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Chemical cues from adult fiddler crabs stimulate molting of conspecific megalopae: evidence from field-caught individuals

James M. Welch[1,2,*], Kathleen A. Reinsel[1,2], Emily E. Waddell[2], Alexander J. Wright[2], Richard B. Forward Jr.[2]

[1]Department of Biology, Wittenberg University, Springfield, OH 45502, USA
[2]Nicholas School of the Environment, Duke University Marine Laboratory, Beaufort, NC 28516, USA

ABSTRACT: In mid-Atlantic estuaries, 3 species of fiddler crabs (*Uca pugilator*, *U. pugnax* and *U. minax*) co-occur, with their adults occupying different habitat types separated by salinity and sediment size. There is evidence that selective settlement is responsible for this separation, but the mechanism for selection is unknown. We examined the effect of chemical cues from adult fiddler crabs on the metamorphosis of field-caught fiddler crab megalopae. Previous research found that cues from adult conspecifics accelerate metamorphosis of lab-reared megalopae. We tested the hypothesis that chemical cues from conspecifics would also stimulate molting in field-caught fiddler crab megalopae. Individual megalopae were held in estuarine water (control) or water in which adult crabs had been incubated; the time each megalopa took to metamorphose was recorded. Over a 10 d incubation period, 40 to 60% of the test megalopae molted into juveniles. Only *U. pugilator* accelerated molting but did so in water that contained any adult cue, indicating that this species may not be very selective. A significantly higher proportion of megalopae of all 3 species molted in conspecific water than in estuarine water or water containing odors of other species. This indicates that chemical cues from conspecific adults are important in regulating molting and that the stimulation of molting to occur may be as important as acceleration of the timing of molting. This stimulation, in conjunction with a behavioral change to terminate flood-tide transport, is likely important in selective settlement of fiddler crab megalopae.

KEY WORDS: Larval settlement · Selective settlement · Multiplex PCR · *Uca* · Metamorphosis

INTRODUCTION

Fiddler crabs are important consumers in temperate and tropical salt marsh, sandflat, and mangrove ecosystems (Crane 1975). In estuaries of the mid-Atlantic coast of the USA, 3 species of fiddler crabs (genus *Uca*) commonly occur: *Uca pugilator* (Bosc, 1802), *Uca pugnax* (Smith, 1870), and *Uca minax* (Le Conte, 1855). In general, these 3 species share a common larval life history: their zoeae are exported to offshore waters where they develop into megalopae, which subsequently reinvade the estuary (Epifanio 1988). However, adults of these species are segregated by habitat in estuaries. *U. minax* is found in low salinity areas, especially brackish salt marshes, *U. pugnax* in moderate to high salinity salt marshes with muddy sediments, and *U. pugilator* in moderate to high salinity sandflats and sandy areas of salt marshes (Teal 1958, Miller & Maurer 1973). Since larvae of all 3 species migrate offshore (Epifanio 1988) but return to different habitats, there must be one or more processes that lead to the separation of the adult populations. Many investigators have found some evidence for selective settlement (O'Connor

*Corresponding author: jwelch@wittenberg.edu

1993, Brodie et al. 2005, Welch et al. 2015). However, the mechanism driving the selection has not been determined.

One possible mechanism for selection of settlement sites by megalopae is stimulation of molting upon reaching a suitable habitat, or alternatively, delay of metamorphosis in the absence of a suitable site (e.g. Wolcott & De Vries 1994). For fiddler crabs, this metamorphic molt is an important step in settlement, since upon completing it they lack the ability to swim. Therefore, they have limited ability for secondary dispersal as juveniles, unlike swimming crabs that can move among habitats after metamorphosis to the juvenile stage (e.g. Reyns et al. 2006). Because of the importance of this step, a number of previous investigators have studied this issue (Christy 1989, O'Connor 1991, 2005, O'Connor & Judge 1997, 1999, 2004, O'Connor & Gregg 1998, O'Connor & Van 2006). For *U. pugilator*, molting was stimulated (measured by a shorter time to metamorphosis) by sediment from adult habitats (Christy 1989), water with adult crabs (O'Connor 1991), or a combination of the two (O'Connor 1991). Similarly, for *U. pugnax,* extracts of adult crabs (O'Connor 2005), seawater conditioned by adult crabs (O'Connor & Gregg 1998), or sediment conditioned by adult crabs (O'Connor & Van 2006) accelerated molting. Additionally, in a set of field experiments, O'Connor & Judge demonstrated that placement of either *U. pugnax* or *U. minax* megalopae in adult habitats (salt marshes) accelerated molting to the first crab stage and that the effect diminished rapidly with distance from the habitat (O'Connor & Judge 1997, 1999, 2004). This indicates a strong effect of habitat cues on the molting of fiddler crab megalopae, providing one possible mechanism for selective settlement in these species.

However, all of these studies were conducted using laboratory-reared megalopae from known-species adults. This was necessary because at the time it was impossible to distinguish megalopae of the 3 species (O'Connor 1990), so the only way to know what species a megalopa belonged to was to either have reared it from hatching from known adults or to grow it to the fifth crab instar, at which point its identity could be determined by examining the setation of the maxillipeds. However, it is now possible to use molecular techniques to identify the species of field-caught fiddler crab megalopae (Behum et al. 2005, Welch et al. 2015). Previous studies have shown differences in growth and development rates of crab larvae in laboratory and field environments (Epifanio et al. 1991, Welch & Epifanio 1995). Because megalopae that develop in the field have experienced variations in salinity, temperature, light, and chemical cues that laboratory-reared larvae have not, they may respond differently to chemical cues from potential settlement sites. Therefore, we conducted this experiment to determine whether cues from adult fiddler crabs stimulated molting in wild-caught fiddler crab megalopae. Our hypothesis was that chemical cues from adult conspecific fiddler crabs would stimulate molting but that cues from other species would not.

MATERIALS AND METHODS

Field collection of megalopae and adult fiddler crabs

We collected megalopae for the experiments with a 0.75 m diameter 333 µm plankton net deployed from a platform under the Pivers Island Bridge in Beaufort, North Carolina, USA (34° 43.20′ N, 76° 40.40′ W) from June to August 2014 and 2015. Since fiddler crab megalopae use flood tide transport to travel upstream into estuarine habitats (De Vries et al. 1994, Forward & Tankersley 2001), the net was deployed for ~1 h surrounding the time of maximum nocturnal flood current. *Uca* megalopae were separated from the organisms collected in the net and held in estuarine water (salinity = 34 ppt) until the start of the experiments. Estuarine water was collected by bucket from the pump house dock at the Duke University Marine Laboratory and filtered through a 5 µm bag filter. Megalopae were not separated according to molt stage and were held for between 8 and 12 h prior to the start of the experiment.

Adult *U. pugilator* were collected from a sandflat in the Rachel Carson Estuarine Research Reserve (34° 42.71′ N, 76° 40.47′ W). Adult *U. pugnax* and *U. minax* were collected from the Bell Creek Salt Marsh (34° 47.39′ N, 76° 40.14′ W), ~10 km from the Duke University Marine Laboratory. Crabs from each location were held separately in large (122 cm) diameter tanks with dripping estuarine water (salinity = 34 ppt) and sediment from the collection site. Crabs were held at approximately 25°C in ambient light (14 h light:10 h dark). Crabs were fed by the periodic addition of fresh sediment from their collection sites. A haphazard mixed-sex group of crabs of each species was used to prepare the odor waters each day; crabs were returned to the holding tanks following odor water preparation. For all species, we held many more crabs than were needed each day. Therefore, crabs were used multiple times for odor prepa-

ration, but the same individual crabs were not used each day.

Odor water preparation

Species-specific odor waters were prepared on each day of the experiments. Estuarine water (defined above) was used as the control treatment as well as the basis for the odor waters. Approximately 25 grams of adult crabs were soaked for 1 h in 500 ml of 5 µm-filtered estuarine water. Each odor water was then filtered through a 500 µm sieve to remove any particulates left behind by the crabs during the incubation. Fresh odor waters for each of the 3 test species were prepared each day between 08:00 and 10:00 h.

Experimental set-up

For each experiment, individual megalopae were placed in 20 ml scintillation vials with 10 ml of one of the water treatments. Vials were held in groups of 64 in an 8 × 8 array, with the placement of treatments systematically varied in each row. Either 1 or 2 groups of 64 were tested at one time. The arrays were maintained at 25°C and the ambient 14 h light:10 h dark cycle for 10 d. Light was about 15×10^{15} photons cm^{-2} s^{-1} from daylight and cool white fluorescent lamps. Each day megalopae were transferred to clean vials with newly prepared water to prevent degradation of the chemical cues. Megalopae were fed newly hatched *Artemia* nauplii daily. Microscope observations were made 4 times each day at 09:00, 13:00, 18:00 and 23:00 h. Any megalopa that had molted or died at each observation period was preserved individually in 95% ethanol, and the date and time recorded. At the end of 10 d, any megalopae that had not molted were also preserved in 95% ethanol for species identification.

Uca species identification

Each megalopa and juvenile used in the experiment was identified to species using the method of Welch et al. (2015). DNA was extracted from each individual (Estoup et al. 1996) followed by multiplex PCR. The size of the amplicon determined by gel electrophoresis identifies the species of the individual (Welch et al. 2015).

Data analysis

For each crab species, the average time to molt in each odor treatment was compared with separate 1-way ANOVAs (Sokal & Rohlf 1981). In addition, for *U. pugilator*, the data for the 3 odor treatments were pooled and compared with the times to molt for those in the control treatment using a *t*-test (Sokal & Rohlf 1981). Since *U. minax* megalopae only molted in 2 of the treatments, the times to molt for those exposed to *U. pugnax* and *U. minax* odors were also compared with a *t*-test. For each crab species, we also used a *z*-test for proportions (Walpole 1974) to compare the proportions of megalopae that molted in each treatment with the proportions that molted in the control treatment, as well as in the other odor treatments.

RESULTS

Allocation of species to treatments and survival in experiment

A total of 896 individual megalopae were tested. Of those, 105 (11.7%) were not identified at the completion of the experiment because they failed to amplify in the PCR. Almost a third of the unidentified megalopae had died, and many of those had partially decomposed before being removed at the next observation time. Overall mortality (including the unidentified animals) was 11.1%. The unidentified animals were omitted for the remainder of the data analysis.

Because megalopae were assigned to treatments before their identities were known, we assessed the distribution of individuals of each species to each treatment after the identifications were complete (Table 1). Within each species, the distribution of individuals among treatments was not different from a uniform distribution (χ^2 test for goodness of fit, all $p > 0.05$; Table 1). Of the 791 megalopae used in the experiment, 78 were *U. minax*, 267 were *U. pugilator,* and 446 were *U. pugnax* (Table 2); this species composition is consistent with previous plankton samples

Table 1. Number of megalopae of each *Uca* species tested in each odor treatment

Treatment	*U. pugilator*	*U. pugnax*	*U. minax*
Control	63	109	16
U. pugilator odor	75	101	25
U. pugnax odor	71	112	20
U. minax odor	58	124	17

Table 2. Number of megalopae of each *Uca* species that molted, remained megalopae or died during the experiment. Unidentified animals omitted

Species	Total tested	No. molted (%)	No. remained megalopae (%)	No. died (%)
U. pugilator	267	176 (66)	56 (21)	35 (13)
U. pugnax	446	113 (25)	310 (70)	23 (5)
U. minax	78	7 (9)	62 (79)	9 (12)
Total	791	296 (37)	428 (54)	67 (9)

at this site (Reinsel et al. 2015). Of the identified megalopae, 37.4% molted, 54.1% remained megalopae, and 8.5% died (Table 2).

Time to molt

Most of the megalopae that molted did so early in the experiment (Days 3–5), with the number molting dropping in later days. The average time to molt for *U. pugilator* megalopae ranged from ~120–140 h (Fig. 1a) and was not significantly different among odor treatments by ANOVA ($F_{3,172}$ = 2.465; p = 0.06). *U. pugilator* megalopae exposed to any crab odor treatment, however, molted significantly earlier (mean = 115.3 vs. 132.9 h) than those exposed to estuarine water alone (*t*-test, crab odor vs. control; t_{172} = 2.265; p = 0.02). The time to molt for *U. pugnax* megalopae was also not significantly different among treatments (ANOVA $F_{3,107}$ = 1.333; p = 0.26) and ranged from ~60 h in the control to ~120 h in *U. pugilator* odor water (Fig. 1b). Very few *U. minax* megalopae molted at all during the experiment (7 ind. total), and those that did so molted in the *U. pugnax* (3 ind.) and *U. minax* (4 ind.) odor treatments (Fig. 1c). Those exposed to *U. pugnax* odor molted after an average of 120 h of exposure, while those in conspecific odor water did not molt until significantly later, after >200 h (*t*-test; t_5 = 5.871; p = 0.002).

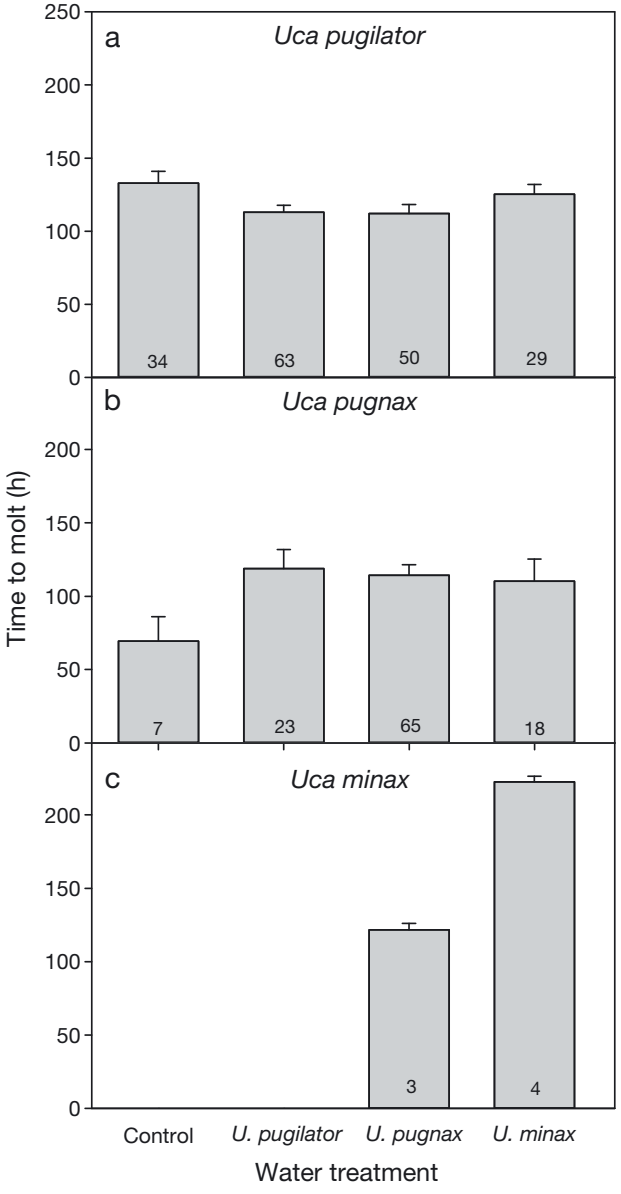

Fig. 1. Mean time to molt (±SE) for megalopae of (a) *Uca pugilator*, (b) *U. pugnax*, and (c) *U. minax* exposed to estuarine seawater (control) or seawater with different adult chemical cues. Numbers in bars represent number of crabs that molted in each treatment

Effect of treatment on molting frequency

Overall, 75.9% of the *U. pugilator* megalopae that remained viable for 10 d molted during the experiment, with at least 60% molting in each of the treatments (Fig. 2a). Significantly more (93%) molted in conspecific odor water than in any other treatment (z > 2.0; p < 0.05 for all tests). Slightly fewer (>80%) molted in *U. pugnax* odor water, but significantly more than in the control (z = 2.26; p = 0.012) or in *U. minax* odor water (z = 2.19; p < 0.014).

In contrast to *U. pugilator*, very few (< 30% of those identified) *U. pugnax* megalopae molted in any treatment except in conspecific odor water, in which >60% molted (Fig. 2b). This was significantly more than in any other treatment (z > 5.0; p << 0.001 for all tests). Although lower percentages molted in the *U. pugilator* and *U. minax* treatments (24 and 15%, respectively), significantly greater proportions molted in those treatments than in the estuarine water control (z > 2.0; p < 0.05 for both).

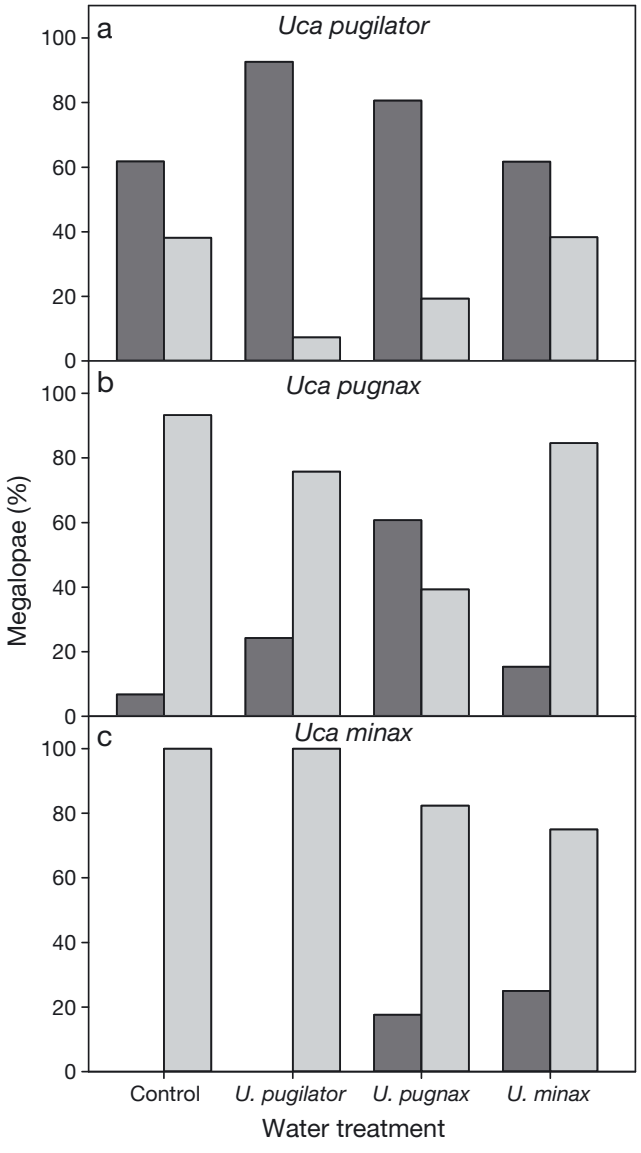

Fig. 2. Percent of megalopae of (a) *Uca pugilator*, (b) *U. pugnax*, and (c) *U. minax* that molted (dark gray bars) and remained megalopae (light gray bars) over 10 d of exposure to estuarine seawater (control) or seawater with adult chemical cues. Different letters represent statistically significant differences in percent molting by *z*-test for proportions. Megalopae that died during the experiment were excluded from percentage calculations

Very few (7 out of 69, 10.1%) of the *U. minax* exposed to any treatment molted at all during the experiment; those that did were exposed to either conspecific or *U. pugnax* odor water (Fig. 2c). The proportion that molted in conspecific odor water was significantly higher than in the control ($z = 1.94$; $p = 0.026$) but not significantly different from the proportion that molted in *U. pugnax* odor water ($z = 0.52$; $p > 0.05$).

DISCUSSION

The 3 species of fiddler crabs on the USA East Coast are generally found in separate habitats with different sediment types and salinities. There is some evidence that this habitat separation is driven by selective settlement by the megalopae (Behum et al. 2005, Brodie et al. 2005, Welch et al. 2015), but the mechanism driving this selection has not been determined. In this study we examined whether chemical cues from adult fiddler crabs would stimulate molting by conspecific fiddler crab megalopae. If those cues do stimulate molting, then this stimulation could be part of the mechanism underlying selective settlement by the megalopae. Our results indicate that chemical cues do stimulate molting of conspecific field-caught megalopae, but we did not detect the acceleration of molting noted by previous investigators.

In none of the 3 species was there a significant difference between the time to molt in conspecific water and the time to molt in other waters (Fig. 1). For *Uca pugilator* (Fig. 1a), many megalopae molted in all water types. The mean time to molt was similar in water with cues from *U. pugilator* and *U. pugnax*, slightly longer in *U. minax* water, and longest in estuarine water alone, but these differences were not significant (ANOVA, $p = 0.06$). However, the mean time to molt for *U. pugilator* megalopae in any crab water was significantly shorter (115.2 vs. 132.9 h) than megalopae in estuarine water (*t*-test, $p = 0.02$). This indicates that molting in *U. pugilator* is accelerated by any crab odor over estuarine water.

For *U. pugnax* (Fig. 1b), relatively few megalopae molted except in conspecific water. The mean time to molt was shortest in estuarine water, but this is based on only 7 megalopae. Those megalopae may have been at the end of their ability to delay metamorphosis and so molted even without a cue (Wolcott & De Vries 1994). Very few *U. minax* megalopae molted in the experiment, and those that did molt molted at the very end of the experimental time period (Fig. 1c). It is likely that *U. minax* requires lower salinity water to molt, since its adult habitat is in lower salinities.

A number of investigators have examined the effects of chemical cues on metamorphosis of laboratory-reared fiddler crab larvae (Christy 1989, O'Connor 1991, 2005, O'Connor & Judge 1997, 1999, 2004, O'Connor & Gregg 1998, O'Connor & Van 2006). All found that the time to molt was shorter in the presence of chemical cues from adult conspecific crabs or from appropriate adult habitat. The most likely explanation for our inability to detect this effect is that our megalopae were caught in the field and were thus likely a

mixture of cohorts of different ages and perhaps different levels of competence to molt. Lab-reared megalopae are taken from the same cohort, with a known age, and have experienced the same physical and chemical conditions throughout their larval development. Therefore, they should reach competence at similar ages in the absence of a stimulus. Field-caught megalopae are almost certainly a mixture of ages and have experienced variations in light, chemical cues, and temperatures during their development. Therefore, any acceleration of molting may have been overwhelmed by the difference in time necessary to reach competence to molt. This limitation could perhaps be overcome by molt-staging the larvae, but we did not do so since it is difficult to do in fiddler crab megalopae because of their small size.

While we did not measure a shortening in time to molt with conspecific cues, we did see a significant stimulation of the occurrence of molting by conspecific cues relative to estuarine water or congeneric cues (Fig. 2). In *U. pugilator*, 93% of the megalopae in conspecific water molted (Fig. 2a). However, many megalopae (>60%) molted in the other treatments as well. This indicates that while *U. pugilator* is most stimulated to molt by conspecific cues, it is also stimulated by congeneric cues, meaning that it may well be less selective than the other species.

In contrast, *U. pugnax* megalopae molted most often in water with conspecific cues, and relatively few (<25%) molted in any other treatment. This indicates that *U. pugnax* may be highly selective. This agrees with the findings of O'Connor & Judge (1999), who found that molting of *U. pugnax* megalopae in field-deployed microhabitats decreased rapidly with distance from the adult habitat. Fewer than 10% of the *U. minax* megalopae in our experiment molted, but more molted in conspecific water than in the other treatments, and no megalopae molted in estuarine water or water with cues from *U. pugilator* (Fig. 2c). It is possible that *U. minax* simply delayed metamorphosis due to the salinity of the water used in the experiment, since their adult habitat is in lower salinity parts of estuaries. Future studies will examine this possibility. It is also possible that *U. minax* megalopae reenter the estuary in a less competent state than the other 2 species, since they would necessarily have to travel farther up the estuary to reach adult habitats. The fact that *U. minax* molted only in water with conspecific cues or with cues from *U. pugnax,* whose habitat it sometimes overlaps, indicates some ability to avoid settling with *U. pugilator.*

Many prior investigators have examined the effects of chemical cues from adult fiddler crabs on molting of fiddler crab megalopae, but this is the first study to do so with field-caught megalopae instead of laboratory-reared megalopae. This distinction is important because previous studies have shown physiological differences between laboratory- and field-reared crab larvae (Epifanio et al. 1991, Welch & Epifanio 1995), and fish larvae (MacKenzie et al. 1990, Duffy & Epifanio 1994, Duffy et al. 1996). As they move from hatching in estuaries to early development in offshore waters to reinvasion of estuaries as megalopae, field-caught larvae have experienced natural variations in light, temperature, salinity, and chemical cues that are impossible to replicate in the laboratory. Therefore, it is likely that their responses to chemical and other cues may be different from laboratory-reared larvae. Our finding that chemical cues from conspecific crabs stimulate molting in field-caught megalopae indicates that these cues may indeed be important in settlement site selection by the megalopae.

The fact that we observed a 'molt or do not molt' signal rather than a clear acceleration of molting, as found in the experiments with laboratory-reared animals, fits the transport model of these megalopae. As a megalopa is transported up the estuary using selective tidal stream transport (STST, De Vries et al. 1994), it will be transported near potentially suitable habitats, and at the end of flood tide, it will rest on or near the bottom. If it is an inappropriate habitat, the megalopa can simply swim again on the next flood tide and continue its transport up the estuary. If the habitat is appropriate, however, it can choose to settle and metamorphose, in which case it has to stop its STST behavior prior to the next flood tide. The acceleration of molting shown in experiments with laboratory-reared megalopae typically shortens the megalopal duration by 1–2 d (O'Connor & Gregg 1998) as opposed to a normal duration of 8–10 d. In order to remain in a habitat without transporting away on the next flood tide, however, a megalopa would have to change its behavior to suppress the STST behavior within 6 h (before the beginning of the following flood tide). This is similar to the rapid behavioral change that allows planktonic larvae to adhere to coral reefs as they are transported past them (Hadfield & Koehl 2004, Koehl & Hadfield 2004). The on/off switch for molting may trigger this behavioral shift in fiddler crab megalopae as molting is initiated.

Thus, the stimulation of molting by chemical cues from adult conspecifics is likely important in selective settlement by fiddler crab megalopae. This study confirms that the stimulation of molting observed in laboratory-reared larvae also exists in field-caught megalopae. It is likely that the complete process involves a

behavioral change to interrupt the flood-tide transport behavior of the megalopae, which allows them to remain in the habitat long enough for molting to take place. It is likely that this combination of physiological and behavioral responses to chemical cues drives selective settlement of fiddler crab megalopae.

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Editorial responsibility: Joseph Pawlik, Wilmington, North Carolina, USA

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