Impacts of low salinity on settlement by competent larvae of *Pycnopodia helianthoides*

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ABSTRACT

Freshwater input from terrestrial environments likely impact much of the biogeographic range of the sunflower sea star *Pycnopodia helianthoides*, particularly its planktonic larvae. Larvae that encounter these water masses need to be able to tolerate freshened seawater. In general, echinoderm larvae have poor tolerance to low salinity, but how changes in salinity affect the settlement and metamorphic success of *P. helianthoides* is not known. Larvae were exposed to an algal settlement cue within four salinity treatments ranging from 20–30‰–control for a duration of 8 days. No difference of overall survival occurred between treatments. By 6 h, settlement occurred in salinity ≥ 24‰. Mean settlement (%) increases steeply from 20‰ to ~24–27‰ indicating this decreased salinity range is likely where larvae delay their settlement. With time, salinity performance curves show the probability of all salinity/settlement cue groups to undergo a metamorphic response approaches 1, indicating settlement rate rather than absolute ability to settle under low salinity conditions may influence patterns of new recruits. Juveniles were first noticed at 177 h exposure and were only found in control and 27‰ treatments. These results suggest that varying salinity levels, commensurate with levels likely experienced by larvae in nature, impact absolute settlement and settlement rates in *P. helianthoides*. We provide important insight into how salinity conditions in the plankton may drive recruitment patterns in the endangered sunflower sea stars benthic habitat.

1. INTRODUCTION

The sunflower sea star, *Pycnopodia helianthoides* (Brandt, 1835), is a large predatory sea star found in the northeastern Pacific Ocean from Baja California to the Alaskan Aleutian Islands. Beginning in 2013, a global outbreak of sea star wasting disease (SSWD) severely reduced the population size and distribution of *P. helianthoides* by up to 94% from 2013 to 2022 (Heady et al., 2022). Given the star's ecological role as a keystone predator, the sharp declines in population size could negatively impact the whole ecosystem (Hermosillo-Núñez, 2020; Paine, 1966; Power et al., 1996). Adults were once common in rocky low intertidal and subtidal habitats across their entire distribution, but are now rare in most of their original range (Harvell et al., 2019; Heady et al., 2022). *P. helianthoides* larvae are planktotrophic and probably have wide dispersal as a result of their long planktonic period (Thorson, 1950; George et al., 2021). The recovery of *P. helianthoides* along its distribution relies on successful larval dispersal as well as settlement and metamorphosis. When coupled with the effects of climate change, the future success of larvae may depend on their ability to tolerate varying abiotic factors such as rising sea surface temperatures and shifts in salinity (George et al., 2021).

In the Salish Sea, the Fraser River serves as the main input of freshwater (Khangaonkar et al., 2017), although glacially-fed rivers and high, seasonal rainfall are other key sources of freshwater. Areas with historically high salinities like the Salish Sea may experience an increasing number of reduced salinity (27‰ and below) events as rising temperatures melt ice caps and increase the rate of snow melt (Bashevkin et al., 2016). Specifically, areas directly beneath and around the Fraser River discharge plume will experience an increase in reduced salinity events. In fact, shifts in seasonality of maximum river discharge from summer to spring as a result of increased snowmelt are already being observed in the Fraser River and Salish Sea (Riche et al., 2014). This shift in river discharge has cascading effects for the marine ecosystems downstream, such as shifting phytoplankton blooms (Riche et al., 2014). The shift in maximum river discharges from summer to spring could have detrimental effects on the larvae of marine invertebrates which reproduce in the spring (George et al. 2021). This combination of more severe storms, shifts in snowmelt, resulting maximum river discharge, and variance in upwelling events as a result of climate change may expose larvae to more salinity variance throughout their planktonic period (George et al., 2021). Species in the Salish Sea would benefit from having larvae with tolerance to reduced salinity in order to prolong individual and species survival (George et al., 2021).

Many echinoderm larvae have been found to be intolerant to reduced salinity (Thorson, 1946; Fenchel, 1965; Binyon, 1972; Watts et al., 1982; Roller & Stickle, 1985). The settlement and metamorphosis portion of the *P. helianthoides* life cycle is an important link to adulthood that may be affected by reduced salinity conditions. It is not yet understood how being exposed to reduced salinity impacts settlement, metamorphosis, or eventual success of juvenile *P. helianthoides*.

Previous investigations indicate that some species of echinoids and asteroids vary in their response to and tolerance of reduced salinity (Clements et al., 2022; George et al., 2021; Stickle & Diehl, 1987). While the water vascular system of adults demonstrates intolerance to reduced salinity, it is poorly understood how affected late-stage larvae are by reduced salinity events. Roller and Stickle (1985) looked at the effect of salinity on the tolerance of early-stage development larvae of four echinoderm species (*Strongylocentrotus droebachiensis*, *S. purpuratus*, *S. pallidus*, and *Pisaster ochraceus*) and found that larvae exposed to salinities less than 22.5‰ had delays in development and 20‰ had abnormal morphology. Limited work has been done on settlement and metamorphosis in the brachiolaria stage larvae of asteroids, especially with respect to low salinity. Previous studies into *Dendraster excentricus* included data on larval development and metamorphosis in low salinity (22‰) conditions, concluding slower development and reduced success of metamorphosis when compared to high salinity (32‰) treatments (George & Walker., 2007). Another study on the crown-of-thorns sea star *Acanthaster* spp. showed deleterious effects on larvae in low salinities (Clements et al., 2022).

Work on *P. helianthoides* larvae under different environmental conditions is relatively recent and focuses on general captive rearing conditions and effects of temperature (Pineda, 2023; Hodin et al., 2021). The work done by Hodin et al. (2021) on egg-to-egg rearing in *P. helianthoides* has greatly advanced the understanding of the planktonic duration and potential aspects of settlement in sunflower sea star larvae. Work on the impacts of different environmental conditions on the larvae of *P. helianthoides* is lacking. This area of research has been well-studied in the larvae of another dominant intertidal species in the Salish Sea, *P. ochraceus*, but there is relatively little literature on the impacts of the environment on *P. helianthoides* settlement (Hodin et al., 2021). A relevant area of research on sea star species inhabiting the rocky intertidal of the Salish Sea aims to understand how reduced salinities (< 24‰) may impact settlement and metamorphosis as the rate of reduced salinity conditions increases in the surrounding area. Hodin et al. (2012) identifies the branching coralline algae *Calliarthron tuberculosum* as a strong cue for settlement in competent larvae of *P. helianthoides*. Other cues that incite settlement responses are mussels, urchins, and adult biofilm (biofilm accumulated from adult tanks) (Hodin et al., 2021). The potential impacts of reduced salinity on *P. helianthoides* larvae settlement and metamorphosis in the presence of settlement cues such as *Calliarthron tuberculosum* have yet to be studied. It is critical to understand the effects of reduced salinity in an increasingly variable Salish Sea on larvae of *P. helianthoides*. Forming an understanding of how an increase in reduced salinity events impacts larval *P. helianthoides* will further elucidate the impact climate change may have on the recovery of this species from SSWD. Most importantly, understanding the impact of reduced salinity on settlement and metamorphosis will indicate future success of this endangered species. This study aims to understand the effect low salinity conditions have on settlement and metamorphosis in competent *P. helianthoides* larvae.

2. MATERIALS AND METHODS

Adult *P. helianthoides* with ~25 cm body diameter, were collected by colleagues from intertidal dock pilings and by SCUBA from 3–20 m depths from waters around San Juan Island (48.54°, -123.01°). Animals were collected with permission from the director of Friday Harbor Laboratory (FHL) who manages the permit for collection of *P. helianthoides* for the San Juan archipelago (state statute: House Bill 68, R.C.W.28B.20.320). These animals were transported in cooling boxes filled with ambient seawater and transferred to 1 m-deep aquaria with high flow of unfiltered seawater (~9–12°C) within 1 h of collection. Individuals were fed ~30–120 g wet weight of *Mytilus trossulus* every ~2–3 d.

2.2. Spawning and rearing

The sex of the adults was first determined after gonad biopsy via small 1 cm^2 incisions at arm junctions, following Hodin et al. (2021). One spontaneous spawn was also observed in at least three males and one female, and fertilised gametes were successfully collected. For surgical fertilisations, two females and two males were biopsied as above. Yielded testes were stored in a tube on ice until use. Ovaries were rinsed gently in 1 mm filtered seawater (FSW) and placed in a dish of the maturation hormone 1-methyl-adenine (10^{-5} M in FSW). All fertilisations occurred in March 2023.

For both biopsy and natural spawn events, fertilisations were confirmed microscopically to have > 90% success. The embryos were rinsed several times to remove excess sperm and placed in 2 L beakers of 1 mm FSW. For surgical fertilisations, gametes from all males and females were mixed to increase genetic variability. Experimental vials containing larvae from various male/female crosses and fertilisations were not monitored separately (see below).

The embryos (~1 ml^{-1}) were reared in 3 L glass beakers of 1 µm FSW and maintained at 11–13°C (± 1°C) in flow-through aquaria. At 5 days post-fertilisation (dpf), larvae had developed a complete digestive tract, indicating the feeding bipinnaria stage. For the first 3.5 months, larvae were fed 2500 cells ml^{-1} of the live microalgae *Rhodomonas* sp. once every 3 d after every water change (~95% of the water was replaced; Hodin et al., 2019). 2 d prior to use in experiments, cultures were fed a mix of 2500 cells ml^{-1} *Rhodomonas* sp. and 3000 cells ml^{-1} of *Dunaliella tertiolecta*. Water changes were done by gentle reverse filtration. Water parameters (temperature ~12°C, salinity 30‰) were measured with a graduated glass thermometer and a refractometer (Tiaoyeer Brix ATC) every other day. The water in culture jars was gently agitated with slow-moving stirring paddles to suspend larvae while maintaining temperature and dissolved oxygen within culture water (Strathmann, 2014).

After ~1 month, the larval cultures were split into ~0.25 larvae ml^{-1} (Hodin et al., 2021). Larvae were raised to competent brachiolariae, identified in culture by a brachiolaria attachment complex and a developed juvenile rudiment. For experiments, 3-month-old larvae with well-developed rudiments (Hodin et al., 2021) (Fig. 1, see below).

2.3. Salinity effects on competent brachiolaria larvae settlement

To test the effects of a low salinity exposure on larval settlement, competent larvae were removed from culture and exposed for 201 h to one of four different salinity treatments 20, 24, 27, and 30‰ with or without an algal settlement cue (Fig. 2). A salinity of 30‰ was chosen as a control and the other three levels represent reduced salinities that may impact *P. helianthoides* habitats on the west coast of North America (Riche et al., 2014; Heady et al., 2022) when their larvae are in the plankton (Hodin et al., 2021), such as the San Juan Island region of the Salish Sea (Bashevkin et al., 2016; Khangaonkar et al., 2019).

For each replicate, 10–11 competent larvae were randomly assigned to one of the four salinities with/or without an algal settlement cue. To make the decreased salinity treatments, 1 μm FSW was diluted with deionised water. All treatments were maintained at 12°C (±SE = 1°C, n = 12) and pH 7.8–7.9. These parameters were evaluated with a graduated glass thermometer and a handheld pH monitor (pH pen, Fisherbrand). Prior to placement in the experimental treatment dishes, larvae were transferred into 100 ml of their designated experimental treatment salinity FSW to wash. Larvae were then pipetted to their experimental glass custard dish, containing 100 ml water at the experimental salinity. Washing ensured that salinity levels in the experimental dishes were not changed by pipetting of larvae. Brachiolariae were assigned randomly to a dish with one of the four salinity treatments with or without an algal settlement cue (Fig. 2).

Thalli of *Calliarthron tuberculosum* (Postels & Ruprecht) E.Y. Dawson, 1964 is a known settlement cue for *P. helianthoides* (Hodin et al., 2021). These algae were collected from Cattle Point, San Juan Island (48.45°, -122.96°) in June 2023, transported to the laboratory, and maintained in unfiltered, flow-through seawater aquaria at ambient temperature (~12°C) until use. Prior to placement of the branch into treatment dishes, epifauna were removed from branches and branches were then washed in DI water for 30 s to kill any remaining contaminating organisms. Branches were monitored closely for signs of post freshwater stress bleaching (i.e., bleached frond tips) and discarded if present. To prepare the settlement cue, 0.6 g branches of *C. tuberculosum* were weighed out using a digital scale (OHAUS). Treatment dishes were randomly dispersed throughout the sea table with running seawater to maintain the temperature of the treatment water within the dishes. The treatment dishes were covered with aluminum foil to minimise evaporation and minimise contaminants.

Larval settlement was scored by observing dishes with a dissecting microscope at 6, 28, 52, 105, 172, and 201 h after larvae were placed in the dishes. The number of competent late-brachiolaria remaining in the water column (= swimming larvae, Fig 1A, B), individuals that had attached to *C. tuberculosum* or the well dish and that had commenced metamorphosis (= metamorphosing larvae, Fig 1C–E), and newly metamorphosed, freely moving juveniles were counted (Fig. 1E). Percentages were calculated for each time point from the proportion of swimming larvae, larvae undergoing metamorphosis, and juveniles versus the starting number of larvae at the beginning of the settlement assay for each replicate. Mean percentages (and variances) were calculated from three replicates of each salinity at each time point. Water changes (~30%) were carefully performed every 3 d with a glass pipette under a microscope to ensure no larvae were lost and to remove any filamentous fibres. At 6 d, the dishes were carefully scrubbed prior to water change to remove noticeable biofilm build-up.

2.4. Statistical analyses

Data were analysed using R (version 4.0.5; R Core Team, 2021). Data on the total survival (mean %: swimming larvae + larvae undergoing metamorphosis + juveniles) after 201 h in the settlement assay were visualised in a column plot. After arcsine transformation, these data were analysed with two-way analyses of variance (ANOVA) using the *lm* function (*stats*). The effects of a 201 h (~8 d) exposure to low salinity (20, 24, 27, and 30‰) and algal settlement cue (present, not present) as well as their interaction were modelled as fixed effects on total survival (%). The response variable was homoscedastic (Levene's test, $p > 0.05$). Visual inspection of the Q-Q plots indicated the normality assumption was not met; however, ANOVA is robust to violations of this assumption in isolation (Quinn & Keough, 2002), so data were not further transformed.

Data on larvae undergoing a metamorphic response (mean %: larvae undergoing metamorphosis + juveniles) in the settlement assay were visualised at six intervals between 6 and 201 h of exposure in a box plot. Juveniles were observed at 177 and 201 h of exposure, and these data were visualised as means (%, ± SE) in dot plots within the respective metamorphic response box plot panels.

To assess the probability of larval settlement at 6 times points between 6 and 201 h of exposure to the settlement cue (1 = settled, 0 = not settled), the sum scores of competent brachiolariae that were undergoing and successfully completed metamorphosis were converted into a proportional data frame out of the starting larval number for each replicate. Salinity (20, 24, 27, and 30‰), algal settlement cue (cue, no cue), time exposed to the settlement/treatment assay, and their interactions were modelled using repeated measures binomial generalised linear mixed effect models (GLMMs) with a logit link function (*lme4*) on the proportional data frame. Replicate ID was included as a random effect in the model formula to deal with the correlation between non-independent observations between levels of time. Models were computed with nAGQ = 25 to enhance model accuracy. To account for model convergence issues, the *glmerControl* function with *bobyqa* optimizer and the *optCtrl* function with 100000 maximum function evaluations were computed in the model call.

Model assumptions were assessed with visual inspection of diagnostic plots (*ggplot2*; Wickham, 2016) and routine tests of homogeneity, multicollinearity, dispersion, linearity, and influential outliers (*DHARMa*; *car*). GLMMs were fitted with estimated marginal means and 95% confidence intervals (CIs). These, and post hoc comparisons for multiple comparisons, were calculated using the *emmeans* package. Type-II Walds Chi-squared tests were used to select the best model fit and to assess significant effects of model coefficients (fixed effects). Salinity performance curves for the probability that competent *P. helianthoides* larvae initiate a metamorphic response were constructed with the best-fit model and 95% CIs with respect to competent brachiolariae settlement using *ggplot2*. To reduce false discovery rates associated with multiple comparisons, p-values ($a = 0.05$) were adjusted using the *BY* procedure following the Benjamin–Yekutieli procedure (Benjamin and Yekutieli, 2001).

3. RESULTS

3.1. Survival of competent larvae in the settlement assay salinity treatments

The mean total survival (%) to 201 h of competent *P. helianthoides* brachiolariae ranged from 90–100% for larvae exposed or those not exposed to an algal settlement cue regardless of salinity level (Fig. 2, Table 1). Two-way ANOVA indicated no significant effect of the salinity, settlement cue, or the interaction of salinity and settlement cue on the mean survival (%) of *P. helianthoides* brachiolariae after 201 h (Fig. 2, Table 2).

3.2 Low salinity effects on P. helianthoides *larval settlement*

An initial response at 6 h of exposure was observed in all larval groups with an algal settlement cue in salinities of 24‰ or higher (mean±SE: 24‰: 33.4±18.6%; 27‰: 56.7±14.5%, 30‰: 41.8±1.8%) (Fig 3, Table 3). By 52 h, groups exposed to an algal settlement cue in ≥ 24‰ approached 100% of larvae undergoing a metamorphic response (mean range: 86.7%, ±13.4 SE – 96.7%, ± 3.4 SE, in 24‰ and 27‰, respectively; see Fig. 3, Table 3). These increases in larvae undergoing a metamorphic response represent a 2.5-, 1.7-, and 2.1-fold increase in settlement for 24, 27, and 30‰ treatments from 6 h of exposure, respectively (Fig. 3, Table 3). For larvae exposed to a settlement cue and 20‰, settlement was low compared to larvae in higher salinities (mean at 6 h exposure: 6.7%, ±3.4 SE; mean at 52 h exposure: 23.4%, ±8.8 SE; see Fig. 3, Table 3). The difference in these cue/20‰ larvae undergoing a metamorphic response between 6 and 52 h of exposure represents a 3.5-fold increase in settlement (Fig. 3, Table 3). While settlement in 20‰ and 24‰ was generally lower over time compared to 27‰ and 30‰, all larvae that were exposed to the cue approached 100% settlement by 201 h (mean percentage±SE: 83.4±3.4% and 20‰ to 100±0% at 30‰; see Fig. 3, Table 3). When the settlement cue was absent, minimal settlement occurred over the 201 h, regardless of which salinity level (Fig. 3, Table 3). These larvae in lower salinities (20 and 24‰) and without settlement cue took much longer to settle over time compared to the higher salinity treatments (27 and 30‰) .

Salinity, algal settlement cue, and time had a significant influence on the probability of the occurrence of settlement (salinity: $\chi^2 = 20.3$, df = 3, $p < 0.001$; cue: $\chi^2 = 107$, df = 1, $p < 0.001$; time: $\chi^2 = 140.8$, df = 5, $p < 0.001$; Table 4). The three-way interaction and all two-way interactions between salinity, cue, and time did not significantly influence the model fit to brachiolariae settlement data (Table 4).

The performance curves (Fig. 4) at 6 h of exposure show a 0 predicted probability for larvae to undergo a metamorphic response in the 20‰/cue treatment. Higher salinities were predicted to have higher probability values for a larva to undergo a metamorphic response (Fig 4). The probability of settlement increases steeply to 24–27‰, indicating this salinity range approximates the settlement tolerance tipping point for deleterious impacts on settlement (Fig. 4). With time, the probability values for a larva in an algal settlement cue to undergo a metamorphic response approach 1. For groups not exposed to an algal settlement cue, the probability of a larva undergoing metamorphosis is negligible (Fig. 4).

Free-moving juvenile *P. helianthoides* were first noticed in our treatments by 177 h of exposure to the settlement cue. The majority of juveniles were observed in the 30‰ treatment with a settlement cue present (mean at 177 h: 30‰: 48.2 ±9.2% SE; mean at 201 h: 71.2±9.5% SE). Juveniles were observed in 27‰/cue treatment group (36.7 ±3.3% SE) by 201 h. No juveniles were observed in the 20‰ or 24‰ treatments by this time.

4. DISCUSSION

Salinity is one of the primary factors that influences the distribution and abundance of a species (Kinne, 1964). Echinoderms are particularly affected by environmental fluctuations in salinity (Russell, 2013). Despite the importance of characterising life stages from early larvae to juveniles, few studies have investigated the impact of salinity on the settlement of echinoderm larvae. Many studies have looked into the effect of a changing climate on other species, including echinoderms. One effect of a changing climate is shifts in maximum river discharge (Riche et al., 2014). These shifts lead to variance in salinity, and increase the abundance of reduced salinity events in the Salish Sea. As climate change increases temperatures, there may be effects on larvae due to increased freshwater influxes. Echinoderms, especially asteroids are documented as having poor osmoregulatory responses (Binyon, 1972; Roller and Stickle, 1985). To understand how reduced salinity impacts the once abundant *P. helianthoides*, it is necessary to understand the effect reduced salinity has on larvae. The recent discovery that coralline algae serves as an effective settlement cue provided an opportunity to study the impacts of salinity on settlement of *P. helianthoides* larvae.

4.1 Survival

Our study found no effect of salinity on survival of larvae of *P. helianthoides* over the 201-h duration when larvae experienced salinities as low as 20‰ (Tables 1, 2). Roller and Stickle (1985) found effects on embryos as well as morphology of early stage bipinnaria larvae and developmental rates in low salinities (less than 22.5‰). Despite having adverse effects on morphology, no mortality was noted over a 32 d period, even at 20‰ (Thorson, 1946; Fenchel, 1965; Binyon, 1972; Watts, 1982). Studies on late-stage 8-arm plutei of *Dendraster excentricus* found that short-term exposure to low salinity (22‰) led to increased settlement, but drops of 10‰ or more in salinity led to survival rates of 71–97% across treatments (George & Walker, 2007). Despite experiencing a 10‰ drop in salinity, there was no noted mortality in our study. A later study by Roller and Stickle (1993) looked at the effect of salinities and temperatures (18 and 20°C) on the settlement and metamorphosis of the Atlantic green sea urchin, *Lytechinus variegatus*. Larvae at salinities lower than 30‰ did not metamorphose, and mortality increased as salinity decreased (Roller & Stickle, 1993). This was not seen in our study as all treatments containing settlement cue experienced metamorphosis, albeit at a delayed rate in reduced salinities. Although all salinity treatments in our study had high survival rates, longer duration studies examining the impact of long-term exposure of juveniles and settled larvae to low salinities are needed to conclude whether mortality is correlated with salinity.

4.2 Settlement

Rate of settlement varied across treatments by exposure time; after the initial 6 h of exposure, only larvae from the 24–30‰ salinity treatments containing settlement cue had begun to settle. Individuals in the 27‰ salinity treatment had higher initial rates of settlement after the 6 and 28 h exposure periods (Fig. 3, Table 3). George and Walker (2007) noted an increase in settlement rate after a short-interval of exposure to low salinity in larvae of *Dendraster excentricus*. Settlement increased at 27‰ but was delayed at the 24‰ and 20‰ salinities. This may suggest larvae of *P. helianthoides* are able to survive large reductions in salinity but still experience detrimental effects at low salinities.Mean settlement increased in the 30‰ treatment from the 6 h exposure period to the end of the exposure period of 201 h (Table 3). It is not understood why the 27‰ treatment had higher initial settlement, but began to decrease after more time elapsed. It is speculated that larvae become increasingly more willing to settle after prolonged exposure to deleterious environmental conditions as a means of "escaping" these conditions within the water column. In the case of our study, being exposed to a moderate reduction in salinity (from 30‰ to 27‰) may have led to some of the larvae attempting to "escape" conditions and settling only to find that the reduction in salinity persisted, decreasing this response. Something to consider is there may be a concentration at which the incentive to settle is diminished by the incentive to persist in the free-swimming stage in hopes of being transported to an area with improved conditions.

In favourable conditions (30‰), it was expected that settlement and metamorphosis would occur at a greater rate. As exposure time increased, settlement at higher salinities also increased. Settlement at the end of the exposure period was highest at 30‰, followed by 27‰ and 24‰ with the least settlement at 20‰ (Fig. 3, Table 3). This suggests that despite settlement rates varying amongst treatment salinities, there is an effect of reduced salinity on the success of settlement and time to metamorphosis. This could be attributed to a likely increase in metabolism in response to reduced salinity, leading to a delay in metamorphosis as more energy is put towards life-sustaining functions as opposed to functions pursuing metamorphosis. In the absence of settlement cue, settlement was greatly delayed in the 24‰, 27‰, and 30‰ treatments and did not occur at all in the 20‰ treatments (Fig. 4). This suggests a strong relationship between settlement cues and settlement for larvae of *P. helianthoides*. There is also indication that larvae ready to settle at 20‰ were unable to settle in the absence of the settlement cue. The only treatment in which the majority of larvae settled and metamorphosed was the 30‰ treatment containing the algae settlement cue. After 165 h, 30‰ was the only treatment that yielded free-moving juveniles (Fig. 5). A distinct difference between the 30‰ and 27‰ treatments containing the settlement cue in terms of number of larvae that eventually metamorphosed into free-moving individuals is seen as 71.2% of individuals underwent metamorphosis in the 30‰ treatment as opposed to 36.7% of individuals in the 27‰ treatment. This has been noted in a variety of echinoderm larvae (Roller & Stickle, 1993; George & Walker, 2007; Clements et al., 2022).

Attachment of larvae on the glass was noted across all treatments containing the algae settlement cue. We attributed this to biofilm residual from other settled larvae in the dish, as the larvae were observed settling near each other (Hodin, *pers. comm.*). The likely cause of a larvae choosing to attach to glass could be attributed to the dissipation of the olfactory cue given off by the algae settlement cue into the surrounding area of the dish.

A limitation of our study is that it did not consider interactions of salinity with other environmental variables that might also impact the settlement of larvae. George et al. (2021) examined the combined effects of temperature and salinity on particle ingestion by *P.*

ochraceus and *D. excentricus* larvae to address this particular limitation. To our knowledge, this interaction has not been explored in *P. helianthoides*, and is a potential future direction to consider. Another relevant question for future research would be to identify the range of salinities over which settlement can and cannot occur in *P. helianthoides* larvae. The concept of reduced salinities has much in common with effects of climate change and more extensive work into the mixed effects of factors such as salinity, temperature and hypoxia on larvae of *P. helianthoides* is necessary to understand the effects of climate change on the future success of this species.

5. CONCLUSIONS

Expected salinities of 24‰, 27‰ and 30‰ with the presence of the alga, *C. tuberculosum*, successfully induced settlement and metamorphosis in *P. helianthoides* within 6 h of exposure. The absence of the cue delayed the settlement of the planktotrophic brachiolariae.

Low salinity can be a major threat for coastal marine invertebrates, especially those with poor abilities to osmoregulate their extracellular fluids (Kinne, 1971; Stickle & Diehl, 1987). Competent larvae of *P. helianthoides* placed at 20‰ demonstrated a weak deployment of a well-developed adhesive attachment. Furthermore, a great number of larvae in the 20‰ began to metamorphose without attaching to the algae cue. In fact, *P. helianthoides* larvae showed a delay of 22 h in settlement at the lowest salinity of 20‰ when exposed to *C. tuberculosum*. This observed behavior is crucial as larvae may face this environmental shift in nature, which increases the probability of predation and mortality.

To predict the future of marine invertebrate populations, it is important to document the effects of a changing climate, specifically, increasing temperatures and decreasing salinities (George et al., 2021). Most importantly, *P. helianthoides* is an endangered species, and conducting this experiment provided a better understanding of the response and settlement succession of competent larvae within low salinities.

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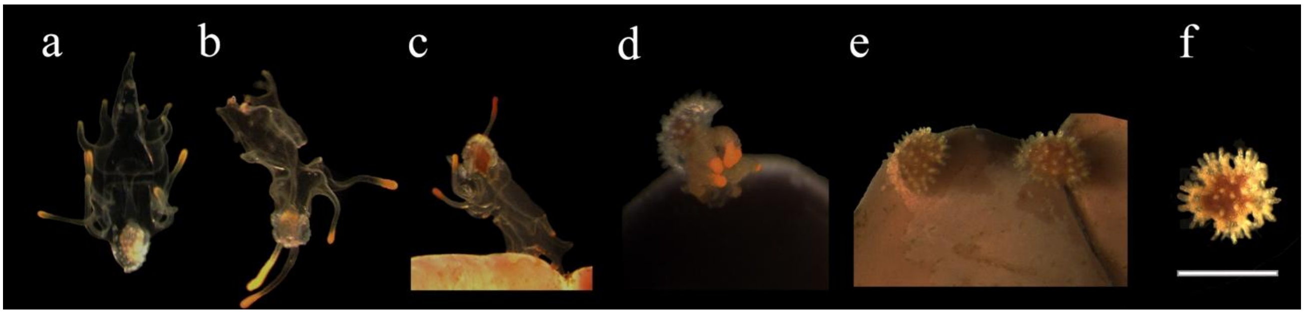

Fig. 1. Stages of *Pycnopodia helianthoides* sea star metamorphosis. **A,B** competent brachiolaria larvae, **C–E** metamorphosing larvae (**C** recently attached to substrate; **D** resorbing larval body and developing primordial juvenile; **E:** metamorphosis near completion), **F** 5-arm juvenile ~6 h after completing metamorphosis. Scale bars represent 1 mm.

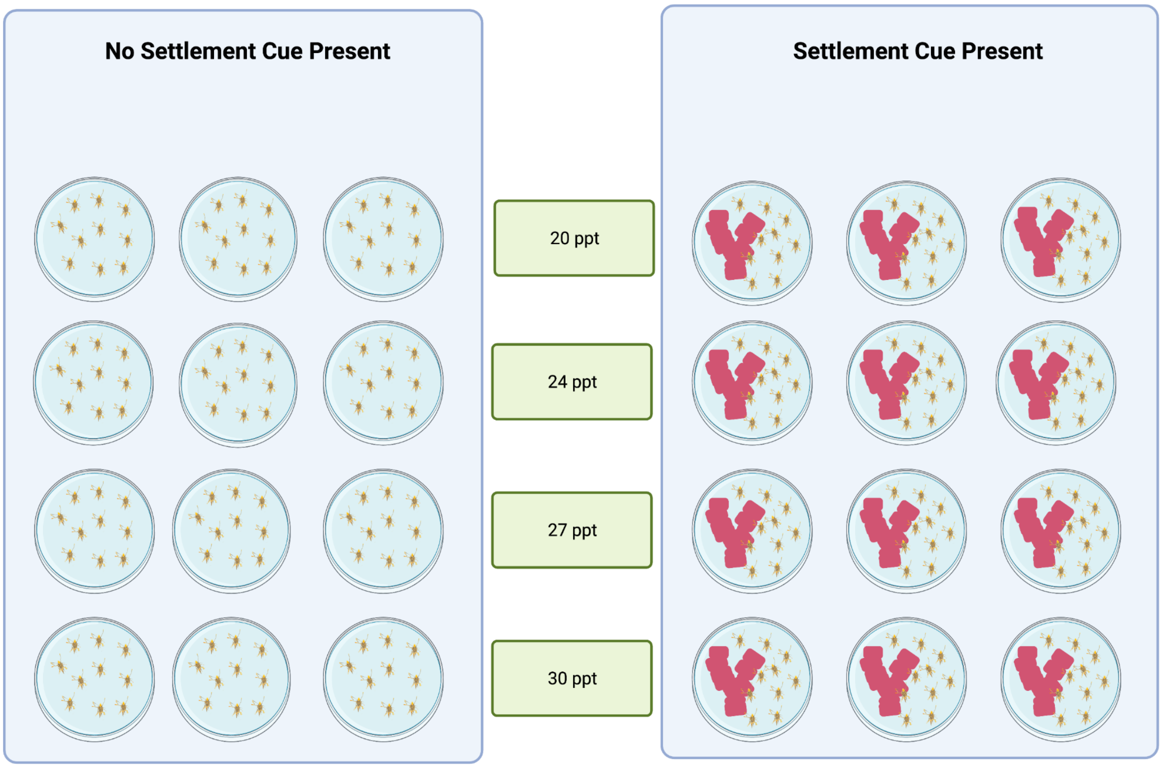

Fig 2. Experimental set-up. 10 brachiolaria larvae were handpicked from culture and placed into a custard dish with an algal settlement cue (0.6 g of *Calliarthron tuberculosum*) present or absent, with 100 mL water at one of four salinity levels: 20, 24, 27, and 30‰–control. Larval were exposed to treatments for 201 h and observations were made at 6, 28, 52, 105, 177, and 201 h. Diagram created using BioRender.

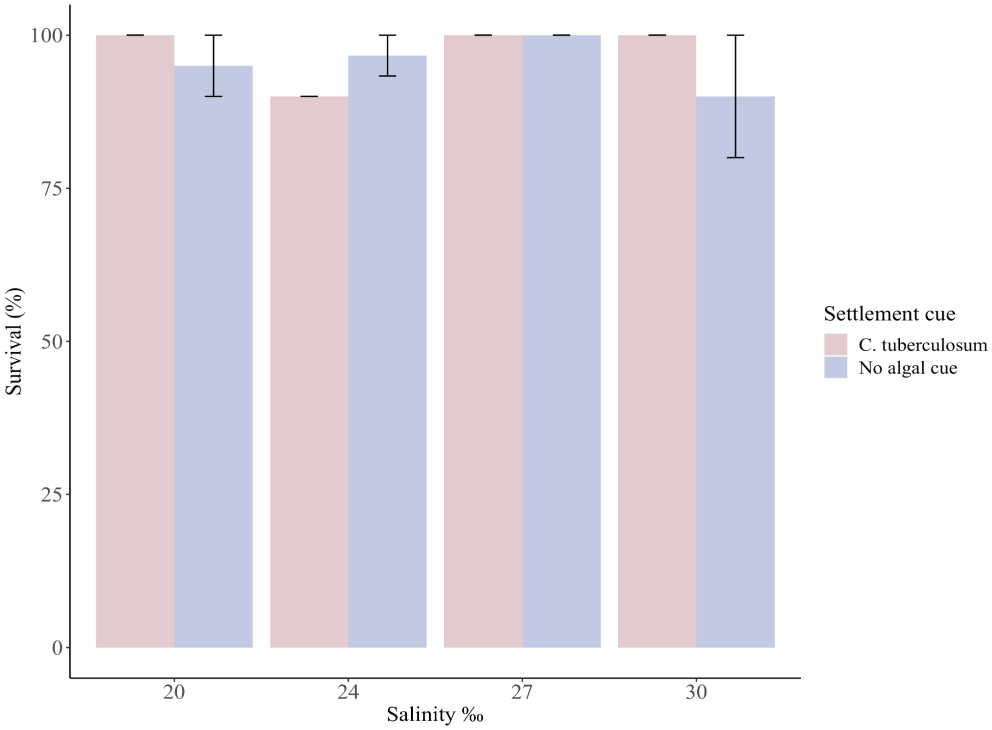

Fig 3. Mean percentage of competent brachiolariae total survival (survival = remaining larvae + larvae undergoing metamorphosis + number of larvae that metamorphosed to the juvenile stage) at 201 h of exposure to the settlement assay in reduced salinity (20, 24, 27 or 30‰ – control). Columns indicate mean % (±SE) from 10–11 larvae used per replicate. Column colours indicate whether larvae were exposed to a 0.6 g branch of *Calliarthron tuberculosum* (pink), a known settlement cue, or to no cue (blue). Means (SE, n) are available in Table 1.

Table 1. Survival of competent brachiolariae (percentage survival = remaining larvae + larvae undergoing metamorphosis + number of larvae that metamorphosed to the juvenile stage/ total larvae at start of study) at 201 h of exposure to the settlement assay in reduced salinity (20, 24, 27 or 30‰ – control). Values are mean % (SE, n) from 10–11 larvae used per replicate.

Settlement cue treatment	**Salinity (‰)**			
	20	**24**	**27**	**30**
Algal cue	100 (0, 3)	90 (0, 3)	100 (0, 3)	90 (1, 3)
No algal cue	95 (4.1, 2)	96.7 (3.4, 3)	100 (0, 3)	100 (0, 3)

Table 2. Two-way analysis of variance (ANOVA) to test for mean differences in arcsine transformed percentages of competent brachiolariae total survival (remaining larvae + metamorphosing + juveniles) after 201 h of exposure to the settlement assay in reduced salinity. Salinity level (four levels: 20, 24, 27 or 30‰) and exposure to an algal settlement cue (two levels: exposure to cue, no cue exposure) were included in the analysis as fixed effects.

Factors	**df**	**SS**	**MS**	**F**	**p-value**
Salinity	3	0.144	0.048	2.084	0.145
Cue	1	0.005	0.005	0.236	0.634
Salinity x Cue	3	0.152	0.051	2.197	0.131
Error	15	0.345	0.023		

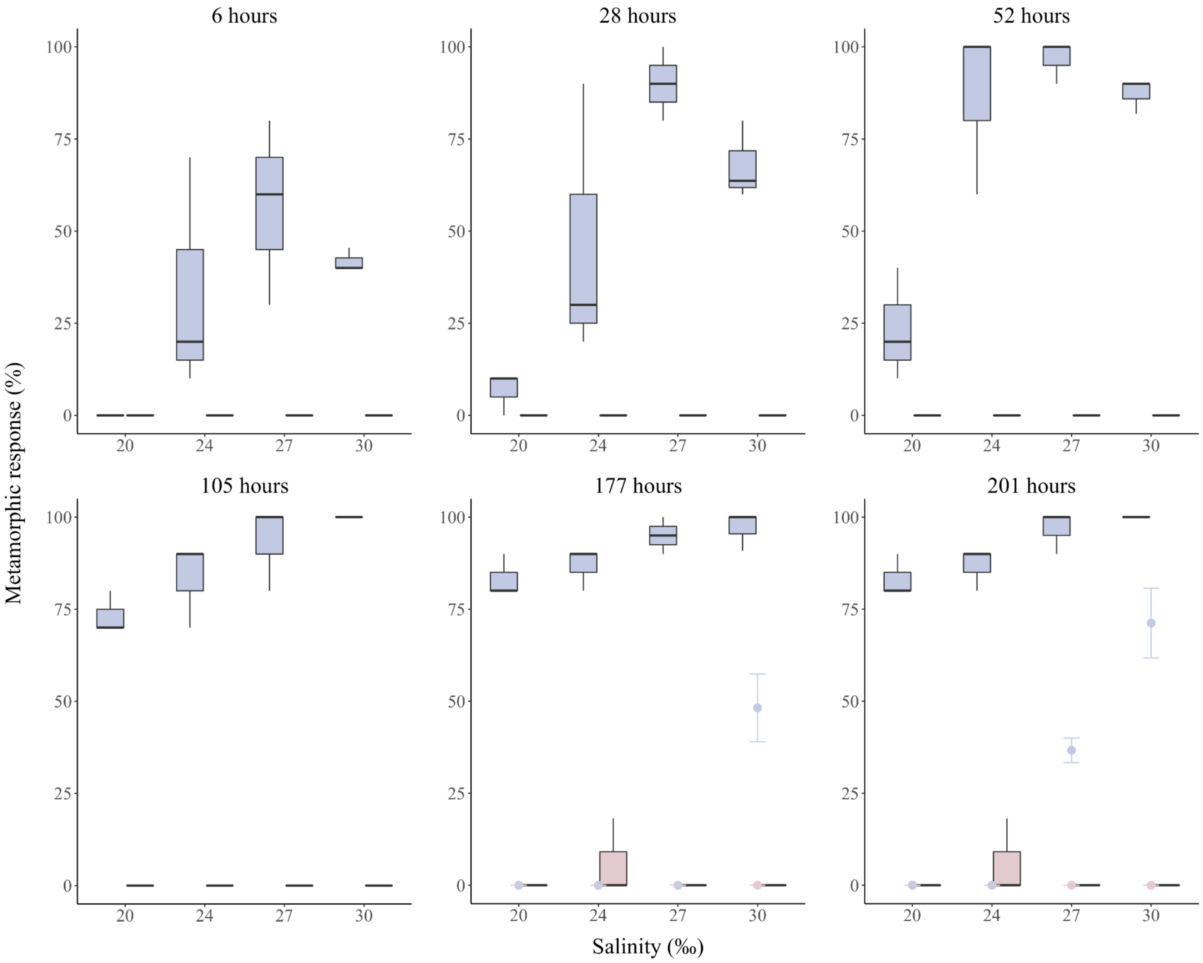

Fig 3. Percentage of competent brachiolariae settlement (%; settlement = larvae undergoing metamorphosis + number of larvae that successfully metamorphosed to the juvenile stage) at 6–201 h exposure to the settlement assay in reduced salinity (20, 24, 27 or 30‰ – control). Box colours indicate whether larvae were exposed to an algal settlement cue (blue) and no cue (pink). The box plots show treatment medians (bolded horizontal line) and interquartile ranges (25th–75th percentiles, shades boxes); maximum-minimum ranges (lines). At 177 h, the first juveniles were noticed in our treatment dishes. Mean % of juveniles (±SE) at, and after, this time point are indicated by coloured points (pink: algal settlement cue; blue: no cue). Means and SE (10–11 larvae, n = 2–3 replicate dishes) are provided in Table 3 (total settlement %) and Table 5 (juveniles %).

Table 3. Percentage of competent brachiolariae settlement (%; settlement = larvae undergoing metamorphosis + number of larvae that successfully metamorphosed to the juvenile stage) at 6–201 h exposure to the settlement assay in reduced salinity (20, 24, 27 or 30‰ – control). Values are mean % (SE, n) with n = number of replicate dishes with 10–11 larvae each.

Exposure time to settlement cue (h)	**Settlement cue treatment**	**Salinity (‰)**			
		20	**24**	**27**	**30**
6	Algal cue	0 (0, 3)	33.4 (18.6, 3)	56.7 (14.5, 3)	41.8 (1.8, 3)
	No algal cue	0 (0, 3)	0 (0, 3)	0 (0, 3)	0 (0, 3)
28	Algal cue	6.7 (3.4, 3)	46.7 (21. 9, 3)	90 (5.8, 3)	67.9 (6.2, 3)
	No algal cue	0 (0, 3)	0 (0, 3)	0 (0, 3)	0 (0, 3)
52	Algal cue	23.4 (8.8, 3)	86.7 (13.4, 3)	96.7 (3.4, 3)	87.3 (2.3, 3)
	No algal cue	0 (0, 3)	0 (0, 3)	0 (0, 3)	0 (0, 3)
105	Algal cue	73.4 (3.4, 3)	83.4 (6.7, 3)	93.4 (6.7, 3)	100 (0, 3)
	No algal cue	0 (0, 3)	0 (0, 3)	0 (0, 3)	0 (0, 3)
177	Algal cue	83.4 (3.4, 3)	86.7 (3.4, 3)	96.7 (3.4, 3)	97 (3, 3)
	No algal cue	0 (0, 2)*	6.1 (6.1, 3)	0 (0, 3)	0 (0, 3)
201	Algal cue	83.4 (3.4, 3)	86.7 (3.4, 3)	96.7 (3.4, 3)	100 (0, 3)

	No algal cue	0 (0, 2)	6.1 (6.1, 3)	0 (0, 3)	0 (0, 3)

*1 replicate lost to contamination

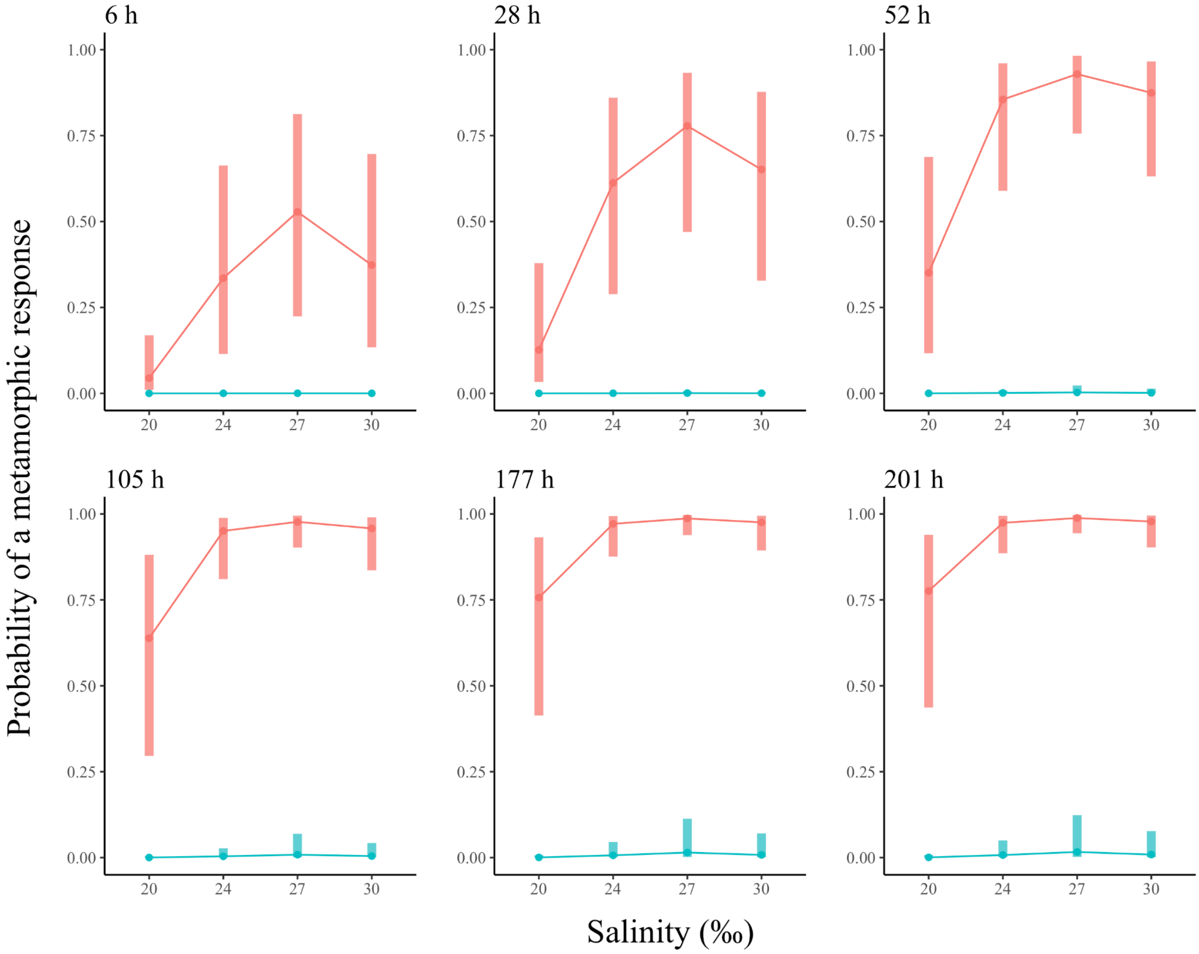

Fig. 4. Salinity performance curves for the probability competent *Pycnopodia helianthoides* larvae will initiate a metamorphic response when exposed to a 0.6 g *Calliarthron tuberculosum* settlement cue (red lines and bars) and for those not exposed to a settlement cue (blue lines and bars) over 201 h. Curves were constructed from generalised linear mixed effect models (GLMMs) on proportional binomial data (1 = larvae responded metamorphically, 0 = no metamorphic response) with salinity treatment (salinity levels: 20, 24, 27 and 30‰), exposure to an algal settlement cue (cue levels: cue, not cue), and time exposed to the settlement assay (time levels: 6, 28, 52, 105, 177, and 201 h) as fixed factors. Replicate Id was included in the models as a random effect. The best generalised linear mixed-effect model fit (Tables 4) was used to generate the curves. The bars indicate 95% confidence intervals (CIs) adjusted with a BY correction method on p values. CIs that do not overlap indicate significant differences.

Table 4. Type II Wald chi-squared test results on binomial-error distributed generalised linear mixed-effect models (GLMMs) for model selection. GLMMs were used to predict the probability of competent brachiolaria larvae undergoing a metamorphic response (1 = initiated a metamorphic response, 0 = did not initiate metamorphosis) as a function of salinity (levels: 24, 27 and 30‰), exposure to an algal settlement cue (levels of cue: algae present, algae not present) and hour of exposure to the settlement cue (levels of time: 6, 28, 52, 78, 105, 129, 153, 177, 201) and their interactions. The 20‰ level was not analysed due to contamination of 1 replicate in the controls. All models include the experimental unit (‘id’) as a random effect. The models performed are indicated in the left column. Model terms define the parameters tested. Significant Wald chi-squared statistics (Wald $\chi 2$) regard model parameters that significantly improve the model fit to the data and are indicated in bold ($p \leq 0.05$), as indicated in the best fit model. Due to model convergence issues, salinity and time variables were centred by subtracting the mean and dividing by the standard error.

Model	**Model terms**	**Waldc^2**	**df**	**p**
Salinity x Cue x Time + (1|id)	Salinity x Cue x Time	0	215	1
(Salinity + Cue + Time)^2 + (1|id)	Salinity x Cue	0	3	1
	Salinity x Time	20.6	15	0.15
	Cue x Time	0	5	1
(Salinity + Cue + Time)+(1|id)	Salinity	20.3	3	**< 0.001**
	Cue	107.7	1	**< 0.001**
	Time	140.8	5	**< 0.001**

Table 5. Percentage of competent brachiolariae that successfully metamorphosed to a free-moving juvenile at 177 & 201 h exposure to the settlement assay in reduced salinity (20, 24, 27 or 30‰ – control). Zero juveniles were present in our treatments prior to 177 h and so these data are not presented here. Values are mean % (SE, n) from 10–11 larvae used per replicate.

Exposure time to settlement cue (h)	**Settlement cue treatment**	**Salinity (‰)**			
		20	**24**	**27**	**30**
177	No algal cue	0 (0, 2)	0 (0, 3)	0 (0, 3)	0 (0, 3)
	Algal cue	0 (0, 3)	0 (0, 3)	0 (0, 3)	48.2 (9.2, 3)
201	No algal cue	0 (0, 2)	0 (0, 3)	0 (0, 3)	0 (0, 3)
	Algal cue	0 (0, 3)	0 (0, 3)	36.7 (3.3, 3)	71.2 (9.5, 3)