 cells ml^{-1}, respectively, 2 to 3 times wk^{-1}. The dosage increased to maximum concentrations of 2000, 22811, and 1139 cells ml^{-1}, respectively, as larval size and stage increased (see Schiopu et al. 2006). Algal cultures were reared under continuous illumination at room temperature.

Halocline set-up

Experimental haloclines were set up in acrylic cylinders (Fig. 2) that measured 45 cm tall and 9 cm in diameter. Each cylinder was fitted with a Plexiglas lid with a hole through which 2 tubes, 167 cm long, 0.32 × 0.64 cm (⅛ × ¼ inch), were inserted. One of the tubes was used to introduce 0.45 µm FSW and the other to introduce brachiolaria larvae into the cylinders. Two clamps were installed on each of the tubes; one functioned as an on/off switch and the other was used to regulate water flow. To measure salinity at various depths in the water column, 3-way valves (n = 7) were installed at 5 cm intervals along the side of each cylinder (Fig. 2). Before filling cylinders with seawater or creating haloclines, all 6 cylinders with lids and their respective tubes were placed in a sea table (90 × 59 × 32 cm). Cylinders without haloclines were gravity fed 40 cm of 30‰ FSW at a temperature of ~11 to 12°C. Haloclines (10/20‰, 20/30‰ or

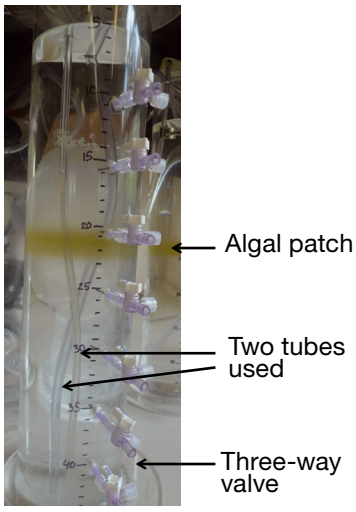

Fig. 2. Column used in all halocline experiments, showing the tubes used to introduce water and larvae into the cylinders, 3-way valves used to collect water samples for salinity, and a food (*Isochrysis galbana*) patch

30/40‰, hereafter 10/20, 20/30 and 30/40) were established by slowly gravity feeding low salinity (10, 20 or 30‰) FSW through the tubing to 15 cm high in the cylinder, followed by high-salinity FSW gravity fed below the low-salinity water until water column height reached 40 cm (see Metaxas & Young 1998a). Several preliminary trials using extra cylinders were run to ensure that flow rate in the tubes remained the same in all experimental cylinders. The surrounding sea table was then filled with seawater that flowed continuously to ensure that the temperature of seawater in the cylinders remained at ~11–12°C. These conditions mimic ambient temperature and salinity conditions at Cantilever Point during the summer. After setting up haloclines, all cylinders were left in the sea table for an hour to stabilize before the introduction of brachiolaria larvae (29–41 d old). For all experiments, only healthy brachiolaria larvae were used. Healthy larvae were those that swam to the surface of the jars within 1 to 2 min after the jars were removed from the system of swinging paddles.

It was difficult to see larvae at the bottom of the cylinders and measure salinity when cylinders were submerged in seawater. An observation chamber was thus made to allow easy cylinder viewing and access. The observation chamber consisted of a glass tank converted to a black box by removing the base and covering the sides with black plastic tarp. The tank was positioned vertically and a fluorescent light provided back-illumination. To determine the number of larvae at various depths in the haloclines, a cylinder was removed from the sea table and gently placed in the observation chamber. Based on repeated observations, larvae were not disturbed during transfer from the sea table to the observation chamber and back.

Experiment 1

Expt 1 was designed to investigate the effect of exposure of embryos and early larval stages to low salinity on larval shape, posterolateral arm length, and the vertical distribution of advanced larval stages.

Expt 1a: Effect of low-salinity exposure on larval growth and survival

In 2011, 2 d after fertilization, the total number of swimming embryos in stock jars (see below) was estimated, and 1800 embryos were placed into each of 12 jars (4 l) containing 2000 ml FSW. Four low-salinity exposure treatments with 3 replicates per treatment were set up with 0, 3, 7, and >25 d of exposure to low salinity (20–21‰) sea water. These treatments were chosen because previous studies indicated that they led to shape changes during development (Pia et al. 2012). Low-salinity seawater was produced by diluting natural filtered seawater (29–31‰) with deionized water. All salinity measurements were made with a portable refractometer.

To determine whether exposure to low salinity affected larval shape and posterolateral arm length, 10 larvae per jar (3 jars per treatment) were taken from each of the 4 salinity treatments during water changes and photographed using a video camera fitted on a compound microscope. The software program ImageJ was used to measure the lengths and widths of each of the 10 larvae per sample, as well as posterolateral arm length when present. Larvae were staged with the aid of descriptions by George (1999) and Pia et al. (2012). Larval survival was estimated 26 d after fertilization by counting all brachiolaria larvae in 50 ml subsamples per jar (3 jars per treatment) for all 4 salinity treatments.

Expt 1b: Effect on the vertical distribution of 29–33 d old bipinnariae and brachiolariae 90 min after introduction

To determine whether prior exposure to low salinity affected the vertical distribution of 29–33 d old *Pisaster* larvae, 4 treatments were set up with 2 replicate cylinders per treatment: controls (20 or 30), a 10/20 halocline, a 20/30 halocline and a 30/40 halocline (Fig. 3A). The salinity of the control varied between 30 and 32‰, depending on the salinity of the seawater pumped into the lab from the San Juan channel. The 20/30 halocline was chosen because stratified conditions with 20‰ sea water at the surface and 30‰ at the bottom are common in the Puget Sound region (Khangaonkar et al. 2011). Furthermore, salinities as low as 21‰ have been reported in this region (Fig. 1B). The 30/40 halocline was chosen because the salinity of seawater that enters the Puget Sound from the Pacific is ~34‰ (Khangaonkar et al. 2011, Sutherland et al. 2011). It is expected that brachiolaria larvae would encounter this salinity at the halocline. Approximately 100 larvae (bipinnariae or brachiolariae) that were never exposed to low salinity were transferred into beakers and gravity fed to the bottom of each cylinder through the installed tubes (see above). Larvae were allowed to swim for 90 min. Each cylinder was then gently removed from the sea table and placed in the observation chamber for larval viewing and counting. Starting at the top of the water column, the numbers of larvae were counted for 30 s in 5 cm increments to the bottom. The salinity of a small drop of seawater taken through each of the 3-way valves (n = 7) installed on the side of each cylinder was then measured with a portable refractometer. Cylinders were immediately returned to the sea table. Cylinders with and without haloclines were set up daily, and the experiment was repeated for brachiolariae from the other salinity treatments (3, 7, and >25 d of exposure to 20–21‰ seawater). For the >25 d exposure treatment, 2 replicates each of controls at 20‰, 20/30 haloclines, and 10/20 haloclines were prepared (Fig. 3A).

Expt 1c: Effect on the vertical distribution of 36–41 d old brachiolariae 90 min and 24 h after introduction

To determine whether time influenced the vertical distribution of larvae in the haloclines, 3 treatments were set up with 2 replicate cylinders per treatment: controls at 30‰, 20/30 haloclines, and 30/40 haloclines. Approximately 150 to 250 larvae that were never exposed to low salinity were introduced into each cylinder, and the vertical distribution of larvae was noted after 90 min and after 24 h. This was repeated using 150 to 200 larvae from the other salinity treatments (3 and 7 d of exposure to 20–21‰ sea water). These experiments were carried out under ambient light conditions.

Experiment 2

This experiment was designed to evaluate the effect over time of prior acclimation to low salinity on

the vertical distribution of 34–36 d old brachiolariae in haloclines with a food patch.

In 2012, 2 d after fertilization, the total number of swimming embryos in stock jars was estimated and 2000 placed in each of 12 jars (4 l) containing 2000 ml FSW. Two salinity treatments, viz. larvae with no exposure to low salinity (30‰) and larvae exposed to low salinity throughout development (>25 d at 20–21‰), were set up, with 6 replicate jars in each treatment.

To determine whether brachiolariae maintain their position within haloclines in the presence or absence of food, 2 replicates of 4 treatments were set up (Fig. 3B). Controls (30‰ FSW) with no food, controls (30‰ FSW) with *I. galbana* dispersed throughout,

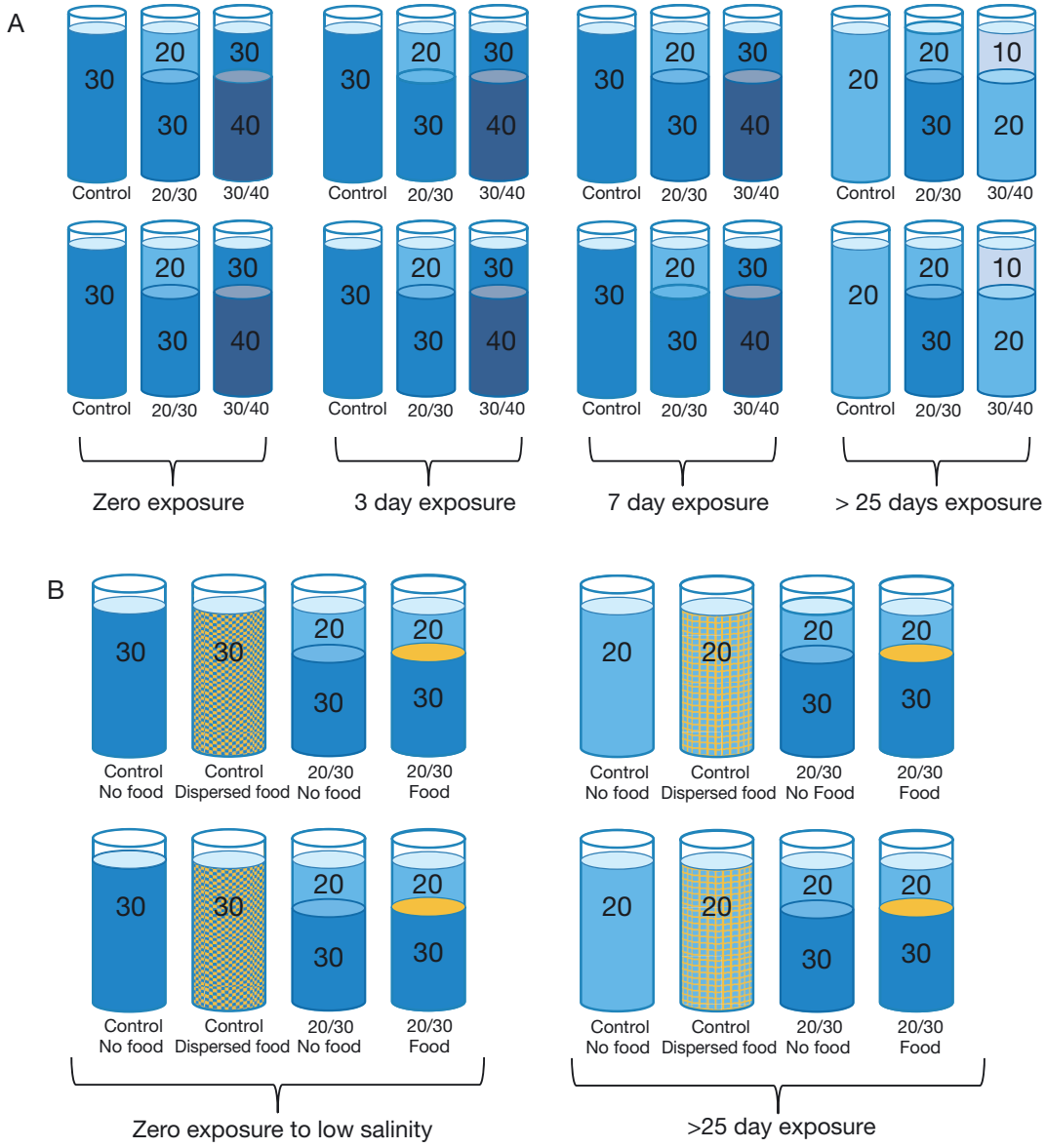

Fig. 3. (A) Expt 1, 2011. Experimental design for the vertical distribution of 29 to 36 d old *Pisaster ochraceus* brachiolaria larvae in cylinders with haloclines ('10/20', '20/30' and '30/40') and without ('20' or '30'). Two replicate cylinders were prepared per treatment. Numbers within each cylinder indicate salinity (‰). The experiment was repeated 4 times, using larvae that had been exposed to low salinity for 0, 3, 7, and >25 d. (B) Expt 2, 2012. Experimental design for the vertical distribution of 34 to 36 d old *Pisaster ochraceus* brachiolaria larvae with haloclines ('20/30') and without ('20' or '30') in the presence and absence of food. Numbers indicate salinity (‰). Food (*Isochrysis galbana*) was dispersed throughout the cylinder (hatched yellow shading) in the no halocline treatments and as a 1 cm patch (solid yellow band) in the 20/30 halocline treatments. The experiment was repeated twice using larvae that were reared in either low salinity (>25 d exposure to 20‰) or 30‰ salinity throughout the experiment

20/30 haloclines with no food, and 20/30 haloclines with a 1 cm food patch at the halocline (initially 2.3 × 10^6 cells ml^{-1} of *I. galbana*, mimicking a thin phytoplankon layer; Metaxas & Young 1998b; Fig. 2). A similar set of 4 treatments was prepared for brachiolaria larvae exposed to low salinity throughout development (>25 d), and the salinity in these controls was 20‰. Cylinders with food patches but no larvae were also set up to determine food patch stability over time. Samples of 1 ml were taken from each food patch, stained with Lugol's solution, and algal cell concentrations determined with a hemocytometer. The cylindrical columns were similar to those used in the previous experiments (see above) but slightly wider (45 cm tall with an inner diameter of 10.3 cm) with 8 (rather than 7) 3-way valves at 40, 35, 30, 25, 20, 17.5, 15, and 10 cm, respectively. Cylinders with and without haloclines were prepared as described above. For halocline treatments with food, the microalga *I. galbana* (cultured in 24.5‰ FSW) was gravity fed into the bottom of the cylinder after the addition of low-salinity water but before the addition of high-salinity water (Metaxas & Young 1998b). For controls, *I. galbana* cultured in either 30 or 20‰ FSW were stirred throughout the water column.

An hour after the haloclines were established, 34 to 36 d old brachiolariae larvae (n = ~100) that were never exposed to low salinity were gravity fed into the bottom of each cylinder. Larvae were starved for 2 to 3 d before use to ensure that larval stomachs were empty.

Larvae were counted in each vertical section after 1, 7, 19, and 24 h. For all experiments, recording the vertical distribution of larvae in a cylinder took approximately 4 to 5 min. Salinity was measured 1 and 24 h after larvae were introduced into the cylinders. The temperature within the cylinder was noted at the end of the experiment. These experiments were carried out in complete darkness and were completed 52 d after fertilization.

Samples of 5 larvae were taken from the halocline in all cylinders with food at 7 and 24 h after introduction. These larvae were then observed under the microscope to determine whether they had empty or full stomachs.

Data analysis

To determine whether salinity affected growth and shape of *Pisaster* larvae, nested analysis of variance (ANOVA) was used. Salinity was a fixed factor and jar was a random factor nested within salinity treatment. To detect significant differences among salinity exposure treatments, pairwise comparisons of the treatments were made using the Tukey HSD test. All data were normally distributed and variances were equal, except for 12 d after fertilization. For Expt 1, a 1-way ANOVA followed by a Tukey HSD test was used to test differences in survival between salinity treatments.

To determine whether prior exposure to low salinity affected the vertical distribution of brachiolariae in cylinders with and without haloclines in both years, larval counts were binned into 4 categories. Binning was based on the salinity profiles of cylinders with haloclines, but cylinders without haloclines were binned exactly the same as halocline cylinders created on the same day. For Expt 1, the 4 categories were surface (0–5 cm depth), above the halocline (5–10 cm), the halocline (10–15 cm), and bottom (15–40 cm). For Expt 2, bigger cylinders led to slightly different binning: surface (5–15 cm depth), halocline (15–20 cm), below halocline (20–35 cm), and bottom (35–45 cm). For Expt 1, we used 2-way contingency tables, with depth and salinity treatment as the 2 factors, to test whether the vertical distribution of larvae with 0, 3, 7, and >25 d of exposure to low salinity differed among the control, 10/20, 20/30, and 30/40 halocline treatments. To ensure that vertical distributions from replicate cylinders could be pooled, 2-way contingency tables with replicate and salinity treatment as factors were run for both years. In most cases, the vertical distributions of larvae in replicate cylinders did not differ and were pooled to determine the effect of salinity exposure treatment on the position of larvae in the cylinders after 90 min. The Pearson chi-squared test was used to report significant differences in larval vertical distribution among salinity treatments. When differences were significant, the Pearson chi-squared test was used to determine where (surface, above the halocline, halocline, bottom) in the water column larval distribution differed. Similar analyses were run to determine whether time (90 min, 24 h) influenced the position of larvae in the cylinders.

For Expt 2, the effect of food patches on larval distributions was analyzed with 2-way contingency tables with depth and food presence as the 2 factors. The Pearson chi-squared test was used to report significant differences for all contingency tables, and the Pearson chi-squared test with time as the main effect and the 4 depth categories as response frequencies was used to determine where (surface, halocline, below the halocline, bottom) in the water column larval distribution differed 1, 7, 19, and 24 h

after their introduction. The Cochran-Mantel Haenszel test (CMH test) based on rank scores was used to determine changes in algal cell concentration in the food patch with and without brachiolariae 7 and 17 h later. The statistical packages Jmp 9.0 and 10.0 were used to analyze data, and Sokal & Rohlf (1995) was used as a reference.

RESULTS

Experiment 1

Expt 1a: Effect of low-salinity exposure on larval growth and survival

Brachiolariae with continuous exposure to low salinity (>25 d) exhibited the greatest morphological differences; they were 8–34% smaller, 14–46% narrower, and had posterolateral arms 55–95% shorter than those observed in the other treatments (0, 3, and 7 d of exposure to low salinity). These differences were observed from Days 5 through 33 for total and posterolateral arm lengths (Fig. 4, nested ANOVA, Tukey HSD $p < 0.05$, Table S1 in the Supplement at www.int-res.com/articles/suppl/m542p123_supp/), and from Days 12 through 26 for larval width (Fig. 4, nested ANOVA, Tukey HSD $p < 0.05$). In addition, larvae exposed to low salinity for 3 d were significantly shorter and narrower than those not exposed to low salinity during development or those exposed to low salinity for 7 d (Fig. 4, from Days 5 to 19 post fertilization, nested ANOVA, Tukey HSD, $p < 0.05$). Eventually, the posterolateral arms for brachiolariae from larvae exposed to low salinity for 3 d and those never exposed to low salinity no longer differed significantly (Days 26 through 40 after fertilization, Table S1, Fig. 4).

Larval mortality differed significantly among treatments 26 d after fertilization; brachiolariae that had never been exposed to low salinity had the lowest mortality (7.4%), those exposed to low salinity for 3 or 7 d were intermediate (36 and 28%, respectively), and those exposed to low salinity throughout development (>25 d) had the highest mortality (59%; Fig. 5, 1-way ANOVA, Tukey HSD, $p < 0.05$).

Expt 1b: Effect on the vertical distribution of 29–33 d old bipinnariae and brachiolariae 90 min after introduction

The vertical distribution of larvae from the 4 salinity exposure treatments differed significantly in cylinders without a halocline ($\chi^2 = 263.2$, $p < 0.0001$). Larvae exposed to low salinity throughout develop-

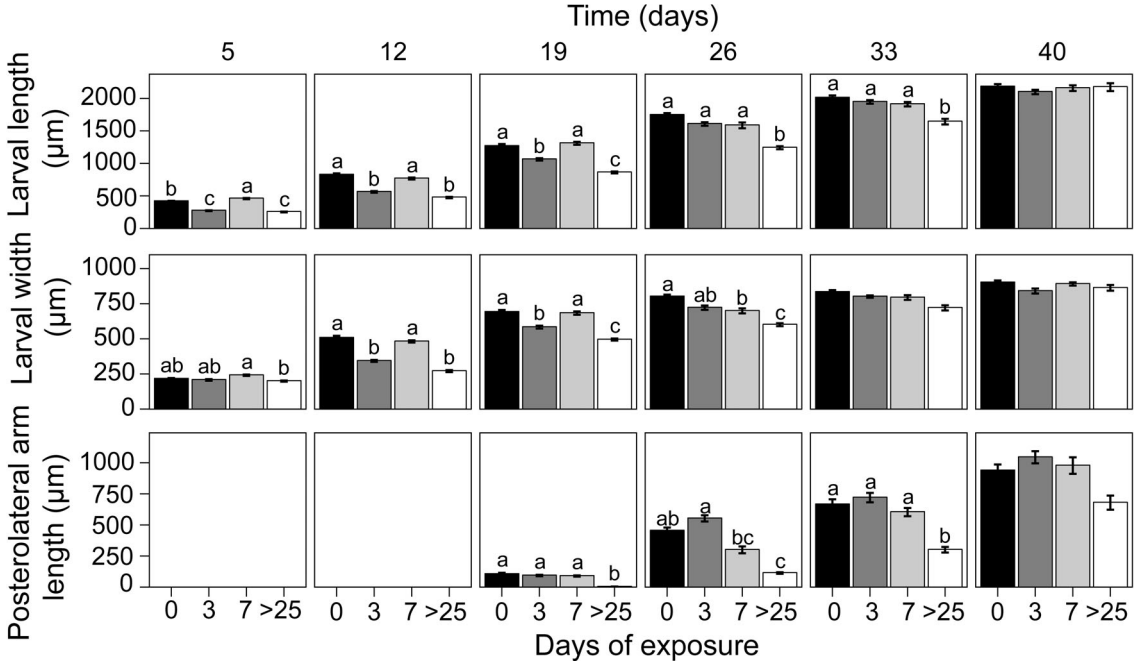

Fig. 4. Larval length, larval width, and posterolateral arm length (µm) for 5, 12, 19, 26, 33, and 40 d old *Pisaster ochraceus* larvae reared in 4 different salinity treatments (0, 3, 7, and >25 d at low salinity). Ten larvae were measured per jar and pooled for statistical analysis. Bars are means ± SE (n = 30). Similar letters indicate no significant differences among the 4 treatments (nested ANOVA, Tukey HSD $p < 0.05$)

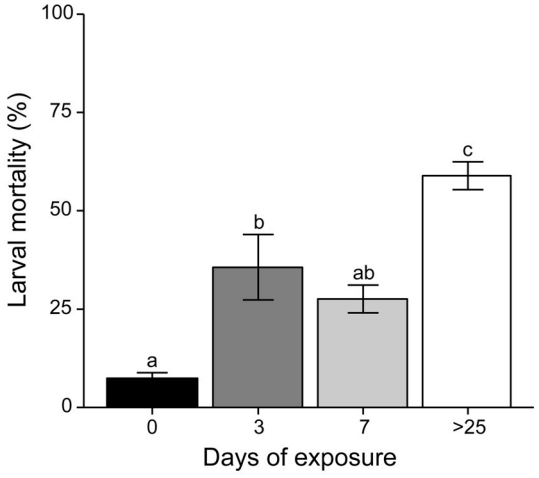

Fig. 5. Mortality of 26 d old *Pisaster ochraceus* brachiolaria larvae with 0, 3, 7, and >25 d of exposure to low salinity. Bars are means ± SE (n = 3 replicate jars per treatment). Similar letters indicate no significant differences among treatments (1-way ANOVA, Tukey HSD, $p < 0.05$)

ment (>25 d) showed behaviors that were substantially different from those that had been subjected to other rearing conditions (Fig. 6). In the absence of a halocline, only 2.8% of these brachiolariae reached the surface compared to 84.3, 63.9, and 29.5% for brachiolariae from the 0, 3, and 7 d low-salinity treatments, respectively (Fig. 6). In general, there was a significantly higher percentage of larvae exposed to low salinity throughout development at the bottom of the cylinders ($\chi^2 = 23.1$, $p < 0.0001$) and a significantly lower percentage at the surface ($\chi^2 = 148.6$, $p < 0.0001$, Table S2 in the Supplement).

In the presence of a 20/30 halocline, 75.5 to 93.4% of brachiolariae from all 4 treatments reached the halocline ($\chi^2 = 29.6$, $p = 0.0001$, Fig. 6). However, a significantly greater percentage of larvae that had been exposed to low salinity for 3, 7, or >25 d were at the bottom of the cylinders compared to those that had never been exposed to low salinity ($\chi^2 = 21.2$, $p < 0.0001$, Table S2).

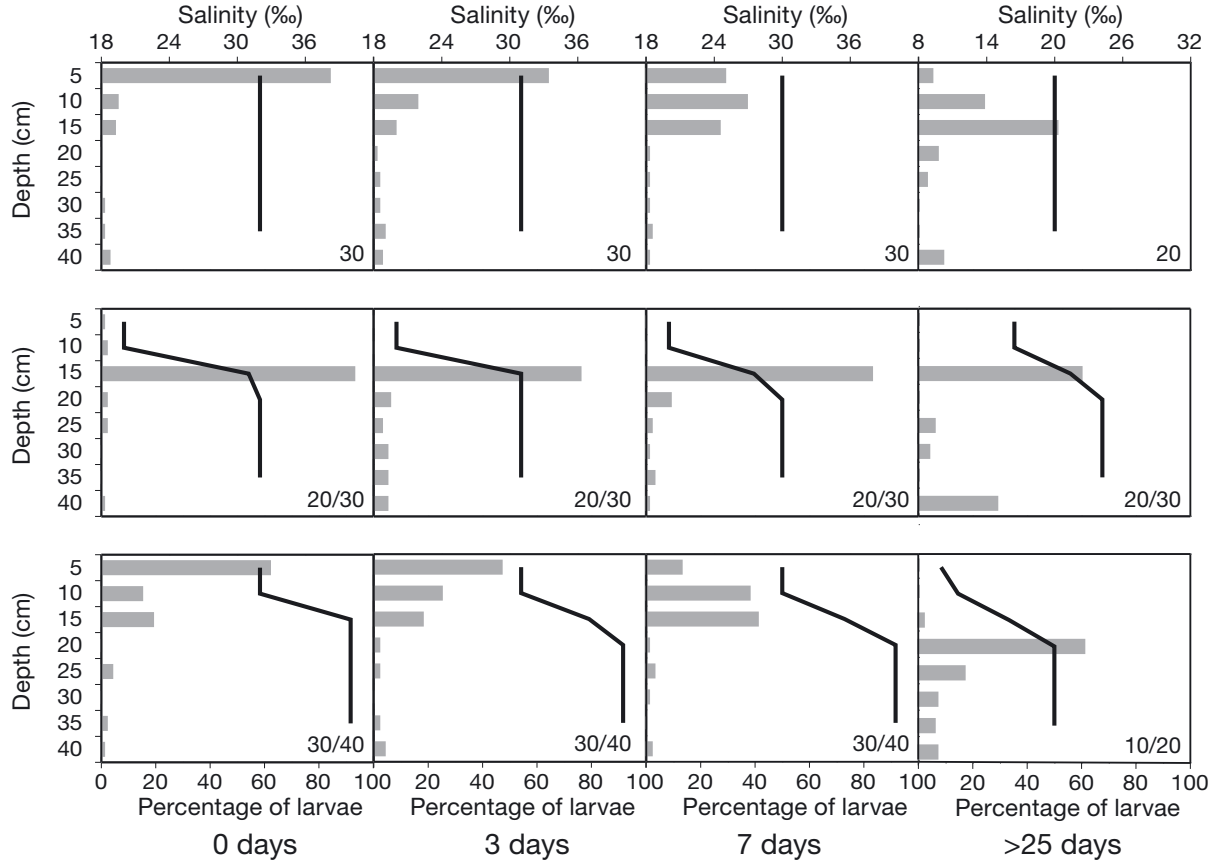

Fig. 6. Vertical distribution of 29–33 d old *Pisaster ochraceus* brachiolaria larvae exposed to low salinity for 0, 3, 7, and >25 d and then placed into cylinders with and without haloclines. Each graph represents the combined results from 2 replicates. Between 100 and 120 larvae were introduced into each cylinder at least 1 h after creation of the halocline. Bars indicate the percentage of larvae at each 5 cm depth bin after 90 min. The solid line indicates mean salinity profile for each halocline treatment: no halocline (top panels, 20 or 30‰ seawater), 20/30 halocline (center panels), and 30/40 or 10/20 haloclines (bottom panels). See 'Materials and methods' for details

In the 30/40 haloclines, larvae exposed to low salinity for 7 d had the greatest difficulty moving to the surface of the cylinders where salinity was 30‰. Only 13% reached the surface in 90 min compared to 60.2% for those that were never exposed to low salinity, and 45.3% for those exposed to low salinity for 3 d ($\chi^2 = 31.0$, $p < 0.0001$). Interestingly, brachiolariae tended to swim into or stay within the salinity layer that they had most recently experienced. The majority of brachiolariae exposed to low salinity for >25 d remained below the halocline in the 10/20 treatment ($\chi^2 = 56.2$, $p < 0.0001$, Table S2).

Expt 1c: Effect on the vertical distribution of 36–41 d old brachiolariae 90 min and 24 h after introduction

Observation of 36–41 d old brachiolaria larvae after 90 min and after 24 h revealed a significant change in their vertical distribution in cylinders without haloclines, 20/30 haloclines, and 30/40 haloclines (Fig. 7).

As expected, in the absence of a halocline, between 75 and 77% of brachiolaria larvae that were never exposed to low salinity were at the surface 90 min and 24 h after their introduction into the cylinders compared to those exposed to low salinity for 3 or 7 d ($\chi^2 = 31.4$, $p < 0.0001$, Fig. 7). A significant percentage (16.6%) of those exposed to low salinity for 7 d were at the bottom of the cylinders 90 min after their introduction ($\chi^2 = 13.9$, $p = 0.0009$).

Brachiolariae from all treatments were originally distributed around the 20/30 halocline 90 min after their introduction ($\chi^2 = 2.7$, $p = 0.6030$, Table S2, Fig. 7). After 24 h, the majority had moved to the surface, with only 1.2% remaining at the bottom of the cylinders for those never exposed to low salinity and 2.1% for those exposed to low salinity for 7 d. However, 12.2% of larvae exposed to low salinity for 3 d were at the bottom of the cylinders after 24 h ($\chi^2 = 18.4$, $p = 0.0001$, Table S2, Fig. 7).

Larvae exposed to low salinity for 7 d continued to have the greatest difficulty moving to the surface in the 30/40 halocline treatment 90 min after their intro-

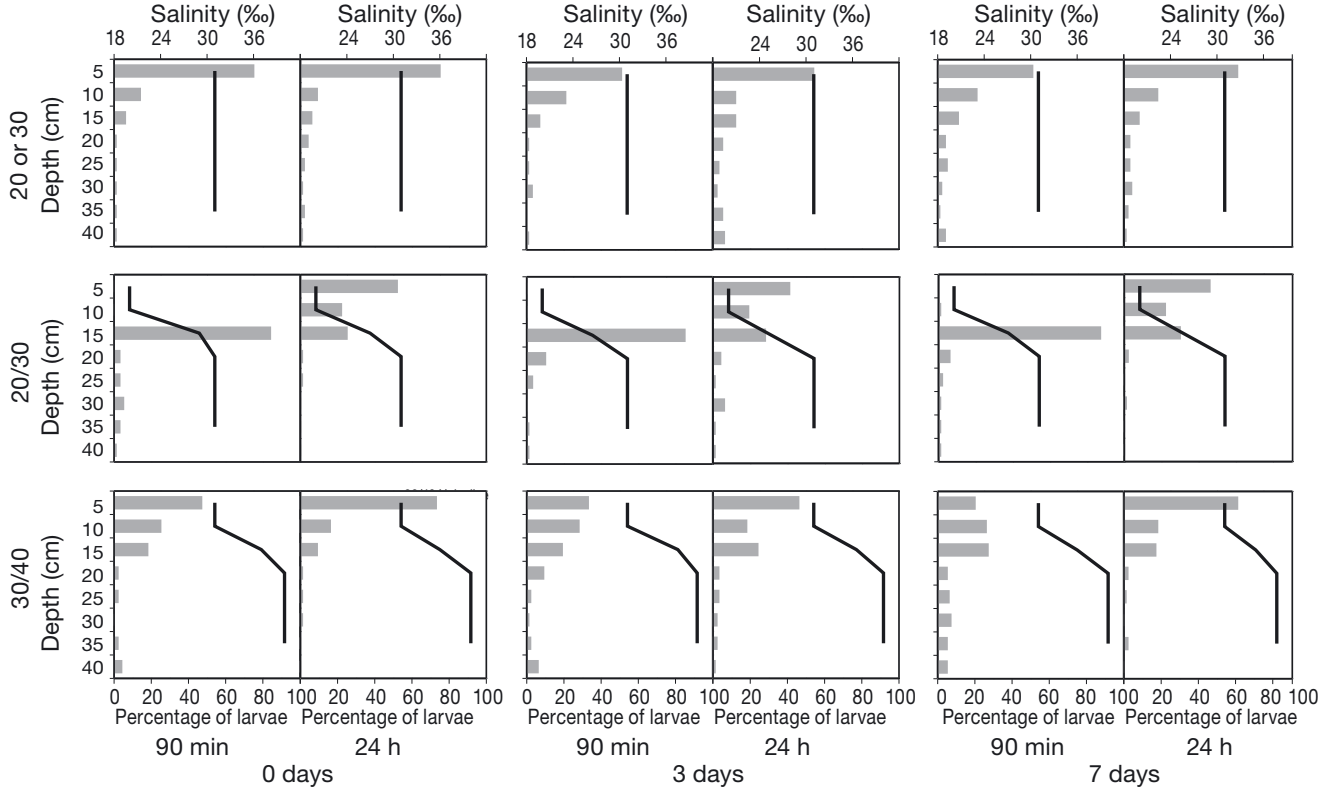

Fig. 7. Vertical distribution of 36–41 d old *Pisaster ochraceus* brachiolaria larvae exposed to low salinity for 0, 3, or 7 d and then placed in cylinders with and without haloclines. Each graph represents the combined results from 2 replicates. Between 100 and 120 larvae were introduced into each cylinder at least 1 h after creation of the halocline. Bars indicate the percentage of larvae at each 5 cm depth bin after 90 min or 24 h. The solid line indicates mean salinity profile for each halocline treatment: no halocline (top panels, 20 or 30‰ seawater), 20/30 halocline (center panels), and 30/40 halocline (bottom panels). See 'Materials and methods' for details

duction (χ^2 = 13.5, p = 0.0012). Only 19.8% had moved to the surface after 90 min compared to 47.3 and 32.7% for larvae exposed to low salinity for 0 and 3 d, respectively. After 24 h, the majority had moved to the surface, with only 2.5% of those that had never been exposed to low salinity remaining at the bottom of the cylinders, 4.1% for those exposed to low salinity for 7 d, and 12.1% for those exposed to low salinity for 3 d (χ^2 = 8.3, p = 0.0159, Table S2, Fig. 7).

Experiment 2

No exposure to low salinity/Halocline absent

The vertical distribution of brachiolaria larvae that had never been exposed to low salinity depended on whether food was present. A significantly higher percentage of larvae were at the surface 1 h after their introduction into cylinders without food than in those with food (χ^2 = 32.9, p < 0.0001). In the presence of food, a significantly higher percentage of larvae were initially at the bottom (χ^2 = 32.9, p < 0.0001), but after 7 h, the majority were at the surface regardless of treatment (Fig. 8A). The percentage at the surface at 19 and 24 h was significantly higher in cylinders with food than in those without food (χ^2 = 37.7, p = 0.0001, and χ^2 = 10.2, p = 0.02, respectively, Fig. 8A,B).

No exposure to low salinity/Halocline present

With haloclines, a significantly higher percentage of brachiolariae that had never been exposed to low salinity remained at the halocline after 1 h in the presence of a food patch than in the absence of a food patch (χ^2= 25.6, p < 0.0001, Fig. 8C,D). A high percentage of these larvae were observed at the halocline in cylinders with a food patch 7, 19, and 24 h after their introduction (χ^2= 149.8, 140.4, and 71.2 respectively, p < 0.0001 in all cases, Table S3 in the Supplement).

Exposure to low salinity/Halocline absent

One hour after introduction, a significantly higher percentage of larvae exposed to low salinity throughout development (>25 d) were at the bottom of cylinders with food compared to cylinders without food (χ^2 = 61.1, p < 0.0001, Fig. 9A,B). By 7 h, a majority of larvae in the control cylinders with food had moved to the surface, significantly more than in cylinders without food (χ^2 = 27.1, p < 0.0001). The percentage of larvae at the surface at 19 and 24 h remained significantly higher in control cylinders with food than in those without food (χ^2 = 20.0, p < 0.0002, and χ^2 = 48.0, p < 0.0001, respectively; Fig. 9A,B).

Exposure to low salinity/Halocline present

Unlike brachiolariae that were never exposed to low salinity, those exposed to low salinity throughout development (>25 d) showed similar behaviors regardless of whether food was present at the halocline, with a significant percentage above the halocline in the presence or absence of food (Fig. 9C,D). At 7 h, the vertical distribution of larvae in haloclines with and without a food patch did not differ (χ^2 = 7.2, p = 0.07). At 19 and 24 h after their introduction, although all larvae from the low-salinity treatment had moved above the halocline, the vertical distribution in haloclines with food patches differed significantly from those in haloclines without a food patch (χ^2 = 55.6, and 28.7, respectively, p < 0.0001). Approximately 90% of these larvae were above the halocline when food was present and only 44 to 60% when food was absent 19 to 24 h after their initial introduction into the cylinders.

These interesting but opposite trends for larvae that had never been exposed to low salinity compared to those that had are summarized in Table S3 and Fig. 10. In the presence of food, a significantly higher percentage of brachiolariae that were never exposed to low salinity were at the halocline 1 h after their introduction (χ^2 = 9.1, p = 0.03, n = 531). These larvae remained at the halocline for the next 19 h, after which the percentage at the halocline decreased as the percentage of those swimming towards the surface increased significantly (χ^2 = 22.3, p < 0.0001). Unlike larvae that were never exposed to low salinity, the percentage of larvae at the halocline for those exposed to low salinity throughout development decreased significantly over time (χ^2 = 34.3, p < 0.0001, n = 538, Fig. 10).

In the absence of food, larvae that were not exposed to low salinity during their development showed similar behavior to those exposed to low salinity throughout development with the exception of 1 h after introduction. In the absence of food, larvae exposed to low salinity throughout development (>25 d) appeared to swim back and forth between the halocline and the surface. In the presence of food at the halocline, the stomachs of these larvae were virtually empty, whereas those of larvae not exposed to low salinity were packed with food.

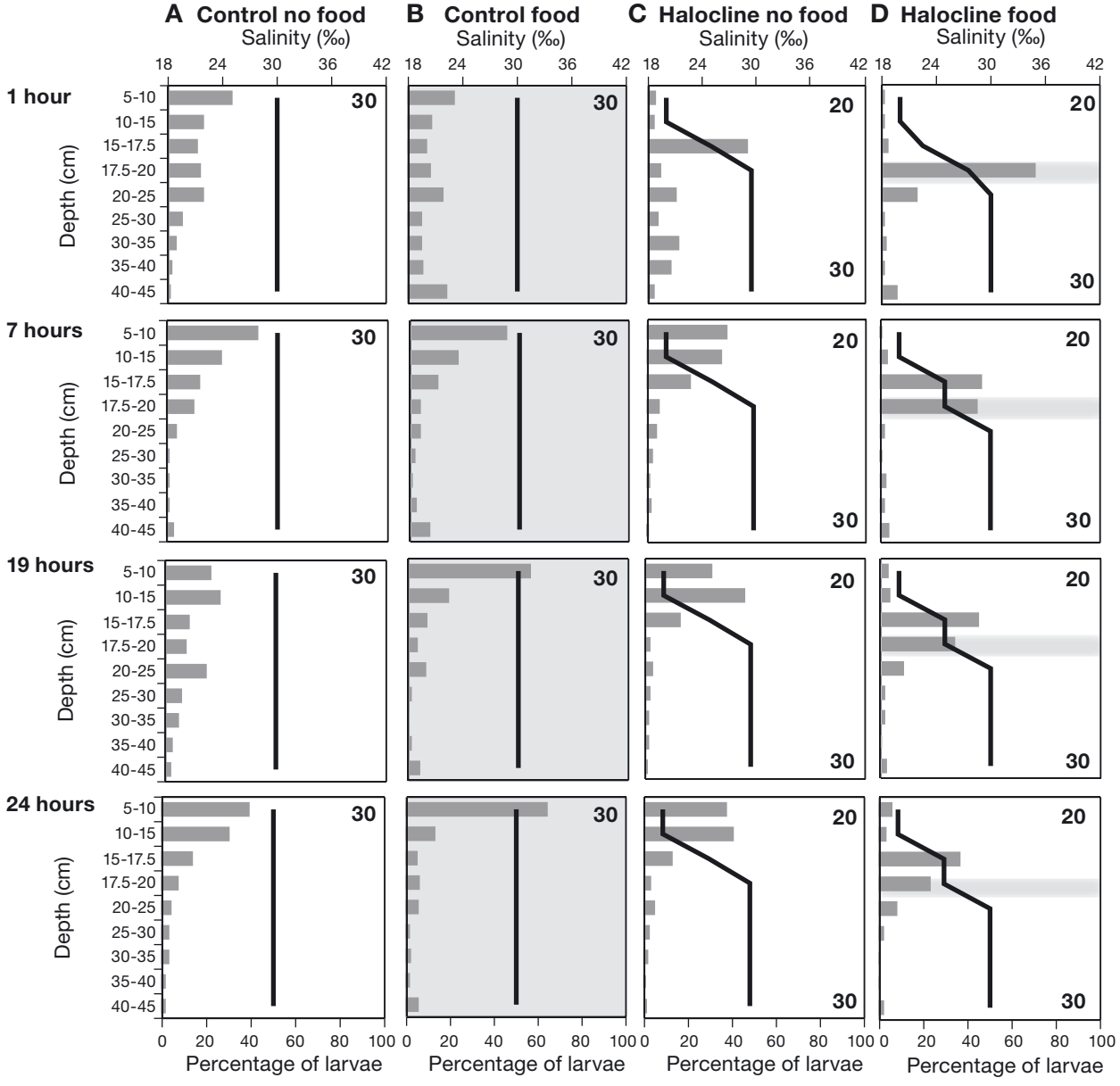

Fig. 8. Vertical distribution of 34–36 d old *Pisaster ochraceus* brachiolariae reared with no exposure to low salinity and then placed in cylinders (A, B) without haloclines (30‰) and (C, D) with haloclines (20/30) in the absence or presence of food. Each graph represents the combined results from 2 replicates. Between 60 and 130 larvae were introduced into each cylinder at least 1 h after creation of the halocline. Bars indicate the percentage of larvae at each 5 cm depth bin after 1, 7, 19, and 24 h. The solid line indicates mean salinity profile for each halocline treatment. The shaded region indicates the initial location of food within the water column. See 'Materials and methods' for details

Algal cell concentration within the food patch differed significantly over time (CMH test, $\chi^2 = 8.0$, p = 0.0046). Between 7 and 17 h, a significant decrease in algal cell concentration was observed at the halocline. This was possibly due to feeding by larvae within the patch and migration of algal cells away from the patch.

DISCUSSION

The results of our study indicated that exposure of early larval stages to low salinity has marked effects on *Pisaster ochraceus* brachiolariae. Consistent with Pia et al. (2012), larvae exposed to low salinity for 3, 7, or >25 d were significantly smaller and had shorter

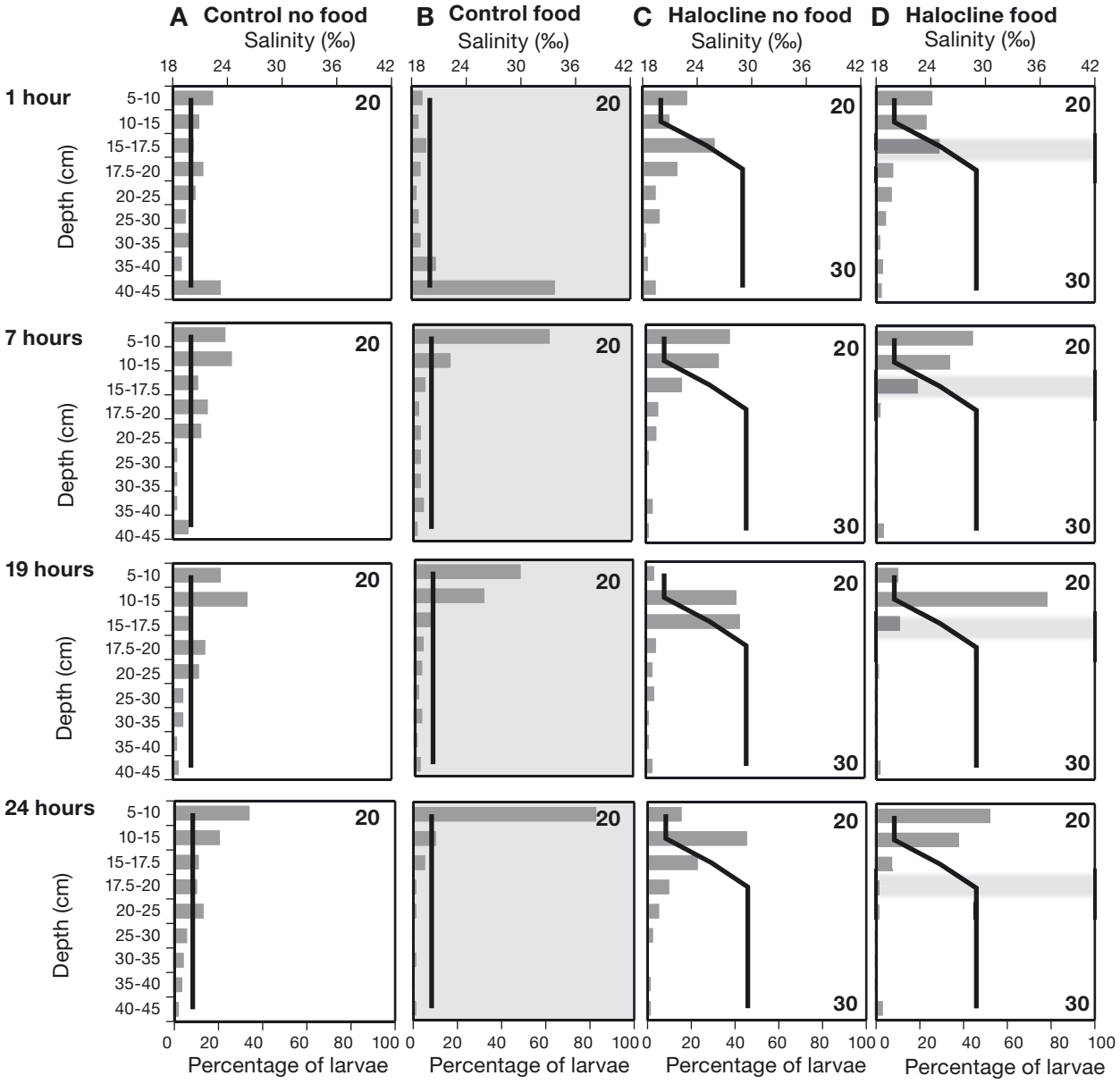

Fig. 9. As in Fig. 8, but for larvae reared in low salinity prior to being placed in cylinders (A, B) without haloclines (20‰) and (C, D) with haloclines (20/30) in the absence or presence of food. Between 50 and 110 larvae were introduced into each column at least 1 h after the creation of the halocline

larval arms, and significantly fewer larvae survived to the brachiolaria stage. Furthermore, morphological differences persisted the longer larvae were exposed to low salinity. This study also documents for the first time that the salinity at which *Pisaster* embryos and early larval stages develop influences their swimming ability. Fewer larvae exposed to low salinity made it to the surface in the presence or absence of a halocline or remained at the halocline in the presence of food.

Larval mortality and morphology

As expected, exposure of early developmental stages of *P. ochraceus* to low salinity (20–21‰) resulted in increased mortality. The highest mortality (59%) was observed for larvae exposed to low salinity throughout development, and the lowest (7%) for those that were never exposed to low salinity during development, despite the fact that all larvae were fed a mixed algal diet that generally promotes

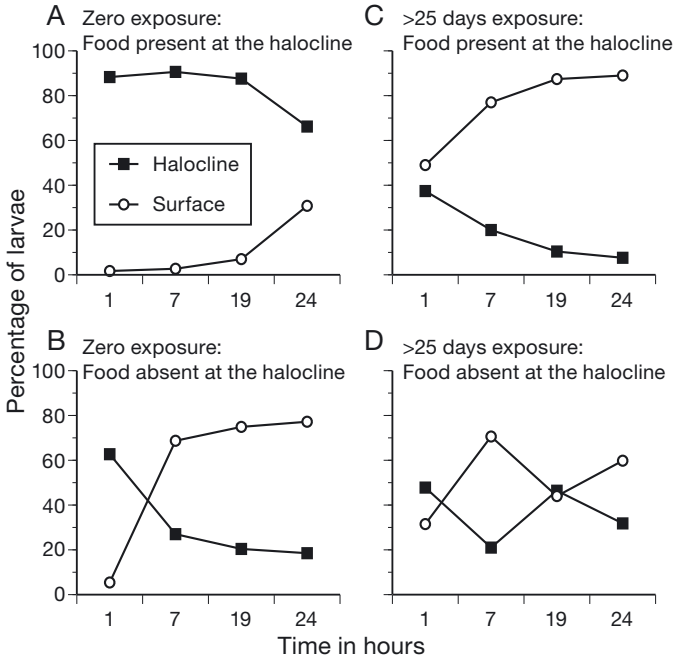

Fig. 10. Percentage of *Pisaster ochraceus* brachiolaria larvae at the halocline or surface as a function of time after introduction into cylinders with a 20/30 halocline. (A) Larvae with no exposure to low salinity remained near the halocline when food was present, but (B) swam to the surface when food was absent. (C) Brachiolariae raised in 20‰ throughout development (>25 d) swam to the surface when food was present, but (D) showed no preference for location when food was absent

optimal growth and survival (Schiopu et al. 2006). These results indicate that if spawning events and early embryonic development coincide with the intrusion of the Fraser River plume into the San Juan Islands, the effects on larval mortality could be substantial. An increase in the magnitude and frequency of low-salinity events during larval development of this species and other sea stars with planktotrophic larval development could lead to large changes in benthic marine communities in the Pacific Northwest (see Menge & Sanford 2013). Since 1950, air and water temperatures in this region have increased by 0.13 and 0.11°C per decade, respectively (Harley 2011). Increased temperatures and the recent widespread wasting disease observed in many adult populations (Hewson et al. 2014, Jurgens et al. 2015), make the findings on survival of the brachiolaria larval stage in low-salinity waters even more troubling.

Our study confirms the results of Pia et al. (2012), who showed that low salinity induces morphological changes in *Pisaster* larvae. In both studies, brachiolariae from larvae reared in low salinity had significantly shorter posterolateral arms. However, the shapes acquired differed between studies. In our study, larvae reared in 20‰ were narrower, whereas in the study by Pia et al. (2012), they were wider. These differences might be due to variation in egg quality between populations (George et al. 1990, George 1996, 1999, Bertram & Strathmann 1998) that might in turn influence early gene expression and variation in larval morphology (Garfield et al. 2013). Regardless, differences in larval morphology have profound effects on feeding and swimming within and among species (Strathmann 1971, Hart 1991, Chan 2012).

Larval morphology and swimming through haloclines

Low salinity affected *Pisaster* larval morphology and the ability to swim through haloclines. Longer exposure of larvae to low salinity resulted in greater effects on larval morphology and a lower percentage of larvae at the surface in the absence of a halocline 90 min after their introduction. Only 3 to 30% of 29 d old larvae exposed to low salinity for ≥7 d reached the surface within 90 min, whereas 64 to 84% of larvae exposed to low salinity for ≤3 d reached the surface in the same time frame. Differences in larval vertical distribution were still noticeable in cylinders with and without haloclines for 36 to 46 d old brachiolariae. These results indicate that prior exposure to low salinity appears to slow down swimming speeds of brachiolaria larvae.

The vertical position of planktonic larvae is influenced by several factors, including stage of development, current speed and direction, and larval swimming (Clay & Grünbaum 2010, Roy et al. 2012). Swimming performance is influenced by larval morphology (Emlet 1994, Grünbaum & Strathmann 2003, Strathmann & Grünbaum 2006, Clay & Grünbaum 2010, Chan 2012), and larval morphology in most echinoderms with planktotrophic larval development changes over time. As they develop, many larvae go from simple to complex forms (Strathmann 1971, Grünbaum & Strathmann 2003, Strathmann & Grünbaum 2006, Chan 2012). In *P. ochraceus,* the embryos initially develop into a bipinnaria with less developed muscle bands and no larval arms (Mortensen 1920, Strathmann 1971, Lacalli 1996). Eventually they develop into a brachiolaria with 5 pairs of larval arms with well developed muscle bands running through each arm (Strathmann 1971, Lacalli 1996,

George 1999, Pia et al. 2012) that most likely enable faster swimming speeds. Arms are usually directed posteriorly but can also be directed anteriorly for stopping and reversing (Strathmann 1971). The longest of the 5 pairs are the posterolateral arms that can grow to over 2 mm in length (Pia et al. 2012). These latter arms can wave rhythmically back and forth for extended periods of time, causing brachiolariae to rotate when suspended vertically in the water column (Lacalli 1996; see the video in the Supplement at www.int-res.com/articles/suppl/m542p123_supp/). The posterolateral arms were significantly longer in brachiolariae with no or only 3 d of exposure to low salinity. It is possible that swimming towards the surface is much more effective for *Pisaster* larvae with longer larval arms than for those with shorter arms. In the larvae of other echinoderms, e.g. sand dollars, modest morphological changes have important consequences for swimming performance (Clay & Grünbaum 2010).

Thirty-three days after fertilization, the length of the posterolateral arms no longer differed significantly among brachiolariae that were not exposed to low salinity or exposed to low salinity for 3 or 7 d, although residual effects of the salinity treatment remained. Fewer 36 d old brachiolariae from larvae exposed to low salinity for 7 d made it to the surface in cylinders with and without haloclines. This was especially noticeable in the 30/40 haloclines 90 min after they were introduced into the cylinders. Despite increases in arm length, osmotic stress experienced by brachiolariae early during development might have long lasting effects, e.g. hampering their ability to vertically migrate from waters that are too saline. Similar observations were noted for 36 d old brachiolariae from larvae exposed to low salinity for 3 d. Although the majority of these larvae were initially at the surface 90 min after they were introduced, 12% were at the bottom of the cylinders after 24 h. It is possible that these brachiolariae were also experiencing osmotic stress or that they were searching for food at the bottom of the cylinders or sinking because they were more advanced in development.

Ultimately, changes in morphology over time might improve swimming. During development, larval morphologies might converge to a shape that enhances swimming by brachiolariae initially exposed to low-salinity waters in Puget Sound for varying lengths of time. Longer larval arms will increase speed and might enable brachiolariae to escape predators, vertically migrate, and locate and capture prey in the water column.

Vertical distribution of brachiolariae in the presence or absence of food

Surprisingly, larvae exposed to low salinity throughout development spent very little time at the halocline in the presence of food, with less than 40% at the halocline 1 h after their introduction and less than 10% after 24 h. An examination of their stomachs revealed the presence of food, but the stomachs were not full. Brachiolariae used in these experiments had been starved for several days before being introduced into the columns, so it is interesting that they did not stay at the halocline to feed. It is possible that they swam quickly to the surface to avoid stressful osmotic conditions at the halocline, or their ability to sense food at the halocline was somehow impaired.

Like all planktotrophic echinoderm larvae, *Pisaster* larvae have a continuous band of ciliated epithelial cells (Strathmann 1971). The coordinated beat of the ciliated band is crucial for larval feeding and swimming. Particles are captured by mechanical stimulation of the cilia (Strathmann 1971, Hart & Strathmann 1995) and their capture and retention varies along different sections of the ciliated band (Hart 1991). If exposing larvae to low salinity results in damage to the ciliated band, then brachiolaria larvae might have a reduced ability to sense food particles in the water column.

Unlike larvae reared in 20‰ salinity for >25 d, those reared in 30‰ salinity swam readily into lower-salinity water at the surface in the absence of food but delayed their movement to the surface when food was present at the halocline. These brachiolariae spent a significant amount of time at the halocline feeding, with over 80% at the halocline 19 h after they were introduced into the columns and over 60% at the halocline after 24 h. Examination of their stomachs confirmed that they were feeding, and the stomachs were packed with food. Metaxas & Young (1998a,b,c) made similar observations for echinoids; larvae remained around the halocline in the presence of food but swam to the surface in the absence of food. Menden-Deuer & Grünbaum (2006) noted that the swimming speeds and turning rates of the protistan predator *Oxyrrhis marina* increased significantly in the presence of a thin phytoplankton layer. They observed that predator abundance was 20 times higher within the *Isochrysis* layer than in the absence of this layer. They also observed that predators had strong vertical components of swimming directions in the absence of a food layer but stronger horizontal components of swimming directions in the

presence of a horizontal layer of food. It is possible that different larval shapes and larval arm lengths lead to different swimming speeds and turning rates of brachiolariae. For instance, a damaged ciliated band and shorter posterolateral larval arms might affect the ability of larvae to sense particles and retain a horizontal position when food is present at the halocline.

When food was absent, brachiolaria larvae exposed to low salinity throughout development appeared to swim randomly to and from the halocline. On the other hand, in the absence of food at the halocline, >60% of brachiolariae that were never exposed to low salinity were at the halocline after 1 h. By 7 h, they had moved to the surface. In contrast to larvae exposed to low salinity throughout development, they did not return to the halocline. Differences in the observed vertical swimming behaviors suggest that larvae initially in high-salinity waters would respond positively to the presence of thin phytoplankton layers at or just below the halocline, whereas those initially in low-salinity waters would not.

The results of our study indicate how *P. ochraceus* larvae might respond to low-salinity surface waters and vertical stratification of the water column in the Salish Sea. The physical conditions in surface waters during spawning events, the initial horizontal and vertical location of embryos and larvae (bipinnariae and brachiolariae) in the water column, and food availability at the surface or halocline all play a critical role in the survival to the brachiolaria larval stage. Larvae of this species have the potential to spend 2 to 7 mo in the water column (Strathmann 1987, Menge & Sanford 2013). The longer poor larval swimmers remain in the water column, the greater the possibility of developing longer arms and becoming better swimmers; however, this is dependent on their vertical distribution in the water column and food availability (see Drake et al. 2015). Furthermore, poor swimmers would have an increased risk of being eaten (Thorson 1946) and being exposed to multiple low-salinity events during the late spring and summer months in the Salish Sea (Riche et al. 2014, present study). Future studies should use mathematical modeling and empirical data to address the effect of flow on swimming and the vertical position of sea star larvae in haloclines. In light of the recent wasting disease along the west coast of the US (Hewson et al. 2014, Jurgens et al. 2015), knowing the effect of the disease on the quality of the gametes produced by surviving populations is of extreme importance. The ability of larvae from these gametes to survive multiple low-salinity events is also of equal importance. The data obtained from these studies will give us insights into the future of sea star populations in the Pacific Northwest.

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