Larval settlement and metamorphosis in a marine gastropod in response to multiple conspecific cues

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Abstract

Larvae of the marine gastropod *Crepidula fornicata* must complete a transition from the plankton, where they are highly dispersed, to an aggregated group of benthic adults. Previous research has shown that selective settlement of larvae on conspecific adults is mediated by a water-borne chemical cue. However, variable experimental conditions have been used to study this cue, and standardization is needed in order to investigate factors that may have weak effects on settlement. In this study, we developed a time-course bioassay based on a full-factorial design with temporal blocking and statistical analysis of larval settlement rates in the lab. We tested this bioassay by examining settlement in the presence of an abiotic cue (KCl), and biotic cues (water conditioned with adult conspecifics and conspecific pedal mucus). Results confirmed settlement in the presence of both KCl and adult-conditioned water, and discovered the induction of settlement by pedal mucus. This optimized, standardized bioassay will be used in future experiments to characterize the complex process of larval settlement in *C. fornicata*, particularly to measure components of potentially small effect.

Introduction

An important challenge in ecology is understanding how broadly-dispersed propagules locate sites where they will survive as adults. Many marine invertebrates with complex life cycles have vastly different distributions in their adult and larval stages. Planktonic larvae can disperse tens to hundreds of kilometers during their larval period, while the benthic adults are often sedentary and aggregated in spatially restricted habitats (Cowen & Sponaugle, 2009). Many larvae exhibit selective settlement, where the planktonic-benthic transition is mediated by physiological responses of larvae to physical or chemical cues associated with suitable sites for adults (Krug & Manzi, 1999) or from the adults themselves (Zimmer-Faust & Tamburri, 1994).

The terminology used to describe the process of transitioning from a larva to a juvenile is variable. Terms here are consistent with Pawlik (1992), where settlement refers to the entire process of transitioning from a planktonic larva to a benthic juvenile, while metamorphosis is used to indicate irreversible developmental changes that prevent a larva from returning to its previous planktonic lifestyle. Therefore, the developmental process of metamorphosis is contained within the ecological process of settlement.

Larvae may use physical or chemical cues to settle selectively in appropriate habitats, avoid inappropriate habitats, or delay metamorphosis until appropriate cues are sensed (Morello & Yund, 2016; Pechenik & Eyster, 1989; Thorson, 1950; Woodin, 1986). Environmental cues known to cause larval settlement are diverse and may be associated with biofilms (the gastropod *Crepidula onyx*, Zhao & Qian, 2002), organisms that provide food or habitat (the hydroid *Proboscidactyla flavicirrata*, Donaldson, 1974; the soft coral *Alcyonium siderium*, Sebens, 1983), conspecifics (the barnacle *Semibalanus balanoides*, Gabbott & Larman, 1987; the polychaete worm *Phragmatopoma californica*, Jensen & Morse, 1984; the oyster *Crassostrea*

61 *virginica*, Zimmer-Faust & Tamburri, 1994), or avoidance of organisms with negative impacts
62 (the polychaete worm *Pseudopolydora kempi,* Woodin, 1985).
63 The larvae of the calyptraeid gastropod *Crepidula fornicata* (Linnaeus, 1758) have a
64 planktonic period of two to four weeks (Collin, 2003), which allows for long larval dispersal
65 distances. Within a single location larvae also have a wide spatial distribution, demonstrated by
66 plankton tows of a single estuary that found larvae present in areas without adults (Rigal et al.,
67 2010). In contrast, the sedentary adults are patchily distributed within intertidal and shallow
68 subtidal habitats (Henry et al., 2010; Hoch & Cahill, 2012). Within these habitats adults exhibit
69 a clumped distribution due to their tendency to form large, semi-permanent mating groups called
70 stacks (Collin, 1995). The presence of small juveniles aggregated on adults demonstrates
71 recruitment to these stacks (McGee & Targett, 1989; Cahill, 2015).
72 Selective settlement of *C. fornicata* larvae in response to cues has been studied in the
73 field. Juveniles preferentially aggregate on adults and increased adult density increases larval
74 recruitment (McGee & Targett, 1989; Bohn et al., 2013a,b; Cahill, 2015), consistent with the
75 idea of a cue associated with conspecific adults. Additional support for the role of settlement
76 cues comes from laboratory studies. Larvae metamorphose in response to dissolved or
77 suspended cues, including increased concentrations of potassium chloride (KCl; Pechenik &
78 Heyman, 1987), dibromomethane from coralline algae (Taris et al., 2010), and seawater
79 conditioned with conspecific adults (Pechenik & Heyman, 1987; Pechenik & Gee, 1993; Bohn et
80 al., 2013b). This is also consistent with a conspecific waterborne cue, though the nature of the
81 cue remains unknown. In some studies of settlement in *C. fornicata*, adult-conditioned seawater
82 was prepared in the same vessel in which larvae were tested (Penniman et al., 2013),
83 confounding the effect of the adult-conditioned water with any potential effect of pedal mucus

84 produced by adult snails. Molluscan pedal mucus is known to affect settlement rates in other
85 marine invertebrate larvae such as barnacles (Johnson & Strathmann, 1989; Holmes, 2002).
86 The existence of a conspecific waterborne cue that induces settlement in *C. fornicata* has
87 been supported using a variety of experimental designs and assay conditions (e.g. Pechenik &
88 Heyman, 1987; Pechenik & Gee, 1993; Bohn et al., 2013b), but the chemical nature and
89 biological mechanism of this cue remain unknown. Further characterization of the cue requires
90 repeatable assays that control for biological, technical, and statistical sources of variation in
91 measurements of the settlement process. Biological variability is due to larval age, genetic
92 differences among larvae, and differences among egg masses in survival, growth, and
93 development (Hilbish et al., 1999). Technical variability stems from protocols that differ in
94 biotic and abiotic conditions known to affect larval settlement, including the mass of adults used
95 to produce the cue or the density of larvae in a trial (Pechenik & Heyman, 1987; Pechenik &
96 Gee, 1993; Padilla et al., 2014). An additional, statistical source of variability is introduced by
97 analysis of settlement based on data collected at a single time point. Using this procedure, the
98 results are sensitive to the form of the mathematical function assumed for settlement rate and
99 dependent on which time point is selected (e.g. performing an ANOVA on the proportion of
100 larvae settled at a single timepoint assumes a constant rate of settlement through time).
101 Following the observation that *C. fornicata* distributions differ between the planktonic
102 larval and benthic adult life stages, and building on previous work with conspecific cues, we
103 aimed to provide a standard by which we can then investigate the mechanism responsible for this
104 ecological transition. We developed a bioassay using a fully factorial, randomized block design,
105 which accounted for biological variability. Technical variability was minimized by optimization
106 and control of biotic and abiotic experimental conditions. To address the problem of statistical

variability, we introduced an analysis that estimated settlement rates as a single parameter by fitting an exponential function using multiple observations from regular intervals. Measuring settlement through time with rates also allows for differentiation among cues of different induction strength and response times. This analysis improved the ability of experiments to repeatably detect changes in settlement rates in the complex larval settlement system of *C. fornicata*.

Methods

Crepidula fornicata collection and husbandry

We collected adult *Crepidula fornicata* from Crab Meadow Beach (Northport, New York, USA: 40°55'46"N, 73°19'38"W) in July 2013 and returned them to the lab the same day. No permit is required for collecting this species in New York State, and animals were not returned to the field. Adult females were removed from their substrates to check for incubating egg capsules. Capsules with larvae that were ready to hatch (stage IV veligers *sensu* Leroy et al., 2013) were selected and hatched by physically agitating them in a bowl of filtered seawater at room temperature. Larvae from three females were combined for rearing in cultures of 800 ml of 1 µm-filtered seawater (FSW) at a concentration of one larva per four ml. We collected seawater from an underground well at Flax Pond Marine Laboratories, Old Field, New York, USA (40°57'49"N, 73°08'26"W). We fed larvae 40,000 cells/ml of the alga *Isochrysis galbana* (clone T-ISO) daily. We maintained larval cultures at 20°C and replaced FSW via reverse filtration every three to four days. We tested for larval competence (ability to metamorphose) every two days once the larvae developed shell brims (Pechenik, 1984) and were at least 750 µm long (Pechenik & Heyman, 1987). Competence was tested by placing 12-24 larvae (1-2 larvae from

each culture) in 20 mM KCl solution for 8 hours. It is not possible to work with a larval culture that has reached 100% competence, because at this point many larvae have spontaneously metamorphosed and are no longer available for experiments. We therefore started the experiment within 24 hours of the larvae reaching 75% competence (75% of larvae metamorphosed in response to KCl; Pechenik & Heyman, 1987), which occurred 19 days post-hatch.

Preparation of test solutions

We tested three factors for induction of larval settlement: seawater conditioned with conspecific adults, conspecific pedal mucus, and 20 mM elevated KCl. Prior to the start of the experiment, we acid-cleaned all glassware for ten minutes in 10% concentrated HCl, rinsed them in deionized water, and then autoclaved them.

To create adult-conditioned seawater (ACW), we placed 100 g of adult *C. fornicata* (shell and wet tissue mass) and one liter of FSW into a beaker, then oxygenated the water for twelve hours. Large epibionts (e.g. barnacles, macroalgae) were removed from the shells, but shells were not otherwise treated. One liter of FSW without *C. fornicata*, to be used as a control, was also oxygenated. After twelve hours, adults were removed and ACW and FSW were filtered to 40 μm with a Nitex mesh filter. Temperature, dissolved oxygen, salinity, and pH were measured at all preparation steps. The biotic and abiotic parameters for the bioassay (temperature, salinity, trial length, mass of adults used for cue preparation, etc.) are listed in Table 1. These values were chosen based on optimization studies for each parameter where settlement was measured over a range of values. The optimal value was chosen based on settlement rates. When multiple

152 values gave similar settlement rates, we made our choice based on logistical concerns (details in
153 Fig. A1).

154 We prepared pedal mucus treatments (replicates with pedal mucus left by adult snails;
155 PMG) at the same time that ACW was prepared. Individual replicates of the experiment were
156 conducted in 60 ml drinking glasses (shot glasses or shooters; hereafter "glasses"). For each
157 PMG replicate, a single small (~15 mm) adult *C. fornicata* was added to all glasses for 12 hours
158 at the same time that the ACW was prepared. Glasses were filled with 35 ml FSW and covered
159 to prevent evaporation and snail escape. All snails began the 12 h period at the bottom of the
160 glass, and so even though some crawled above the water line during this period, they still left
161 mucus footprints in the glasses. During the ACW preparation, glasses not receiving the PMG
162 treatment had adults removed and were acid-cleaned and autoclaved to remove the mucus. To
163 prevent desiccation of the mucus, we did not remove adults from glasses receiving the PMG
164 treatment until immediately before the start of the experiment; these glasses were then drained

165 For KCl treatments, we prepared a concentrated solution of 200 mM KCl in distilled
166 water, which was stored until use in the experiments (following Pechenik and Heyman 1987).

167

168 ***Bioassay***

169 Because larvae are expected to become more likely to metamorphose as they develop, we used a
170 randomized complete block design to account for larval age, using the start date of the
171 experiment as a blocking factor (i.e. blocks were run through time). All blocks used the same
172 batch of larvae and therefore larvae in the later blocks were older. All blocks were run within a
173 ten-day period. All three factors (ACW, PMG, KCl) had two levels (present or absent) and were
174 tested using a factorial design (eight possible treatments; Fig. 1). The treatment combination

where all factors were absent corresponded to FSW and served as a negative control. Each treatment combination had three replicate glasses for 24 replicates per block (72 replicates total).

Each glass in the bioassay contained 20 ml of the test solution (Fig. 1). To make the KCl treatments, we added 2 ml of the concentrated KCl solution to the test solution of the replicate (either FSW or ACW; Fig. 1) for a final KCl concentration of 20 mM elevated above FSW. Ten larvae were then individually pipetted into each glass. Larval growth and development in many marine larvae, including *C. fornicata*, varies among cultures (rearing beakers). This variation was accounted for by placing one larva from each rearing beaker (ten total beakers) into each replicate glass. The same set of rearing beakers was used in all three blocks of the experiment.

Every 12 hours, we counted the number of larvae metamorphosed in each replicate, and recorded any mortality. Metamorphosis was measured by the loss of the velar lobes, meaning that it was an irreversible step in development. We removed metamorphosed juveniles and dead larvae from the trial at each time point. To limit bacterial growth and the buildup of waste products, after 24 hours we replaced the test solution in the glasses with clean glasses containing new ACW, KCl, and PMG prepared as described above, and individually pipetted larvae into the clean glasses. The total time of the experiment was 48 hours, which included five time points and two different preparations of test solutions. Larvae were not fed during the experiment.

Data analysis

We conducted an analysis based on settlement rates rather than the proportions of larvae settled at a fixed time point, since proportions are sensitive to the underlying mathematical function of settlement rate and the timepoint selected for the analysis. We modeled larval settlement by

197 predicting the proportion of larvae settled *(y)* at each timepoint *(t*, in hours*)* using the cumulative
198 distribution function for the single-parameter exponential model:
199 $y = 1 - e^{-\lambda t}$.
200 Given a constant probability of settlement, the waiting times for a single individual to
201 settle (t_i) in a given treatment are exponentially distributed. Note that this model assumes that all
202 larvae in the experiment are developmentally capable of settling (competent), although trials
203 began when only 75% of larvae were competent; it was not possible to wait for 100%
204 competence due to high rates of spontaneous settlement under these conditions (see above).
205 Incomplete competence will not affect the overall results if competence is equal in all treatments,
206 a reasonable assumption given our random assignment of larvae to treatments. The exponential
207 distribution is defined by the single parameter λ, which can be estimated as
208 $\hat{\lambda} = n / \Sigma_{i=1}^{n} t_i$.
209 However, because not all larvae settled during the first 48 hours, we calculated λ incorporating
210 Type I censoring with the following equation:
211 $$\hat{\lambda} = \frac{r}{\Sigma_{i=1}^{r} t_i + T(n-r)},$$
212 where n is the total number of larvae tested and r is the number of larvae that settle during the
213 bioassay. Thus, $(n - r)$ is the number of non-metamorphosed larvae at time T which represents
214 the end of the bioassay (fixed at 48 hours for all experiments). By modeling larval settlement in
215 this way, the settlement rate for each replicate could be summarized with a single value ($\hat{\lambda}$) that
216 used time-course data and also accounted for Type I censoring.
217 To analyze the experimental data, we corrected the number of larvae tested (n) for
218 mortality (average mortality per block = 2%, or approximately five larvae; mortality was
219 consistent among treatments) and then calculated $\hat{\lambda}$ for each replicate. We used data from the

first block of the experiment to calculate the correlation between the predicted number of larvae settled for each replicate at each timepoint (based on λ) and the observed number of larvae settled.

The full experiment was then analyzed using $\hat{\lambda}$ as a response variable in an analysis of variance. Blocks were treated as random effects and experimental treatment factors (ACW, PMG, KCl) were treated as fixed effects. The treatment where all factors were absent was equivalent to FSW and served as a control (Fig. 1). Planned comparisons were conducted of each factor against this control (H_0: KCl = FSW, ACW = FSW, and PMG = FSW). The significance of planned comparisons was assessed using the 95% confidence intervals of the mean difference between the treatments. Confidence intervals that did not overlap with zero indicated a significant difference between the treatment and the control (Motulsky, 2010).

Finally, in order to compare our results using rates to results that would be obtained by using proportions, we calculated an ANOVA on the arcsine-squareroot transformed proportions of larvae settled at each timepoint in the analysis (five separate ANOVAs). Statistics were conducted using JMPIN (Version 4.0.4, © SAS Institute 2001) and R 3.0.1 (R Core Development Team, 2013).

Results

Modeling settlement rates

The proportion of larvae expected to settle over successive twelve-hour intervals was predicted by modeling settlement with the rate parameter λ from the exponential distribution. The fit of the estimated values of λ ($\hat{\lambda}$) to the observed cumulative proportions of larvae settled is illustrated with data from the first block of the experiment (Fig. 2). The correlation of predicted

and observed values was high (overall $r = 0.930$, $p < 0.001$) and consistent across treatments (Fig. 2A). The slope of the best-fit line of predicted and observed data was less than one (slope = 0.810 ± 0.030 SE), indicating that the model slightly underpredicted at most time points (Fig. 2B). Modeling settlement as the rate parameter λ was a more informative statistical analysis than using proportions of larvae settled at a given time. Performing the analysis on arcsine-transformed proportions using ANOVAs yielded inconsistent results, such that the significance of both main effects and interaction terms depended on the time point selected for the analysis (Table 2, Table A1).

Larval settlement rates

All factors showed an increased settlement rate (λ) relative to the filtered seawater (FSW) control (Fig. 3,4). The linear model contained two statistically significant treatment effects (KCl, $F_{1,62} = 19.43$, $p < 0.001$; KCl*ACW, $F_{1,62} = 14.57$, $p < 0.001$; Table 3), with block effects through time accounting for 24.6% of total variation. The strength of the artificial cue was likely responsible for significant interaction effects (KCl*ACW, Table 3), as complete induction by KCl allowed for no additional effect of the ACW treatment.

The mean settlement rates (λ, in units of ln(number larvae settled per hour)) for KCl, ACW, PMG, and FSW were 0.01796, 0.01218 0.007234, and 0.000715, respectively. The 95% confidence intervals of the difference in mean settlement rates between each treatment factor and the FSW control did not overlap with zero (difference in λ between KCl - FSW = 0.01075 - 0.02374; ACW - FSW = 0.005248 - 0.017572; PMG – FSW = 0.002583 - 0.010456). This indicates that all factors induced settlement in *C. fornicata* (Fig. 4).

Discussion

This experiment confirmed larval settlement induction in *Crepidula fornicata* by 20 mM elevated potassium chloride (KCl) as well as adult-conditioned water (ACW) and discovered the inductive effect of pedal mucus (PMG) in the absence of ACW (Fig. 3,4). Induction of settlement in *C. fornicata* by KCl and ACW has been previously reported (Pechenik & Heyman, 1987; Pechenik & Gee, 1993; Bohn et al. 2013b). We reproduced these previous results with statistical significance, validating the time-course bioassay and analysis using the rate parameter λ.

Using λ to measure settlement rather than proportions of larvae settled at a single timepoint allowed for a consistent analysis. When using proportions, the significance of both main effects and interactions varied depending on the timepoint selected (Table 2), making the analysis less robust to variation in experimental duration. The use of λ will be particularly important when measuring potentially weak effects of induction, which induce settlement at a slower rate. The experiment presented here does not allow for any characterization of the chemical cues involved in this complex settlement system. However, the bioassay and analysis now provide a standardized protocol and statistical analysis for the estimation of subtle differences in settlement rates under various treatments.

Pedal mucus is a weak inducer of settlement which was not detectable using the proportions of larvae settled at particular timepoints (Table 2, Table A1), but which we were able to detect by analyzing settlement rates (Fig. 3,4). Previous work has shown that molluscan pedal mucus affects settlement in other organisms (the barnacles *Balanus glandula* and *Semibalanus balanoides*: Johnson & Strathmann, 1989; Holmes, 2002). Additionally, a study of settlement in *C. fornicata* used a combined treatment of adult-conditioned water and pedal mucus to induce

289 settlement (Penniman et al., 2013). However, our study is the first to demonstrate the inductive
290 effect of mucus in the absence of adult-conditioned water.

291 There may be other weak inducers of settlement that can be detected with the analysis of
292 settlement rates. Bacteria and biofilms have often been implicated in larval settlement, including
293 in *Crepidula* species (*C. onyx*; Zhao & Qian, 2002). To limit bacterial growth in the current
294 experiment, larvae were transferred into new water and glasses after 24 hours. There was no
295 accelerated settlement in time points immediately preceding transfers, indicating that any effect
296 of bacterial populations on settlement is overwhelmed by the signal of the conspecific cues in
297 ACW and PMG treatments. However, we did not test explicitly for the effect of biofilms and
298 bacteria, which have been shown to be a weaker effect than conspecific cue in *C. onyx* (Zhao &
299 Qian, 2002). The bioassay can be used to test for this effect, as well as measuring its strength
300 relative to conspecific cues in *C. fornicata*.

301 The sensitivity of the bioassay and analysis also allows for the characterization of the
302 chemical cues involved in *C. fornicata* settlement. Many chemical inducers of settlement are
303 known from other gastropods, including carbohydrates (*Alderia modesta*, Krug and Manzi,
304 1999), metabolites (*Phestilla sibogae*, Hadfield and Pennington, 1990), volatile halogenated
305 organic compounds (*Haliotis discus hannai*, Kang et al., 2004), and peptides (*Adalaria proxima*,
306 Lambert et al., 1997). Work is currently underway to use the bioassay to begin to characterize
307 the cue present in both ACW and PMG (A. Cahill and S. Koury, unpublished data).

308 The optimization and standardization of the conditions of the bioassay allowed us to
309 account for several sources of variation in larval settlement. Settlement rates in this study were
310 variable among temporal blocks, with nearly one quarter of the variation in settlement rate
311 explained by block effects. Larval settlement rate is expected to change through time as larvae

become competent during development. The use of a blocked design allowed us to statistically account for this variation while increasing our sample size beyond the number of larvae that could be tested at one time.

Differences in competency among larvae within a block could be explained by differences in larval growth rate (Pechenik & Lima, 1984; Pechenik et al., 1996) due to food availability or temperature (Padilla et al., 2014). However, we controlled variation by rearing all larvae on the same diet and at the same temperature. Variation in larval growth rate of *C. fornicata* is also influenced by sire (Le Cam et al., 2009) and maternal effects (Hilbish et al., 1999). Another potential source of variation among broods was due to the fact that we artificially hatched the larvae, rather than waiting for natural hatching. We attempted to control for this by only hatching very late-stage embryos, but some broods may have been more developmentally advanced than others, leading to higher settlement rates. Due to the size of the experiment and logistical constraints, we were unable to statistically account for these effects (i.e. by blocking according to brood). By randomly mixing larvae from multiple broods, we spread unknown brood-related variation evenly among all treatments and blocks, so such variation does not impact our overall results regarding the different treatments.

Variation in settlement rate among larvae may also explain the discrepancy between the fact that 75% of the larvae tested in KCl were competent before an experiment began, but that settlement was consistently lower than 75% in the KCl treatments under experimental conditions (Fig. 3, Fig. A1e). The KCl used for the competency tests was prepared in the same way as that for the experiment itself. Although the use of KCl solution prepared in DI water reduced the salinity to approximately 26, slightly below values reported as optimal in Table 1, these treatments nonetheless showed high levels of settlement, and larval behavior did not appear

335 impacted. The salinity levels remained near or above the values observed at the collection site in
336 Northport (e.g. salinity in June 2015 was 24.1; A. Cahill, unpublished data).
337 In addition to variation among larvae, there was variation among preparations of both the
338 ACW and the PMG: different adult animals were used for each preparation. The standardization
339 of the abiotic and biotic parameters of the experiment minimized differences among blocks
340 associated with preparation of adult-conditioned water (Table 1, Fig. A1). However, in the
341 absence of a clearly identified chemical that induces settlement, it remains impossible to control
342 the exact concentration of cue delivered to the larvae.
343 Food limitation has also been shown to increase settlement rates in *C. fornicata*
344 (Pechenik et al., 1996). This may have played a role in our experiment, since larvae were not fed
345 during the settlement trials. This food limitation was the same across treatments, and does not
346 explain the high settlement rates in KCl and ACW treatments relative to FSW. It may, however,
347 explain the small number of larvae that settled in the FSW treatment despite the absence of
348 settlement cues (Fig. 3,4). This spontaneous, background settlement potentially due to food
349 limitation should be the same across treatments.
350
351 **Conclusion**
352 We developed an optimized time-course bioassay to estimate larval settlement rates in *C.*
353 *fornicata*. We replicated previous results by demonstrating settlement in response to both
354 elevated concentrations of KCl and a waterborne conspecific cue. For the first time, we
355 demonstrated that pedal mucus from adult conspecifics induces settlement in the absence of
356 adult-conditioned water. Future work using our new bioassay will characterize these cues and
357 investigate other potentially weak inducers of settlement.

Acknowledgements

W. Bruno, A. Pellman-Isaacs, and J. Tay helped with larval rearing. We thank J. Levinton and W. Eanes for lab space and equipment, J. Rohlf for statistical advice, and J. Levinton, D. Padilla, D. Futuyma, F. Viard, W. Eanes, J. Rollins, E. Rollinson, and several anonymous reviewers for comments on previous versions of the manuscript.

References

Bohn K, Richardson CA, Jenkins SR. 2013a. The importance of larval supply, larval habitat selection and post-settlement mortality in determining intertidal adult abundance of the invasive gastropod *Crepidula fornicata*. *Journal of Experimental Marine Biology and Ecology*, 440: 132-140. DOI:10.1016/j.jembe.2012.12.008.

Bohn K, Richardson CA, Jenkins SR. 2013b. Larval microhabitat associations of the non-native gastropod *Crepidula fornicata* and effects on recruitment success in the intertidal zone. *Journal of Marine Biology and Ecology*, 448: 289-297. DOI:10.1016/j.jembe.2013.07.020.

Cahill AE. 2015. Adult density affects larval recruitment in the calyptraeid gastropod *Crepidula fornicata*. *Journal of Experimental Marine Biology and Ecology*, 465: 77-82. DOI:10.1016/j.jembe.2015.01.013.

Collin R. 1995. Sex, size, and position: a test of models predicting size at sex change in the protandrous gastropod *Crepidula fornicata*. *American Naturalist*, 146: 815-831.

Collin R. 2003. Worldwide patterns in mode of development in calyptraeid gastropods. *Marine Ecology Progress Series*, 247: 103-122.

381 Cowen RK, Sponaugle S. 2009. Larval dispersal and marine population connectivity. *Annual*
382 *Reviews of Marine Science*, 1: 443-466. DOI: 10.1146/annurev.marine.010908.163757.
383 Donaldson S. 1974. Larval settlement of a symbiotic hydroid: specificity and nematocyst
384 responses in planulae of *Proboscidactyla flavicirrata*. *Biological Bulletin,* 147: 573-585.
385 Gabbott PA, Larman VN. 1987. The chemical basis of gregariousness in cirripedes: a review. In:
386 Southward AJ, ed. *Barnacle Biology*. Rotterdam: A.A. Balkema, 377-388.
387 Hadfield MG, Pennington JT. 1990. Nature of the metamorphic signal and its internal
388 transduction in larvae of the nudibranch *Phestilla sibogae*. *Bulletin of Marine Science*,
389 46: 455-464.
390 Henry JJ, Collin R, Perry KJ. 2010. The slipper snail, *Crepidula*: an emerging lophotrochozoan
391 model system. *Biological Bulletin*, 218: 211-229.
392 Hilbish TJ, Sasada K, Eyster LS, Pechenik JA. 1999. Relationship between rates of swimming
393 and growth in veliger larvae: genetic variance and covariance. *Journal of Experimental*
394 *Marine Biology and Ecology*, 239: 183-193. DOI:10.1016/S0022-0981(99)00009-X.
395 Hoch JM, Cahill AE. 2012. Variation in size at sex-change among natural populations of the
396 protandrous hermaphrodite, *Crepidula fornicata* (Gastropoda, Calyptraeidae). *Marine*
397 *Biology*, 159: 897-905. DOI:10.1007/s00227-011-1867-4.
398 Holmes SP. 2002. The effect of pedal mucus on barnacle cyprid settlement: a source for indirect
399 interactions in the rocky intertidal? *Journal of the Marine Biological Association of the*
400 *United Kingdom*, 82: 117-129. DOI: http://dx.doi.org/10.1017/S0025315402005234.
401 Jensen RA, Morse DE. 1984. Intraspecific facilitation of larval recruitment: gregarious
402 settlement of the polychaete *Phragmatopoma californica* (Fewkes). *Journal of*

403 *Experimental Marine Biology and Ecology*, 83: 107-126. DOI:10.1016/0022-
404 0981(84)90040-6.
405 Johnson LE, Strathmann RR. 1989. Settling barnacle larvae avoid substrata previously occupied
406 by a mobile predator. *Journal of Experimental Marine Biology and Ecology*, 128: 87-
407 103. DOI:10.1016/0022-0981(89)90094-4.
408 Kang KH, Kim BH, Kim J M. 2004. Induction of larval settlement and metamorphosis of the
409 abalone, *Haliotis discus hannai* larvae using bromomethane and potassium chloride.
410 *Aquaculture*, 230: 249-259. DOI:10.1016/S0044-8486(03)00440-X.
411 Krug PJ, Manzi AE. 1999. Waterborne and surface-associated carbohydrates as settlement cues
412 for larvae of the specialist marine herbivore *Alderia modesta*. *Biological Bulletin*, 197:
413 94-103.
414 Lambert WJ, Todd CD, Hardege JD. 1997. Partial characterization and biological activity of a
415 metamorphic inducer of the dorid nudibranch *Adalaria proxima* (Gastropoda:
416 Nudibrancha). *Invertebrate Biology*, 116: 71-81. DOI: 10.2307/3226971.
417 Le Cam S, Pechenik JA, Cagnon M, Viard F. 2009. Fast versus slow larval growth in an invasive
418 marine mollusc: does paternity matter? *Journal of Heredity*, 100: 455-464. DOI:
419 10.1093/jhered/esp007.
420 Leroy F, Meziane T, Riera P, Comtet T. 2013. Seasonal variation in maternal provisioning of
421 *Crepidula fornicata* (Gastropoda): fatty acid composition of females, embryos, and
422 larvae. *PLoS ONE*, 8: e75316. DOI: 10.1371/journal.pone.0075316
423 McGee BL, Targett NM. 1989. Larval habitat selection in *Crepidula* (L.) and its effect on adult
424 distribution patterns. *Journal of Experimental Marine Biology and Ecology*, 131: 195-
425 214. DOI:10.1016/0022-0981(89)90112-3.

426 Morello SL, Yund PO. 2016. Response of competent blue mussel (*Mytilus edulis*) larvae to
427 positive and negative settlement cues. *Journal of Experimental Marine Biology and*
428 *Ecology*, 480: 8-16. DOI: 10.1016/j.jembe.2016.03.019.
429 Motulsky H. 2010. *Intuitive Biostatistics*, 2nd ed. New York: Oxford University Press.
430 Padilla DK, McCann MJ, McCarty-Glenn M, Hooks AP, Shumway SE. 2014. Effect of food on
431 metamorphic competence in the model system *Crepidula fornicata*. *Biological Bulletin*,
432 227: 242-251.
433 Pawlik JR. 1992. Chemical ecology of the settlement of benthic marine invertebrates.
434 *Oceanography and Marine Biology, an Annual Review*, 30: 273-335.
435 Pechenik JA. 1984. The relationship between temperature, growth rate, and duration of
436 planktonic life for larvae of the gastropod *Crepidula fornicata* (L.). *Journal of*
437 *Experimental Marine Biology and Ecology*, 74: 241-257. DOI:10.1016/0022-
438 0981(84)90128-X.
439 Pechenik JA, Eyster LS. 1989. Influence of delayed metamorphosis on the growth and
440 metabolism of young *Crepidula fornicata* (Gastropoda) juveniles. *Biological Bulletin*,
441 176: 14-24.
442 Pechenik JA, Gee CC. 1993. Onset of metamorphic competence in larvae of the gastropod
443 *Crepidula fornicata*, judged by a natural and an artificial cue. *Journal of Experimental*
444 *Marine Biology and Ecology*, 167: 59-72. DOI:10.1016/0022-0981(93)90184-P.
445 Pechenik JA, Heyman WD. 1987. Using KCl to determine size at competence for larvae of the
446 marine gastropod *Crepidula fornicata* (L.).*Journal of Experimental Marine Biology and*
447 *Ecology*, 112: 27-38. DOI:10.1016/S0022-0981(87)80012-6

448 Pechenik JA, Lima GM. 1984. Relationship between growth, differentiation, and length of larval
449 life for individually reared larvae of the marine gastropod, *Crepidula fornicata*.
450 *Biological Bulletin*, 166: 537-549.
451 Pechenik JA, Estrella MS, Hammer K. 1996. Food limitation stimulates metamorphosis of
452 competent larvae and alters postmetamorphic growth rate in the marine prosobranch
453 gastropod *Crepidula fornicata*. *Marine Biology*, 127: 267-275. DOI:
454 10.1007/BF00942112.
455 Penniman JR, Doall MK, Pires A. 2013. Neural correlates of settlement in veliger larvae of the
456 gastropod, *Crepidula fornicata*. *Invertebrate Biology*, 132: 14-26. DOI:
457 10.1111/ivb.12014.
458 R Core Team. 2013. *R: A language and environment for statistical computing*. R Foundation
459 for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-
460 project.org/
461 Rigal F, Viard F, Ayata S-D, Comtet T. 2010. Does larval supply explain the low proliferation of
462 the invasive gastropod *Crepidula fornicata* in a tidal estuary? *Biological Invasions*, 12:
463 3171-3186. DOI : 10.1007/s10530-010-9708-9
464 Sebens K P. 1983. Settlement and metamorphosis of a temperate soft-coral larva (*Alcyonium*
465 *siderium* Verrill): induction by crustose algae. *Biological Bulletin*, 165: 286-304.
466 Taris N, Comtet T, Stolba R, Lasbleiz R, Pechenik JA, Viard F. 2010. Experimental induction of
467 larval metamorphosis by a naturally-produced halogenated compound (dibromomethane)
468 in the invasive mollusc *Crepidula fornicata* (L.). *Journal of Experimental Marine*
469 *Biology and Ecology*, 393: 71-77. DOI:10.1016/j.jembe.2010.07.001.

Thorson G. 1950. Reproductive and larval ecology of marine benthic invertebrates. *Biological Reviews of the Cambridge Philosophical Society*, 25: 1-45.

Woodin SA. 1985. Effects of defecation by arenicolid polychaete adults on spionid polychaete juveniles in field experiments: Selective settlement or differential mortality? *Journal of Experimental Marine Biology and Ecology*, 87: 119-132. DOI:10.1016/0022-0981(85)90085-1.

Woodin SA. 1986. Settlement of infauna: larval choice? *Bulletin of Marine Science*, 39: 401-407.

Zhao B, Qian P-Y. 2002. Larval settlement and metamorphosis in the slipper limpet *Crepidula onyx* (Sowerby) in response to conspecific cues and the cues from biofilm. *Journal of Experimental Marine Biology and Ecology*, 269: 39-51. DOI:10.1016/S0022-0981(01)00391-4.

Zimmer-Faust RK, Tamburri MN. 1994. Chemical identity and ecological implications of a waterborne, larval settlement cue. *Limnology and Oceanography*, 39: 1075-1087. DOI: 10.4319/lo.1994.39.5.1075.

485 **Table 1:** Optimized values of abiotic and biotic parameters in the bioassay. Further details about
486 the optimization process can be found in Fig. A1.

Variable	Range Tested	Optimal Value
pH	7.9 – 10.2	8.0 – 8.3
Salinity	25 – 40	27-30
Adult mass used for ACW	4 g – 800 g	100 g
ACW preparation time	1 h – 24 h	12 h
Larvae per replicate 20 ml glass	5 – 20	10
Trial length	8 h – 212 h	48 h
Sampling time intervals	4 h – 24 h	12 h

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Table 2: Heat map illustrating the change in significance through time of main effects and interactions tested, analyzed using ANOVAs on arcsine square root transformed proportions; calculations were done at each time step. Values in cells represent p-values for each factor at each timestep. Dark grey: $p < 0.001$; light grey: $0.001 < p < 0.05$; white: $p > 0.05$. Full ANOVA tables for each timestep can be found in Table A1.

Source of Variation	12 hours	24 hours	36 hours	48 hours
Adult Conditioned Water (ACW)	0.408	0.301	**0.002**	**0.006**
Pedal Mucus Glass (PMG)	0.936	0.949	0.0514	0.058
Potassium Chloride (KCl)	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
PMG*ACW	0.750	0.223	**0.046**	**0.026**
PMG*KCl	0.317	0.598	**0.006**	**0.004**
ACW*KCl	**< 0.001**	**< 0. 001**	**< 0.001**	**< 0.001**
ACW*PMG*KCl	0.207	0.182	0.145	0.096

496 **Table 3:** Analysis of variance table, conducted using rates (λ) as the response variable.

497 Significant effects at p = 0.05 are highlighted in bold.

Source of Variation	df	SS	MS	F	p
Block Effect (Time of experiment)	2	1.74E-03	8.71E-04	-----	-----
Adult Conditioned Water (ACW)	1	6.71E-05	6.71E-05	1.29	0.26
Pedal Mucus Glass (PMG)	1	1.26E-05	1.26E-05	0.24	0.63
Potassium Chloride (KCl)	**1**	**1.01E-03**	**1.01E-03**	**19.43**	**< 0.001**
PMG*ACW	1	1.07E-04	1.07E-04	2.04	0.16
PMG*KCl	1	1.26E-04	1.26E-04	2.43	0.12
ACW*KCl	**1**	**7.59E-04**	**7.59E-04**	**14.57**	**< 0.001**
PMG*ACW*KCl	1	6.51E-06	6.51E-06	0.12	0.72
Error	62	3.23E-03	5.21E-05	0.00	
Total	71	7.06E-03			

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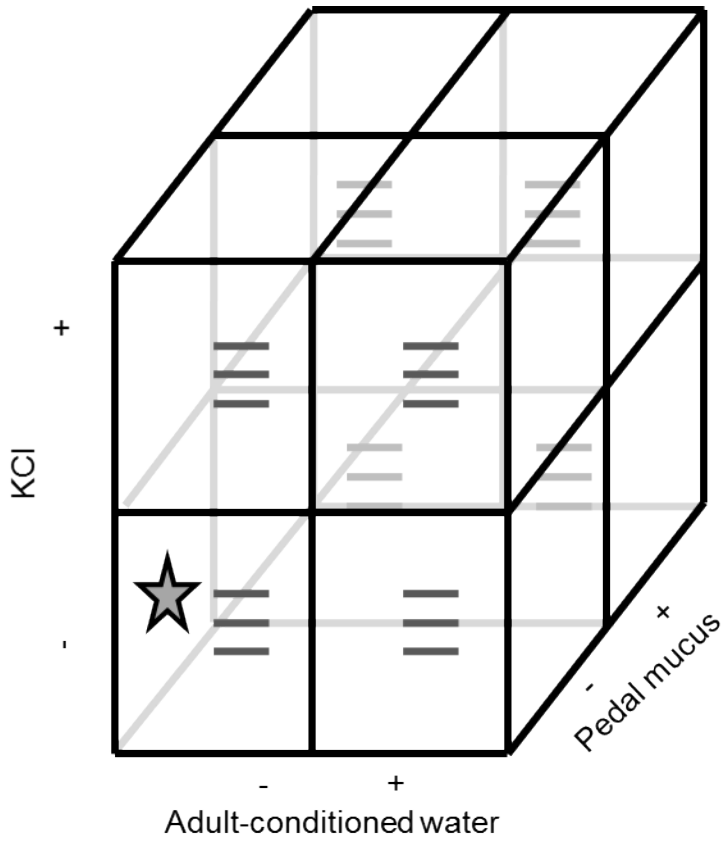

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504 **Figure 1: Experimental design.** The experiment contained three factors (potassium chloride,
505 adult-conditioned water, and pedal mucus), each either present (+) or absent (-), crossed in a
506 factorial design for a total of eight treatments. Each treatment had three replicates (grey lines)
507 with ten larvae in each replicate. The star indicates the treatment where all factors were absent.
508 This treatment was equivalent to filtered seawater and served as the control. This design was
509 repeated in three blocks through time (see details in text).

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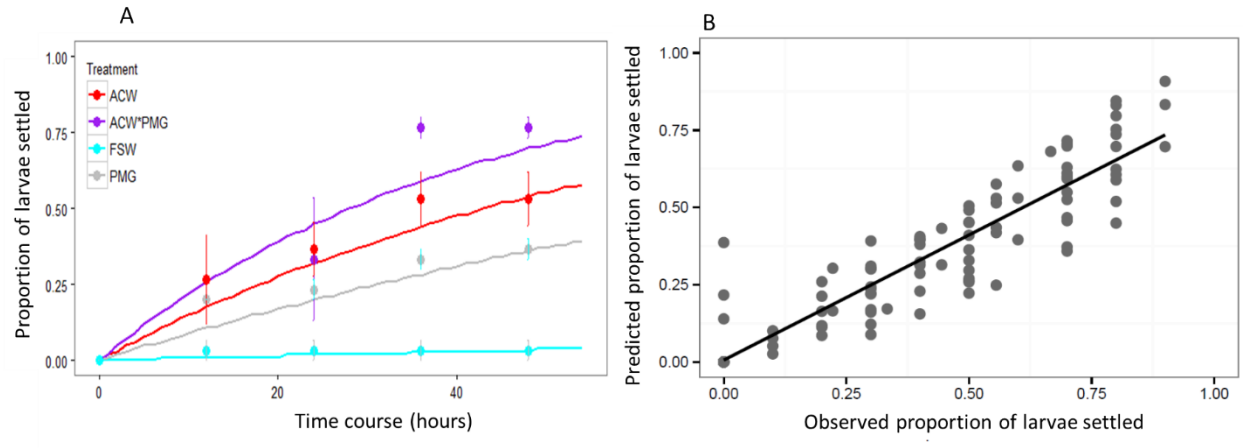

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512 **Figure 2: Fit of λ to observed data.** A) Observed larval settlement (circles) and the values
513 predicted by λ (lines) at each time point, plotted as the proportion of larvae settled at each point.
514 Values calculated based on the first block of the experiment. Error bars represent standard error.
515 There are close correlations between observed and expected values for all treatments: adult-
516 conditioned water (ACW; $r = 0.977$, $p < 0.001$), adult-conditioned water and pedal mucus
517 (ACW*PMG; $r = 0.957$, $p < 0.001$), pedal mucus glasses (PMG; $r = 0.965$, $p < 0.001$), and
518 filtered seawater (FSW; $r = 0.710$, $p < 0.001$). B) Plot of observed versus predicted proportions
519 of larvae settled in each glass at all time points. Values calculated based on the first block of the
520 experiment. The best-fit line is the linear regression to the data ($y = 0.810x + 0.006$; $r = 0.930$; p
521 < 0.001).

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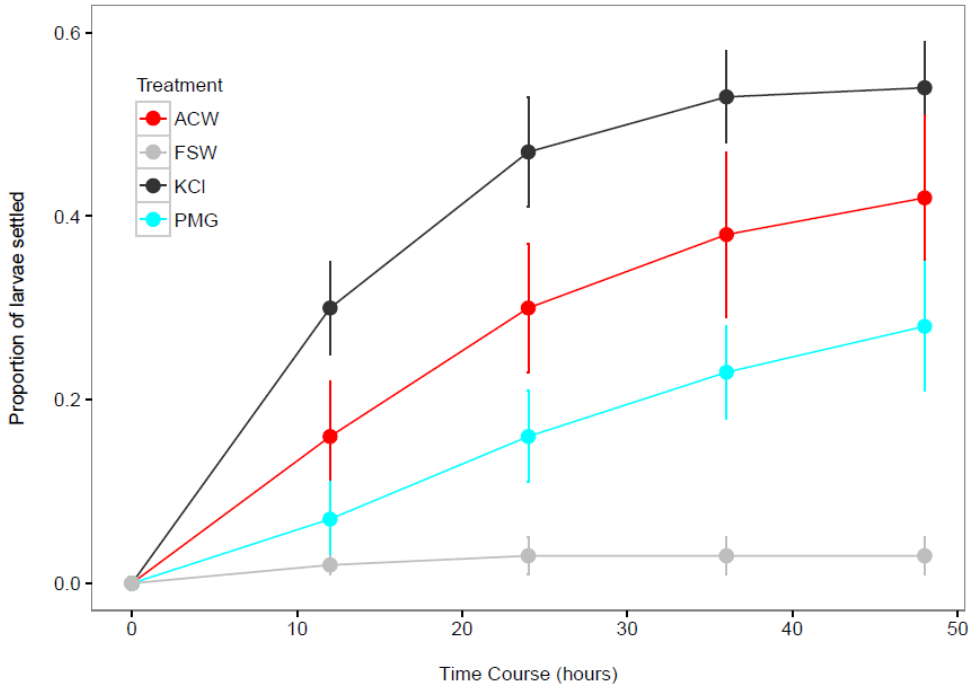

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525 **Figure 3: Settlement rates of larvae in response to different experimental factors.** Time-
526 course data of larval settlement in adult-conditioned water (ACW; red line), filtered seawater
527 (FSW; grey line), potassium chloride (KCl; black line), and pedal mucus glasses (PMG; blue
528 line). Points represent running averages across all three experimental blocks; error bars represent
529 1 SE.

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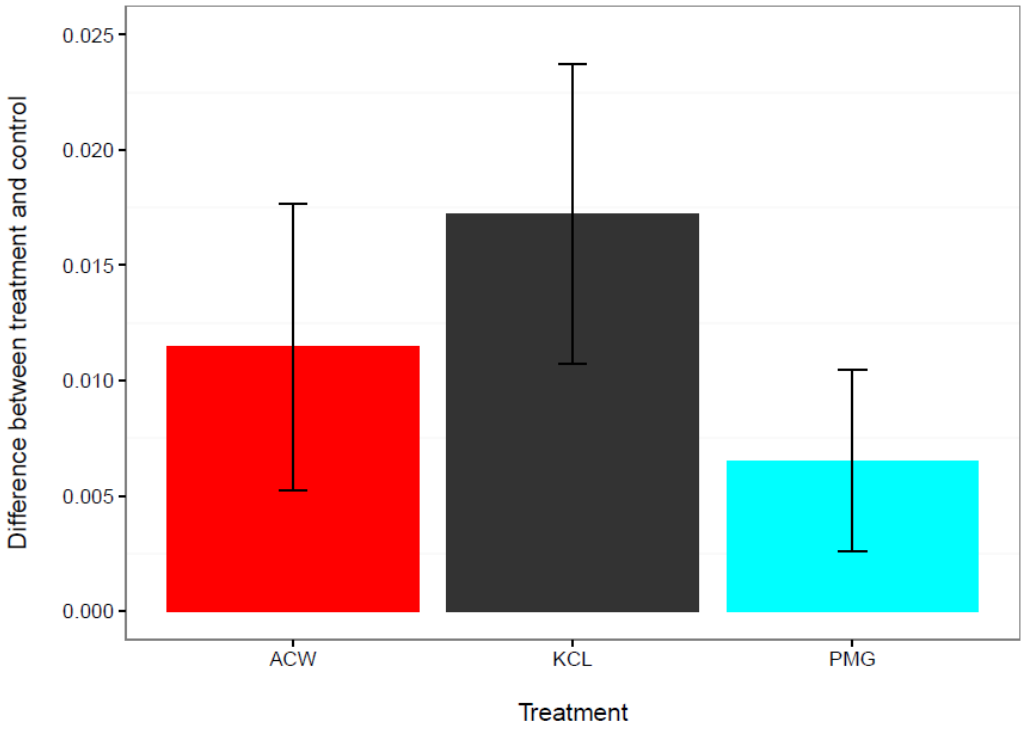

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532 **Figure 4: Comparisons of settlement factors to the** control. The difference in settlement rate
533 (λ, in units of ln(number larvae settled per hour)) between each settlement factor and the control
534 for adult-conditioned water (ACW; red), potassium chloride (KCl; grey), and pedal mucus
535 glasses (PMG; blue). Bar height represents the difference between each treatment mean and the
536 filtered seawater control, and error bars represent the 95% confidence intervals on those
537 differences. Error bars that do not overlap with zero indicate treatments with a significantly
538 higher settlement rate than the control at $\alpha = 0.05$.

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