Response of competent blue mussel (*Mytilus edulis*) larvae to positive and negative settlement cues

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ABSTRACT

Recent work on larval settlement cues has emphasized mechanisms by which larvae exploit individual, positive cues (cues that larvae move toward), often in complex flow fields. Yet in natural systems, larvae of habitat generalists probably respond to multiple settlement cues, including a mixture of positive and negative cues. First, a simple test chamber in which cue dispersal was dominated by diffusion was used to assess whether competent blue mussel (*Mytilus edulis*) larvae responded negatively or positively to cues from a variety of intertidal species. Second, choice experiments tested the responses of larvae offered a mixture of conflicting (positive and negative) cues from the same direction and conflicting cues from different directions. Responses to individual cues were predictable from established ecological interactions. Larvae were attracted to odors from conspecifics, tended to move toward odors from a filamentous alga, avoided odors from two predators of post-settlement mussels, and exhibited little response to odors from an herbivorous gastropod. Negative and positive cues offered from the same direction produced movement both toward and away from the mixture, while offering the combined cues from different directions resulted in net movement that was largely consistent with predictions from the individual cue responses. Larvae presented with a choice between two negative cues mainly remained where they started, in the middle of the test apparatus, and exhibited no choice. These results highlight the need to consider the combined effects of a broad range of cues when evaluating the net effect of cues on settlement in the field.

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1. Introduction

Recent work on the odor cues involved in larval settlement decisions has largely focused on how larvae of specific species respond to either a single important cue (Hadfield et al., 2000; Lau and Qian, 2001; Hadfield and Koehl, 2004; Dobrestov and Wahl, 2008; Swanson et al., 2006) or groups of closely related cues (Krug and Manzi, 1999). Such approaches have provided valuable mechanistic information on the processes by which larvae follow cues to reach appropriate habitat, even in complex flows (Tamburri et al., 1996; Hadfield and Koehl, 2004). Larvae of many species (especially habitat generalists), however, probably respond to a complex interaction of multiple cues (Slattery, 1992; Walters and Hadfield, 1996; von der Meden et al., 2010; Dixson et al., 2014). Understanding the role that odor cues play in establishing larger scale settlement and population connectivity patterns requires evaluating the entire suite of available settlement cues and how larvae may integrate multiple, potentially conflicting cues to reach ultimate settlement decisions.

The vast majority of studies of larval chemical settlement cues have explored the action of positive cues — cues which larvae orient toward and that will lead larvae to an appropriate adult habitat (Pawlik, 1992; Lau and Qian, 2001; Hadfield and Koehl, 2004; Dobrestov and Wahl, 2008; Swanson et al., 2006). In a few cases, such cues have been interpreted as resulting in the avoidance of a negative post-settlement interaction. For example, sand dollar larvae may cue on compounds present in the sand of adult sand dollar beds because potential juvenile predators are rare in this habitat (Highsmith, 1982), and ascidian larvae may use cues to preferentially settle on refuge host species to avoid future competition (Grosberg, 1981) or gastropod predation (Young, 1989). But the cues involved are positive ones, in the sense that larvae orient toward them, not away from them.

The exploration of directly negative chemical cues (ones that larvae avoid) has been less comprehensive. A very extensive body of literature addresses compounds that prevent fouling by deterring larvae from settling (Bak and Borsboom, 1984; Walters and Hadfield, 1996; Bers et al., 2006; Khalaman et al., 2008). These studies are generally conducted from the perspective of the host, and so assess benefits to the potential substrate from deterring the settler, rather than the consequences for the settler. Less is known about negative cues from the settler's perspective. Several studies have documented avoidance of future competitors (Young and Chia, 1981; Petersen, 1984; Dixson et al., 2014). Only a very

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limited number of papers have documented responses to negative cues provided by future potential predators, however, and such evidence is limited to crustacean (Johnson and Strathmann, 1989; Boudreau et al., 1993; Welch et al., 1997; Diaz et al., 1999) and fish larvae (Vail and McCormick, 2011). Larvae of these taxa are relatively large, fast swimming, and behaviorally sophisticated compared to larvae of many invertebrate taxa, so evidence that smaller, weaker swimming larvae like bivalves can avoid cues from future predators has been lacking. Because weaker swimming larvae are unable to overcome typical field horizontal velocities (Young and Chia, 1987; Chia et al., 1984), they may have less ability to respond to negative cues. In addition, virtually nothing is known about larval responses to multiple cues from different directions (i.e., hierarchal preference), or the net effect of multiple, possibly conflicting (negative and positive), cues from the same direction. If a larva receives a positive substrate cue and a negative predator cue from the same direction, does the positive cue over-ride the negative one, or vice-versa? Larvae are likely to encounter such conditions during transport and settlement, and thus these behaviors have the potential to effect settlement location.

Assessments of the settlement consequences of multiple, interacting cues will ultimately need to be conducted in the complex flows that are typical of near-bottom hydrodynamic environments (Tamburri et al., 1996; Hadfield and Koehl, 2004). Initial surveys of the different cues affecting an individual species and their relative impacts, however, can arguably proceed more rapidly in simplified hydrodynamic environments. Here, the effects of multiple potential settlement cues, both positive and negative, on the behavior of competent blue mussel, *Mytilus edulis* (L.), larvae are explored in a simple fluid environment where odor cues were disseminated via diffusion. The relative effects of different cues are evaluated first, then behavior in response to the combination of a conflicting pair of cues, and finally the net effect of providing different cues from two opposing directions. Cues included a mix of likely positive (cues from conspecifics and algal habitat; Petersen, 1984; McGrath et al., 1988; Dobrestov and Wahl, 2008; von der Meden et al., 2010; Palanikumar et al., 2011) and potential negative (exudates from future potential predators) signals. Analysis integrated the larval behaviors of moving toward a cue, away from a cue, and exhibiting no choice to develop a more complete understanding of how *M. edulis* larvae interact with combinations of cues during transport and settlement.

2. Materials and methods

2.1. Overview of experiments

Three sets of experiments assessed how larvae responded to different individual cues, responded to conflicting (a combination of positive and negative) cues, and exhibited choice between different cues (hereafter termed response, conflict, and choice experiments respectively). The response experiment tested whether larvae moved toward (a positive cue), away from (a negative cue), or exhibited no preference response (no-choice) to cues from five target species relative to a control of unconditioned seawater. This experiment established whether larval behavior conformed to a priori expectations based on known ecological interactions. The conflicting cue experiment tested larval response to a "mixed" cue (combined positive and negative) from the same direction, which is a scenario that larvae are likely to encounter in the environment. For this experiment, a 1:1 mixture of the target species cues that elicited the highest percentage of positive and negative responses was used as the "mixed" cue and tested against a control. The choice experiment attempted to establish a hierarchy of cue responses for *M. edulis* larvae using choice trials between positive pairs of cues, negative pairs of cues, and a positive cue vs. a negative cue. This experiment tested how larvae might respond if encountering two cues in the same category from different directions, or conflicting cues from the same direction.

2.2. Production of mussel larvae

Larvae used in the experiments were produced in two batches in June and July of 2014. For each batch, approximately 18 adult *M. edulis* (three females and 15 males) were collected at random from a rocky intertidal population with intermediate wave exposure adjacent to The Downeast Institute in Beals, Maine (Lat. 44.481856° N, Long. 67.599981° W) to serve as parents and housed for 24 h in flowing seawater (sourced from an intake suspended ~1 m above a rocky benthos, ~2 m below mean low water, and ~10 m from shore). Based on site characteristics (geography, community composition, substrate, and wave exposure) and previous studies (Menge, 1976), adult mussels were probably subject to intermediate predator exposure prior to collection. Mussels were then removed from the water and exposed to ambient air for a three-hour period before placement in 24 °C filtered seawater to induce spawning. Spawning females were immediately transferred to individual sterilized containers of filtered seawater, while males were removed from the water and wrapped in a moist paper towel. Eggs from multiple female containers were sieved to 150 μm to remove fecal pellets and combined into a single sterilized 20 L container. Male mussels were then transferred to a 3 L container of 24 °C filtered seawater and allowed to complete spawning; 20 mL of sperm/water solution was then added to the container of eggs and the contents homogenized. When 90% of the embryos had commenced cleaving, they were transferred into a 400-L conical tank of lightly aerated 21 °C filtered seawater that was changed every 48 h. Larvae were housed at this temperature throughout development (18 days to pediveliger) and fed a 1:1 mix diet of diatoms (*Chaetoceros muelleri* and *Chaetoceros calcitrans*). These methods have been used successfully to rear mussel larvae at The Downeast Institute for a number of years and produce healthy, rapidly growing juveniles. The spawning and rearing process may have introduced parental effects or inadvertent selection on larvae. To reduce or control such confounding factors, parents were randomly sampled across an area of intermediate predator and conspecific density (S.L. Morello personal observation), and two different batches of larvae were created.

2.3. Cue-conditioned water

Supernatant from the incubation of target species in filtered seawater served as cue-conditioned water during experimentation. To produce cue-conditioned water, target species were collected from the same intertidal rocky shores where mussels were collected for larval production. Separate collections were made for each of the two batches of larvae tested. There were a priori expectations that each target species would yield either a positive, negative, or neutral (no effect) cue based on known ecological interactions with *M. edulis* either during or following settlement. Target species expected to generate positive cues included *M. edulis* adults and the filamentous green algae *Chaetomorpha linum*. Adult *M. edulis* beds serve as recruitment surfaces for conspecific larvae (McGrath et al., 1988) and can indicate ecologically suitable habitat for settlement and growth (Highsmith, 1982; Petersen, 1984). Filamentous algae also serve as important settlement surfaces for mytilid larvae in the Northwest Atlantic (Hunt and Scheibling, 1996, 1998) and elsewhere (Bayne, 1964; Petersen, 1984; Dobrestov and Wahl, 2008; Yang et al., 2007). The algae *C. linum* is a locally abundant and accessible species on which mytilids are known to preferentially settle (Palanikumar et al., 2011) and *M. edulis* has been observed to settle locally on this species (S.L. Morello personal observation). Target species expected to generate negative cues included two locally abundant and well-documented predators of juvenile and adult *M. edulis* — the gastropod *Nucella lapillus* (Menge, 1976), and the crab *Carcinus maenas* (Ropes, 1968). One of these predators, *C. maenas*, is a recent invader of Northwest Atlantic shores (1800s), but also co-occurs with *M. edulis* in its native Northeastern Atlantic habitat (Carlton and Cohen, 2003). The herbivorous gastropod mollusk *Littorina littorea* was selected

as a species expected to generate a neutral cue because it has no known direct interactions with *M. edulis*. Indirect effects are possible, however. *L. littorea* grazing can increase available space on intertidal bedrock for *M. edulis* growth (hence perhaps leading larvae to cue on this herbivore), or decrease the abundance of filamentous algae for *M. edulis* settlement (Lubchenco and Menge, 1978; hence perhaps providing a source of a negative cue). Nevertheless, directional settlement cues from *L. littorea* seemed less likely than for species involved in direct interactions with *M. edulis*.

For each of two batches of cue-conditioned water, three *M. edulis* (mean length ± SE = 5.79 ± 0.18 cm, n = 6), 3.5 g (wet weight) of *C. linum*, five *N. lapillus* (mean length ± SE = 3.44 ± 0.22 cm, n = 10), one *C. maenas* (mean carapace length ± range = 52.75 ± 2.55 cm, n = 2), and five *L. littorea* (mean length ± SE = 2.14 ± 0.05 cm, n = 10) were collected to serve as cue sources. These quantities constituted an approximately equivalent biomass of each target species and were selected in an attempt to roughly control signal concentration when generating cue-conditioned water. It is possible that soft tissue and shell or exoskeleton produce different amounts or types of cue, however, and that the ratios of soft vs. hard parts probably varied among species. Any epibionts were removed and the collected organisms were rinsed in seawater filtered to 1 μm (hereafter, filtered seawater), then left to acclimate in separate sterilized glass containers of filtered seawater at 10 °C for 6 h. Organisms were then transferred to a new sterilized glass container with 600 mL of filtered seawater and incubated at 10 °C for 48 h. Following incubation, the water for each cue species was filtered through 60-micron mesh and into a sterilized glass container to remove organisms and any particulates and then the supernatant (cue-conditioned, water) was stored at 10 °C until use (mean storage time ± SE = 0.91 ± 0.08 days).

2.4. Test chambers

Test chambers consisted of two wells to hold treatment (cue conditioned) and/or control (unconditioned) seawater that were connected by a narrow channel and separated by a smaller central well that was the site of initial larval deployment (Fig. 1). A 13-cm-long channel of width 0.10 cm and depth 0.40 cm was milled into 1.10-cm-thick acrylic plastic sheet (Fig. 1). The narrow width and shallow depth minimized vertical mixing from thermal advection during experimentation and limited larval movement to essentially a single, horizontal, dimension along the channel. The absence of flow precluded boundary layer formation along the walls of the narrow passages. Any small-scale fluid dynamics within the channel (e.g., Brownian motion) would have been trivial relative to larval swimming speeds and acted similarly across all experiments and larvae sampled, and so could not have confounded the experimental results. At each end of the channel, a 0.80 cm deep and 3.25 cm diameter well with a tapered bottom was drilled into the acrylic so that the channel was perpendicular to the

side of each well and connected the two wells (Fig. 1). Wells were arbitrarily labeled A and B to evaluate orientation during the experiments. A third well of depth 0.40 cm (equal to that of the channel) and diameter 0.50 cm was then milled in the center of the channel, dividing the channel in half so that 6.25 cm of channel extended between the center well and each of the side wells (Fig. 1). The total volume of a completed test chamber was 5 mL. All milled and drilled surfaces were washed with dimethyl chloride (a solvent for acrylic) to dissolve any microscopic surface irregularities and ensure smooth surfaces. Lines were then drawn on the underside of the acrylic at 0.50 cm from the edge of the center well. These lines were visible though the top of the acrylic but did not interfere with the channel itself, and served to delimit a central "neutral zone" of 1.50 cm (including the center well) within which larvae were deemed to not have made a choice (no-choice, neither A nor B; Fig. 1). A total of 6 experimental chambers were manufactured. Following production, all test chambers were soaked in filtered seawater for 48 h. to dilute any compounds that might leach out of the acrylic or solvent.

2.5. Experimental protocol

Larvae used in experiments were deemed competent to settle when two criteria were met: 1) individuals from the same cohort had begun to settle in the main culture tank, and 2) larvae had reached the pediveliger stage, but still possessed a velum so they were able to swim. Larvae were collected from the conical tank by sieving ~1 L of water through a 60 μm mesh and backwashing the filtered larvae into a sterile glass Petri dish with filtered seawater. This process produced low densities of larvae that could be handled individually during experimentation. As more larvae were needed (i.e., all larvae had been used in experiments, or larvae had been out of culture >2 h.), the process was repeated.

Experiments were conducted on a level, non-reflective, gray surface that was shaded from direct exposure to ambient light sources (windows, lamps, etc.). A fluorescent lamp with a diffuser was suspended above, and parallel to, the experimentation surface to ensure equal light availability across each channel and minimize potential artifacts from larval phototaxis (Bayne, 1964). Each channel was first filled with 4.5 mL of filtered seawater at ambient temperature and eddies allowed to dissipate for 5 min. One well (A vs. B) was selected using a random number generator and inoculated with 80 μL of ambient temperature cue-conditioned water. Absence of a temperature difference between water sources eliminated the potential for thermally-driven mixing. Propagation of the potential cue down the channel was via diffusion (Welch et al., 1997) rather than advection (as is more typical in nature; Boudreau et al., 1993; Tamburri et al., 1996; Diaz et al., 1999; Dixson et al., 2014) for reasons previously discussed. Filtered seawater was used as a control in response and conflict experiments and 80 μL was placed in the well opposite the conditioned water. In choice experiments, where larvae were offered a choice between two cues and both wells contained a cue, one
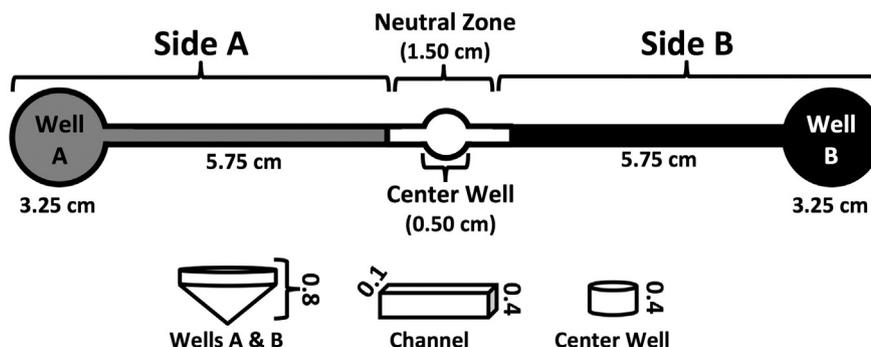

Fig. 1. Design of the test chambers. Channels and wells were milled into an acrylic plate. 3-Dimensional representations of the end wells, channel, and center channel are shown in the lower portion of the figure.

cue was randomly selected (e.g., *M. edulis* vs. *C. maenas*) and placed into well A, and then the second cue into well B. A cue-free control evaluated the larval response to non-cue conditioned water and assessed any potential asymmetry, or procedural, artifacts in the experimental process for all experiments. For this test, two drops of unconditioned filtered seawater were used in place of cue-conditioned water and placed in both wells. The cue-free control was expected to generate a symmetrical response from the larvae if there was no effect of channel geometry, external stimuli, or experimental procedure on larval behavior.

Experiments were initiated by adding a single *M. edulis* larvae to the center well. With the aid of a dissection scope, a competent *M. edulis* larvae and 5 μL total volume of ambient seawater was collected from the glass Petri dish with a pipet. If multiple larvae were collected inadvertently, they were discarded and a new larva collected. The individual larva was then placed in the center well of the channel. The presence of the larva in the center well was confirmed using a handheld digital microscope and external monitor. Larvae were allowed to move freely for 20 min then located using the handheld digital microscope, and the position of the larva in the channel (side A, side B, or neutral zone) recorded. The experimental duration was set at 20 min because preliminary dye experiments indicated that it took this long for the contents of the end wells to diffuse into the neutral zone and start to enter the opposite side of the channel, at which point interpreting larval position relative to a cue would have become problematic. The dye served as a proxy for the tested compounds as a group, though the specific diffusion rate of each individual cue was unknown. Following each experimental trial (replicate), the channel was rinsed with fresh water then filtered seawater, and allowed to dry before the next experiment. Each experiment was replicated a total of 24 times per treatment (testing one larvae per replicate) with replicates interspersed among other experimental trials and distributed across both batches of larvae (batch 1, July 21–25, 2014; batch 2, August 5–8, 2014), except the mixed (*M. edulis* + *C. maenas*) vs. filtered water comparison, which was replicated 20 times using only the second batch of larvae.

2.6. Larval behavior observations

Before designing a quantitative sampling regime, the behaviors of the initial 92 larvae sampled (38.98% of total larvae sampled) were continuously and carefully observed with a real-time feed from a digital microscope mounted on a sliding track (to reduce disturbance to the test chamber). These observations spanned multiple cues and experiments, and allowed us to assess 1) whether larval location at a fixed sampling time could serve as a proxy for overall behavior, 2) if the test chamber imposed movement restrictions imposed on larvae, and 3) how larvae explored the chamber. Larvae typically explored the center well for approximately 8 min (8:02 min ± 0:53 SE) before initially entering the neutral zone of a side channel. During these explorations, larvae passed the entrance to both side channels multiple times. Once larvae traveled beyond the neutral zone, they typically remained on the side of the test chamber they initially entered. Occasionally, larvae traveled partially along a channel and then reversed direction, especially if a cue caused a negative response. Once larvae traveled the length of a channel and entered a side well, the larvae did not return to the channel and/or travel back along the channel in the opposite direction. Overall, the observations suggested that conclusions based on the location of larvae after 20 min would not differ from any based on detailed movement patterns.

Under field conditions, where horizontal velocities typically exceed larval swimming speed, larvae predominantly move in a vertical direction by traveling in concentric spirals (Chia et al., 1984). Nevertheless, larvae were quite capable of swimming horizontally in the test chambers. So while the experiments did not permit larvae to move naturally in an unbounded system, larvae moved quite functionally within the test chamber. Larvae did move in concentric spirals when in the center well, prior to entering either side of the channel. In the channels, larvae

swam horizontally as their primary method of movement, and were largely unimpeded by the channel walls. Occasionally, larvae would crawl along the channel, possibly sampling the water for cue with their foot. This behavior was very temporary, however, and larvae resumed swimming soon afterwards. Larvae swam at relatively constant speeds, with occasional increases in speed that might reflect responses to changed concentrations of cue along a gradient.

2.7. Analysis

All analyses were run in JMP version 9.1.2 (SAS Institute, 1989–2010). The effects of channel orientation (side A vs. B), and construction (variation among test chambers) were tested by comparing the observed frequencies of side A and B results (no-choice results excluded) from all experiments against an expected 1:1 ratio of responses with a Pearson chi-square (X^2) test (hereafter referred to as a Chi-square test), and a Cochran–Mantel–Haenszel (CMH) test stratified by individual test chamber. These tests were used in addition to the cue-free control response experiment as a means of assessing any behavioral artifacts introduced via the channel design and/or experimental procedure. A difference in response (treatment, control, no-choice) between larval batches was also tested by comparing response experiment results for the *M. edulis* cue-conditioned water between larval batches with a two-way Chi-square test. Results from the *M. edulis* cue treatment were used for this analysis because sample sizes were equal (12 vs. 12) for the two batches.

Results from response and conflicting cue experiments (frequency of larvae moving toward treatment water, control water, and not exhibiting a choice) were analyzed together using a combination of a Chi-square test and a correspondence analysis. The correspondence analysis integrates the magnitude of the positive or negative preference relative to the control with the extent to which larvae exhibited any preference vs. no-choice. The Chi-square tested whether differences existed among treatments, and then ordination along the first two axes from the correspondence analysis was used to investigate those differences. Individual treatment results from the response and conflicting cue experiments, pairwise comparison results from the choice experiments (frequency of larvae selecting one cue vs. the other), and cue-free control results, were tested separately against 1:1 ratios of response with either two-way Chi-square tests (hypotheses with no a priori expectation) or exact one-sided binomial tests (hypotheses with a priori directional responses). Individual treatment results for all experiments were further analyzed by testing the frequency of no-choice response against that observed in the cue-free control with a one-sided binomial test. This analysis tested whether individual treatments resulted in lower frequencies of no-choice response than would be expected if no cue were present.

3. Results

All 236 experimental larvae tested were successfully located after 20 min of movement. Larval preference (frequency of selecting the treatment water, control water, or exhibiting no-choice) in the response experiments using *M. edulis* cues did not differ between the two batches of larvae (Chi-square test, $X^2 = 0.91$, p = 0.63), with channel orientation (A vs. B, Chi-square test, $X^2 = 1.22$, p = 0.27), or among individual test chambers (CMH test, $X^2 = 1.19$, p = 0.28). Larval preference did differ among individual experiments (cue-free control and all response and conflict experiments, Chi-square test, $X^2 = 34.94$, p = 0.0005), and patterns of preference conformed to a priori expectations of positive (*M. edulis*, *C. linum*), negative (*N. lapillus*, *C. maenas*), and neutral (*L. littorea*, cue-free control) signals (Figs. 2–3). Most of the variation in the correspondence analysis was along the first axis (72.73%), which separated treatments eliciting positive vs. negative responses in larvae (Fig. 2). The second axis accounted for the remaining variation (27.27%) and separated treatments failing to elicit a choice response in
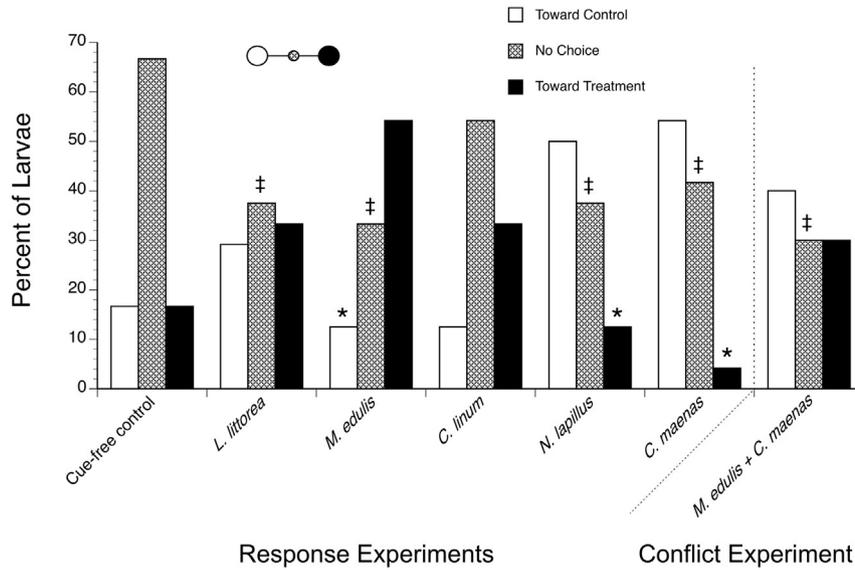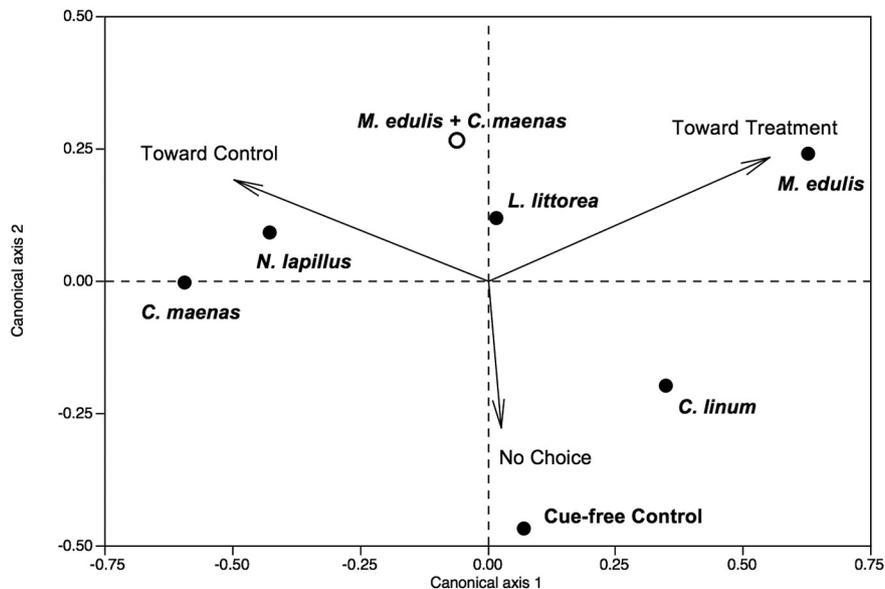
Fig. 2. Results of the response and conflict experiments (*M. edulis, C. linum, N. lapillus, C. maenas*, n = 24; *M. edulis + C. maenas*, n = 20). Response experiments presented larvae with cue-conditioned water and a control of filtered water, while the conflict experiment combined a negative and a positive cue. The cartoon of the test chambers in the upper part of the figure is coded to reflect the larval outcomes depicted in the figure. For visualization purposes the treatment well was arbitrarily assigned to the right side, but during experiments alternated between two sides (see text for details). Experimental outcomes with significantly lower than expected frequencies (toward control vs. treatment relative to a 1:1 response, ★; no-choice response relative to cue-free controls, ‡) are indicated in the figure.

larvae (no-choice; larvae remained in the neutral zone) from those exhibiting choice responses (positive or negative response; Fig. 2.). A greater percentage of larvae moved into *M. edulis* and *C. linum* cue-conditioned water than into control water (Figs. 2–3), though only the response to *M. edulis* cues differed from an expected 1:1 response ratio (Exact one-sided binomial test, *M. edulis*, p = 0.01; *C. linum*, p = 0.11) and elicited a lower than expected (lower than observed in the cue-free control) frequency of no-choice responses (Exact one-sided binomial test, *M. edulis*, p = 0.0009; *C. linum*, p = 0.1401). A lower percentage of larvae ended in *N. lapillus* and *C. maenas* cue-conditioned water relative to control water (Figs. 2–3). Both results differed significantly from an expected 1:1 ratio (Exact one-sided binomial test, *N. lapillus*, p = 0.02; *C. maenas*, p = 0.0009) and elicited a lower than expected frequency of no-choice responses (Exact one-sided binomial

test, *N. lapillus*, p = 0.0032; *C. maenas*, p = 0.0103). No preference was observed for *L. littorea* cue-conditioned water, or for the A vs. B chamber in the cue-free control (Figs 2–3; two-way Chi-square test, *L. littorea*: $X^2 = 0.07$, p = 0.80; cue-free control: $X^2 = 0.0$, p = 1.0), but more larvae exhibited a response (both positive and negative) in the *L. littorea* treatment than expected based on cue-free controls (Exact one-sided binomial test, p = 0.0032).

In the conflict experiment, similar percentages of larvae ended in cue conditioned (*M. edulis + C. maenas*) and control seawater, with a slightly higher percentage in control seawater, indicating that the net effect of the mixed cue was not distinctly positive or negative (Figs. 2–3; two-way Chi-square test, $X^2 = 0.29$, p = 0.59). In addition, larvae exhibited a lower than expected frequency of no-choice responses (Exact one-sided binomial test, p = 0.0009), indicating

Fig. 3. Results of the correspondence analysis. Closed circles represent values from response experiments while the open circle represents the value from the conflict experiment. Vectors represent the relative contribution of larval outcomes (toward treatment, toward control, and no-choice) to ordination patterns.

that under these conditions, movement in one direction or the other exceeded non-movement. Choice experiments between positive pairs and negative pairs of cues did not indicate any discernable hierarchy of cue preference (Fig. 4; Chi-square test, M. edulis vs. C. linum, $X^2 = 0.25$, p = 0.62; N. lapillus vs. C. maenas, $X^2 = 0.0$, p = 1.0), but positive pairs elicited a lower than expected frequency of no-choice responses (Exact one-sided binomial test, M. edulis vs. C. linum, p = 0.0009; N. lapillus vs. C. maenas, p = 0.0677). Comparisons of positive vs. negative cues maintained the pattern observed in response experiments (Fig. 4; M. edulis vs. C. maenas, Exact one-sided binomial test, p = 0.003), and also produced a frequency of no-choice responses equivalent to cue-free controls (Fig. 4; M. edulis vs. C. maenas, Exact one-sided binomial test, p = 0.0677).

4. Discussion

4.1. Avoidance of future risk

Though a positive directional response of M. edulis larvae to waterborne cues (a significant response to adult M. edulis and positive trend to filamentous algae) is not a novel result, the negatively taxic response of mussel larvae, or any bivalve larvae, to cues from a post-settlement predator (N. lapillus, C. maenas) has not previously been demonstrated. This result augments other larval behavior studies that demonstrate avoidance (negative) responses during settlement that are of future, rather than immediate, ecological benefit to the individual. These benefits range from selecting habitat with fewer potential competitor species (Young and Chia, 1981; Petersen, 1984, Dixson et al., 2014) to other examples of reducing the risk of future predation (Johnson and Strathmann, 1989; Boudreau et al., 1993; Welch et al., 1997; Diaz et al., 1999; Vail and McCormick, 2011). These behaviors need not involve cognition on the part of larvae, but rather the outcome of selective pressures exerted by predators, or competitors, of future life stages. Examples of avoidance of future risk now span diverse taxa with a range of larval swimming capabilities (vertebrates, crustaceans, bryozoans, cnidarians, and mollusks), suggesting that long-term risk avoidance may be common in the settlement processes of marine taxa. Additionally, the observed patterns of larval preference conformed to a priori expectations based purely on known ecological interactions involving M. edulis,

suggesting that a diverse suite of species cues may play relatively predictable roles in the behavior of marine larvae.

4.2. Cue reliability, availability, and exploitation by larvae

In taxa with weakly swimming larvae, the ultimate ecological relevance of any behavioral response to a cue is dictated by 1) the reliability with which the cue indicates desirable adult habitat, 2) the availability of the cue, and 3) the capacity of small-scale larval movements to modify ultimate settlement position on a spatial scale relevant to the threat or benefit. The alga C. linum appears to provide the least reliable cue tested here. Because algal abundance is not directly impacted by either mussels or predators, this alga can occur in areas devoid of adult M. edulis, as well as in the presence of potential M. edulis predators, (Lubchenco and Menge, 1978). Moreover, many filamentous algae species, including C. linum, are ephemeral in intertidal systems (Lubchenco and Menge, 1978). As a consequence, they only provide cues for a short period of time, thus reducing overall cue availability. Morphologically, however, C. linum tends to occur in a filamentous tangled mass that is typically interpreted as a desirable surface for initial M. edulis settlement (Bayne, 1964; Seed, 1969) and possible buffer against thermal fluctuations (King et al., 1990; Hunt and Scheibling, 1996). Since M. edulis can engage in secondary settlement, during which mussels may detach from an original settlement point (e.g., ephemeral filamentous algae) in search of more permanent adult mussel beds (Bayne, 1964; Seed, 1969), the limited lifespan of C. linum may not reduce its value as a cue. The alga C. linum is thus a potentially useful, but possibly scarce and unreliable, odor cue for post-settlement success, and tactile cues from this substratum may be adequate to explain M. edulis settlement patterns.

Adult M. edulis cue is arguably a more reliable positive cue than C. linum because it is associated with specific habitat where other mussel larvae have successfully settled and grown to adulthood (e.g. Highsmith, 1982; Petersen, 1984; McGrath et al., 1988). Field experiments in the Downeast region of Maine, where the study took place, demonstrate greater larval settlement around established adult mussels than in mussel-free areas, leading to an emergent fractal structure of soft-sediment mussel beds (Commito et al., 2014). A positive and reliable adult M. edulis cue may thus play an important role in the establishment,
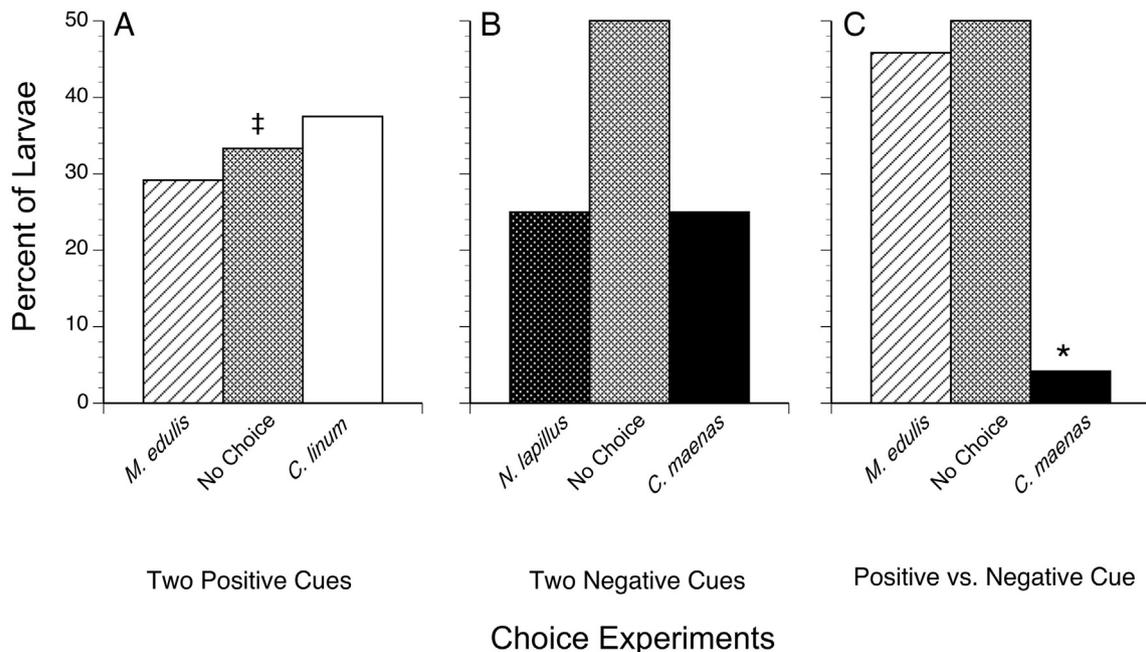
Fig. 4. Results of the conflict experiments (n = 24 per experiment). Larval movement toward positive cues is coded in with lighter patterns, while larval movement toward negative cues is coded with darker patterns (no-choice is intermediate). Experimental outcomes with significantly lower than expected frequencies (cue A vs. cue B relative to a 1:1 response, ★; no-choice response relative to cue-free controls, ‡) are indicated in the figure.

structure, and maintenance of soft-sediment mussel populations in the study region, similar to that recently hypothesized for cues from healthy coral reefs (Dixson et al., 2014).

Cues from future predators (*N. lapillus* and *C. maenas*) might not initially appear to be reliable indicators of unsuitable habitat for post-settlement success. Both *N. lapillus* and *C. maenas* are relatively mobile compared to mussels, and so the current location of a predator is not necessarily a good indicator of that location's future predation pressure. To provide useful information to settling larvae, cues must integrate predator abundance over a larger spatial scale. Gastropod predators deposit mucus as they move, and mucus is thought to be the source of odor cues. For example, mucus left behind by the congeneric gastropod predator *Nucella lamellosa* was avoided by barnacle larvae for up to 3 weeks after initial deposition (Johnson and Strathmann, 1989). Additionally, Ellrich et al. (2015) found reduced barnacle settlement in areas adjacent to *N. lapillus* enclosures compared to gastropod exclusions. As multiple gastropods move around an area, the resulting mucus trails are likely to provide a space and time-averaged cue source that reflects the overall predator density in that area.

Because crabs are more mobile than gastropod predators, and do not leave mucus trails, it is less clear how the odor from *C. maenas* provides reliable information about future habitat quality. Nevertheless, odors from a group of foraging crabs should provide a space-averaged signal that presumably scales with crab density and/or body size. The reliability of this cue is linked to another scaling problem – the spatial scale of dispersal necessary to avoid mobile predators is large relative to the swimming ability of a mussel larva. Bivalve larvae typically swim at speeds on the order of 10^{-4} m s^{-1} (Hidu and Haskin, 1978; Chia et al., 1984) and mussel larvae moving toward cues in the test chambers swam at this speed (unpublished analysis). Swimming speeds are substantially lower than typical horizontal current velocities in the field (up to 1 m s^{-1}; Young and Chia, 1987), so mussel larvae are unlikely to avoid predators simply by swimming along a cue concentration gradient. Vertical current velocities (on the order of 10^{-5} m s^{-1}), however, are typically lower than both horizontal velocities and bivalve swimming speed (Young and Chia, 1987). Ample evidence indicates that bivalve larvae can substantially change their dispersal trajectories by migrating vertically (Raby et al., 1994; Garland et al., 2002; Knights et al., 2006). Hence negative cues from predators appear most likely to be exploited by larvae moving vertically between the benthic boundary layer and overlying water masses. Given a high level of turbulent mixing and proximity to cue sources within the benthic boundary layer, cues may interact with hydrodynamics to be relatively homogenized and concentrated within that near-bottom water and reflect a spatially-averaged density of predators, providing an effective signal of the quality of the habitat a larva is approaching. Vertical movement between the benthic boundary layer and the overlying water mass in response to odors appears to be the most likely candidate for the mechanism by which mussel larvae exploit odor cues from predators.

4.3. No-choice responses and the interpretation of larval behavior

In the simple fluid environment of these experiments, larvae had the option of remaining in the neutral zone and not actively moving toward, or away from, cue-conditioned water (no-choice response). The frequency of this no-choice response, relative to choice responses (directional, whether positive or negative), was useful for explaining variation among different cues (Fig. 3) and was generally associated with a weaker larval preference between the two choices. But no-choice responses are largely un-tested, or ignored, in most larval behavior studies. In many cases, methods of assessing responses to odor cues simply preclude a clear no-choice option (e.g., settlement induction, with settlement assessed as a binary variable, Turner et al., 1994; larval behavior in a Y-shaped or choice flume, with outcomes assessed as proportion of time spent in one channel vs. the other, Dixson et al., 2014). Yet in other studies, the no-choice response exists, but is ignored.

For example, Diaz et al. (1999) allowed blue crab megalopae the option of remaining in a central chamber, rather than choosing among cues in radial chambers, but those larvae were considered "unresponsive" and discarded from the analysis. Studies of larval acoustic cues similarly include a provision for no-choice behavior (two-sided "choice chambers" with a central "no-choice" option), but these data are discarded during analysis, under the assumption that such responses are "ambiguous" and possibly an experimental artifact or indicative of poor larval quality (Tolimieri et al., 2004; Leis and Lockett, 2005; Radford et al., 2007). Arguably, no-choice responses may be negatively correlated with the level of searching activity resulting from a cue, and thus provide useful information when comparing among multiple potential cues or testing for experimental artifacts (e.g., side asymmetry in a test apparatus). This interpretation is supported by the same acoustic cue studies, which consistently comment on the higher frequencies of no-choice response when a cue is absent (controls, no auditory stimulus) relative to when cue is present (e.g. 92% vs. 9% no-choice responses respectively; Leis and Lockett, 2005). The cue-free control similarly generated the highest frequency of no-choice responses, indicating larvae were least actively searching the channel when exposed to naught but unconditioned filtered seawater (Figs. 2–3). By contrast, when a cue was provided in either one or both wells, larvae were more active and generated a lower frequency of no-choice responses (Figs. 2–3).

Cues from *M. edulis* elicited a positive response from larvae and cues from *C. linum* produced a similar trend, though cues from *C. linum* produced a greater frequency of no-choice responses. Larvae were thus less active in orienting toward the *C. linum* cue relative to the *M. edulis* cue, suggesting that *M. edulis* may have a greater effect on larval behavioral responses in the field. By contrast, cues from *N. lapillus* and *C. maenas* produced comparable frequencies of no-choice response, suggesting equivalent effects on behavior. The cue from *L. littorea*, which produced no clear directional response from larvae, was also associated with a relatively low frequency of no-choice responses. This pattern raises the possibility that even a neutral cue may elicit an increase in overall searching activity, perhaps because it is indicative of the proximity of some type of intertidal habitat.

4.4. Complex choice responses

Pairwise contrasts of positive (*M. edulis* vs. *C. linum*) and negative (*N. lapillus* vs. *C. maenas*) pairs of cue in choice experiments also generated variation in the frequency of no-choice responses. Experiments involving two negative cues elicited many more no-choice responses than positive pairs, suggesting larvae were less directional in their movement when confronted with negative cues on both sides and largely avoided swimming and encountering either cue (Fig. 4). Positive cue pairs elicited a lower frequency of no-choice behavior, as larvae actively swam in search of cue sources (Fig. 4). The choice experiment that paired positive and negative cues resulted in active movement toward the positive cue and limited movement toward negative cue, but with a surprisingly high incidence of no-choice responses that were not consistent with the no-choice results in experiments involving positive cues, but similar to those involving negative cues (Fig. 4). Although response experiments suggest a different level of attraction between *M. edulis* cue (greater) and *C. linum* cue (weaker), neither of the choice experiments between positive pairs and negative pairs of cue established a clear hierarchy of preference. Thus, choice experiments suggest larvae may only interpret cues so far as they are positive (attracting) or negative (repelling; Fig. 4). Alternatively, the lack of hierarchal preference during choice experiments may have been an artifact of differences in concentration between cues.

Our attempt to control for cue concentration assumed that cue abundance was a linear function of biomass. Previous work has demonstrated that cue concentration may alter the frequency of positive or negative larval response and may require that concentrations exceed

a threshold level to elicit any response (Turner et al., 1994; Zimmer-Faust and Tamburri, 1994; Krug and Manzi, 1999; Hadfield and Koehl, 2004; Santagata, 2004; Gribben et al., 2011). Consequently, variation in concentration between compared cues could obscure variation in larval preference, and in the absence of information on the dose–response function for each cue, the outcomes of 1:1 comparisons (choice experiments) or combinations (conflict experiment) of exudate are open to alternative interpretation. Such mismatches in concentration may have also confounded conflict experiments, which did not induce a clear unidirectional response in larvae (Figs. 2–3), relative to the clear directional responses when the same cues were offered from different directions (choice experiments; Fig. 4C). If concentration differences did not confound results, however, conflict experiments suggest that positive and negative cues may neutralize each other because the net effect on larvae was similar to that of the neutral *L. littorea* cue (Figs. 2–3). Recent research by Ellrich et al. (2015) demonstrates a similar response whereby barnacle (*Semibalanus balanoides*) settlement is less affected by negative cue from *N. lapillus* when there is an abundance of potentially positive cue present (i.e., high densities of conspecific recruits). A useful next step would be to isolate the specific compounds in the target species that elicit larval responses (Krug and Manzi, 1999), and to test these compounds in varying combinations and concentrations to elicit larval behavioral responses. Alternatively, since identification and isolation of cue compounds can be time-consuming and difficult (Clare, 2011), concentrations might be manipulated by altering the densities or biomass of individual species when generating the cue. The relationship between these relative concentrations and larval responses (e.g., slope) could then be compared among cues. The latter method is also well suited for more complex laboratory manipulations that might test how spatial variation in cue concentrations and combinations may interact with complex hydrodynamics to explain larval behavior in the environment. Such comprehensive assessments of how larvae may integrate complex combinations of odor cues are critical to understanding larval behavior during transport and settlement, and the ultimate consequences of cue distributions for larger scale settlement and population connectivity patterns.

Acknowledgments

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