Role of larval release pheromones and peptide mimics in abdominal pumping and swimming behavior of ovigerous blue crabs, *Callinectes sapidus*

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ABSTRACT

Blue crabs *Callinectes sapidus*, like most decapods, synchronously hatch eggs and release larvae over a very short time period. Synchrony is achieved though vigorous abdominal pumping in response to pheromones from hatching eggs. We hypothesized that these or related pheromones stimulate vertical swimming associated with larval release and ebb-tide swimming during the last few days before egg hatching. We used abdominal pumping and swimming assays to investigate the roles of pheromones. We tested responses of crabs to egg extract containing pheromones, trypsin (an enzyme that generates peptide pheromones), and bradykinin (a peptide pheromone mimic). We delivered test substances directly into the egg mass via capillary tubing. In response to egg extract, ovigerous crabs increased abdominal pumping and vertical swimming, showing native pheromones evoke both behaviors. Delivery of trypsin and bradykinin caused increased pumping but not vertical swimming. These results suggest that pheromones generated from eggs stimulate vertical swimming during ebb-tide transport, but that peptides that induce abdominal pumping are not sufficient to cause swimming. We hypothesize that swimming is stimulated by a blend of molecules that includes these peptide pheromones.

1. Introduction

Larval release in most decapod crustaceans is precisely timed to correspond with tidal, diel, or lunar cycles (Saigusa and Hidaka, 1978; DeVries et al., 1983; Forward, 1987; Morgan and Christy, 1995; Tankersley et al., 2002; Saigusa and Kawagoye, 1997). Fertilized eggs are attached to the pleopods, where they develop for days to months. Egg hatching and larval release are usually highly synchronous within a given release event, lasting only a few minutes (e.g. Saigusa and Hidaka, 1978; Forward et al., 1982; DeVries et al., 1991). At the time of larval release, the female elevates on her walking legs and vigorously flexes (pumps) her abdomen, assisting egg hatching and propelling larvae into the water column. Ovigerous blue crabs, *Callinectes sapidus* Rathbun, typically swim to the surface immediately prior to larval release, with abdominal pumping and larval release taking place at or near the surface.

Synchronous release during specific phases of environmental cycles can decrease predation pressure on both larvae and females (DeCoursey, 1981; Christy, 1982; Morgan, 1990; Morgan and Christy, 1995, 1997; Hovel and Morgan, 1997), maximize the chances of transport to appropriate nursery grounds (Saigusa and Hidaka, 1978; Christy, 1982; Morgan and Christy, 1995), avoid larval stranding (Saigusa, 1981; Christy, 1986), and decrease physiological stress on the larvae (Saigusa, 1981; Forward et al., 1982).

Hatching synchrony in subtidal crabs is explained in a conceptual model, proposed by Forward and Lohmann (1983). In this model, embryos control the time of hatching and females synchronize hatching. First, a few eggs hatch releasing pheromones. The pheromones stimulate vigorous abdominal pumping by the female which causes more eggs to hatch. This positive feedback loop results in eggs hatching over a short interval.

Proteolytic enzymes play a role in crustacean egg hatching and generate peptide pheromones that induce abdominal pumping. Proteolytic enzymes are released from the egg mass around the time of larval release of *Neopanope sayi* (DeVries and Forward, 1991) and *Sesarma hematocheir* (Saigusa, 1996; Saigusa and Iwasaki, 1999; Gusev et al., 2004). Incubation of ovigerous females in exogenous trypsin stimulates abdominal pumping in the crab *Rhithropanopeus harrisii* (Rittschof et al., 1990) and the spiny lobster *Panulirus argus* (Ziegler, 2007). Thus, abdominal pumping is stimulated by peptides generated by proteolytic cleavage of proteins in the egg membranes.

Peptide pheromones released by hatching eggs are similar to peptide pheromones and signals that function in a variety of signaling systems (reviewed by Rittschof, 1993; Rittschof and Cohen, 2004). Larval release pheromones are short neutral-basic peptides, <500 Da, with one or more neutral amino acids preceding arginine or lysine at the carboxyl-terminus (Rittschof et al., 1985, 1989; Forward et al., 1987; Rittschof and Cohen, 2004). The basic amino acid at the carboxyl terminus is generated by the action of trypsin-like endoproteases which cleave

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proteins after the basic amino acids lysine and arginine. Solutions of egg hatch water or aqueous extracts of egg homogenates induce pumping responses (e.g. Forward and Lohmann, 1983; DeVries et al., 1991; Tankersley et al., 2002), as do peptide pheromone mimics (reviewed by Rittschof and Cohen, 2004). However, pure peptides typically induce much weaker responses than egg hatch water or egg extracts. This suggests additional kinds of molecules are involved in the larval release process (Rittschof et al., 1985; Rittschof and Cohen, 2004).

Much of the work on brachyuran larval release pheromones has been focused on two Xanthid crabs (*R. harrisii*: Forward and Lohmann, 1983; Rittschof et al., 1985, 1989, 1990; Forward et al., 1987; *N. sayi*: DeVries and Forward, 1991; DeVries et al., 1991) and one Grapsid crab, *S. hematocheir* (Saigusa, 1994, 1996; Saigusa and Iwasaki, 1999; Gusev et al., 2004). Like these crabs, subtidal blue crabs *Callinectes sapidus* exhibit synchronous egg hatching. Larvae are released around the times of morning high tides and the larval release rhythm is endogenous (Ziegler, 2002). Within a larval release event, blue crabs synchronously release all larvae in a very short period of time (Tankersley et al., 2002; Ziegler, 2002). Water in which eggs have hatched and water in which eggs have been homogenized stimulate abdominal pumping in blue crabs (Tankersley et al., 2002), and blue crab egg hatching follows the conceptual model of Forward and Lohmann (1983).

In addition to larval release behavior, spawning blue crabs swim routinely on falling tides. This ebb-tide swimming behavior increases in the last several days before egg hatching (Forward et al., 2003, 2005; Hench et al., 2004). The purpose of this study was to test the hypothesis that peptide pheromones, generated from the egg mass, induce larval release behavior and swimming behavior in ovigerous blue crabs. We conducted abdominal pumping and swimming assays to test the responsiveness of ovigerous blue crabs to: egg extract; trypsin, which generates peptide pheromones within egg masses; and bradykinin, a peptide pheromone mimic.

2. Methods

2.1. Definitions

Embryos include all of the developmental stages within the egg. Egg is the combination of egg membrane, developing embryo, and all other material enclosed within the egg membranes. Larvae are developmental stages that occur outside of the egg (Giese and Pearse, 1974; Forward and Lohmann, 1983; DeVries and Forward, 1991).

2.2. Collection and maintenance of crabs

Ovigerous blue crabs, *Callinectes sapidus*, were captured at night around the time of low tide using dip nets in the Rachel Carson National Estuarine Research Reserve, Beaufort, NC (34°42.65′ N, 76°40.40′ W). Except after the occurrence of strong storms, salinity in this area is 30–35. Crabs were transported to the Duke University Marine Laboratory individually in buckets containing ~1.5 L of seawater. Crabs were held in 950 L tanks with running seawater and were fed seasonal fish (primarily pinfish, spot, and croaker) daily until used in assays. All crabs were used within 7 days of collections. Carapace width of crabs used in experiments ranged from approximately 110–180 mm.

Each crab was used in either the abdominal pumping or swimming assay and tested twice at each volume or concentration of a test solution. One test was during the time of flood tide and the other test at the time of ebb tide in the field. Beginning with the control, concentrations of each test solution were delivered in order of increasing concentration.

2.3. Preparation of test solutions

2.3.1. Egg extract

The first test solution was an extract of late-stage eggs homogenized in seawater (Forward and Lohmann, 1983) and was used to determine if substances from the eggs stimulate larval release and vertical swimming behaviors. Females possessing late-stage eggs (stages 8–9 of DeVries et al., 1983) were collected and eggs were removed from the pleopods using forceps and a scalpel and frozen at −20 °C. Immediately after thawing, eggs were homogenized with a mortar and pestle in an equal volume of seawater filtered to remove particles >5 μm. The homogenate was centrifuged at 15,000 rpm for 5 min and the supernatant was collected and frozen in 15 mL aliquots at −20 °C until use. This procedure results in a concentration of ~19,000 eggs mL^{-1}. Filtered seawater was used as a control solution in the egg extract experiments.

2.3.2. Trypsin

Trypsin stimulates abdominal pumping in ovigerous mud crabs *Rhithropanopeus harrisii* (Rittschof et al., 1990) and ovigerous spiny lobsters *Panulirus argus* (Ziegler, 2007). Test solutions were bovine trypsin (9820 BAEE units mg^{-1}, T8003, Sigma-Aldrich) in NaCl isotonic to seawater and 1 mM HCl. Two concentrations of trypsin were prepared: ~17,000 BAEE units mL^{-1} and ~44,000 BAEE units mL^{-1}. These concentrations are similar to concentrations of trypsin measured in blue crab egg masses within 12 h of hatching (Hinshaw et al., Duke University, unpublished data). Trypsin solutions were stored in 15 mL aliquots at ~4 °C until use. Control solutions used in trypsin experiments were seawater filtered to remove particles >5 μm and 0.1 mM HCl in a NaCl solution isotonic to seawater.

2.3.3. Bradykinin

Bradykinin is a vertebrate peptide hormone with neutral proline and phenylalanine residues preceding the basic arginine residue at the carboxyl terminus. Bradykinin is biologically active in a number of crustacean signaling systems, including barnacle settlement and crustacean larval-release behavior (reviewed by Rittschof and Cohen, 2004) and is commonly used as a pheromone mimic. A stock solution of 10^{-3} M bradykinin (B3259, Sigma-Aldrich) was prepared in deionized water and frozen at −20 °C in 1.5 mL aliquots until use. The stock solution was diluted to the test concentration (10^{-8} to 10^{-6} M) in 5 μm-filtered seawater immediately before use. The seawater salinity remained relatively constant after addition of the small volume of the bradykinin solution. Seawater filtered to remove particles >5 μm was used as a control solution in bradykinin experiments.

2.4. Behavioral assays

2.4.1. Abdominal pumping assay

The purpose of the abdominal pumping assay was to determine if test solutions stimulate larval release behaviors (i.e. abdominal pumping, Forward and Lohmann, 1983; Rittschof et al., 1985; Tankersley et al., 2002). Preliminary trials indicated little change in pumping responsiveness with embryo developmental stage. Therefore, ovigerous crabs with embryos in all stages of development were used in this assay. Each crab was held in a small plastic aquarium (30 cm × 18 cm) containing ~3 L of aerated, ambient estuarine water filtered to remove particles >5 μm in diameter. To minimize potential responses of crabs to the experimenter, aquaria were illuminated continuously with dim red light, as blue crabs are insensitive to wavelengths >600 nm based on their visual pigments (Cronin and Forward, 1988).

Approximately 50 cm of 0.86 mm inner-diameter polyethylene tubing (PE/6 tubing, Scientific Commodities Inc., Lake Havasu City, AZ) was attached to the carapace using cyanoacrylate glue such that one end of the tubing extended 2–3 mm into the egg mass and the

other end extended outside of the aquarium (Fig. 1). In this way, the test solutions could be delivered directly into the egg mass. Plastic-coated, 18-gauge copper wire was wrapped around the large lateral spines and used to provide support for the tubing and to hold the free tubing away from the crab, preventing the crab from pinching or becoming entangled in the tubing.

In the abdominal pumping assay, each crab was observed for two consecutive 3-min intervals. First, each crab was observed for 3 min in clean seawater and the number of abdominal pumps recorded. This interval established the baseline (unstimulated) abdominal pumping rate (Forward et al., 1987). A test solution or appropriate control solution (see above) was then delivered through the tubing into the egg mass over 5–10 s and pumping activity was quantified for an additional 3 min. This interval established the response (stimulated) pumping rate. A response was classified as positive if pumping activity was greater following delivery of the test solution than before delivery. Observations ceased before the end of the 3-min time period if a positive response was observed. The before and after protocol was used to control for variations in baseline pumping activity with egg stage or time of day (Rittschof et al., 1989; Tankersley et al., 2002). Testing crabs using both the test solution and an appropriate control solution controlled for any effects of the delivery method on abdominal pumping rate. Delivery volumes ranged from 10–500 μL for the egg extract solution. For the trypsin and bradykinin assays, delivery volume was held constant at 100 μL and different concentrations were tested. This delivery volume maximized abdominal response in preliminary experiments using egg extract.

2.4.2. Swimming assay

The purpose of the swimming assay was to determine if the test solutions stimulate vertical swimming behavior. Only crabs with late-stage embryos (Stages 6–9 of DeVries et al., 1983) were used. Each crab was held individually in a large, transparent cylindrical tube (1.23 m tall × 44 cm diameter, Aquatic Eco-Systems model T8) containing aerated, ambient estuarine water filtered to remove particles >5 μm in diameter and illuminated continuously with red light. Approximately 1.6 m of tubing was attached to the carapace as described for the abdominal pumping assay (Fig. 1). Crabs were monitored using a video camera (Panasonic WV-BP330) and time-lapse recorder (Panasonic AG-RT850).

In the swimming assay, each crab was observed for two consecutive 30-min periods. Each crabs was first observed for 30 min before test solution delivery and the number of ascents into the water column was recorded. An ascent was recorded when a crab swa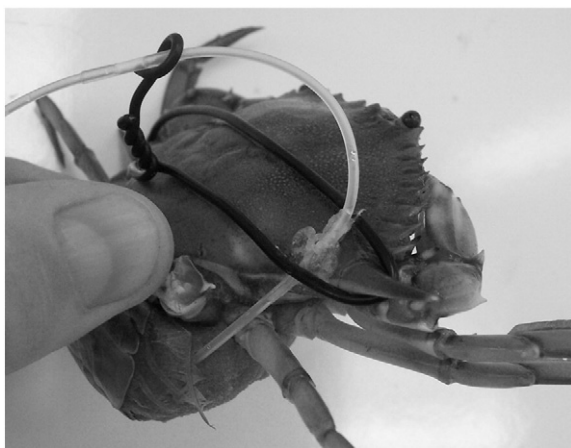m above the bottom 1/3 (~41 cm high) of the column. The test solution was then delivered through the tubing over 5 min, delivering 1/10 of the total delivery volume every 30 s. Ascents were then quantified for 30 min, including the 5 min of delivery time. A response was classified as positive if the number of ascents was greater in the 30 min following delivery of the test solution than in the 30 min before delivery. Delivery volumes ranged from 250–1500 μL for the egg extract solution. For trypsin and bradykinin assays 750 μL of 17,000 BAEE units mL^{-1} trypsin or 10^{-6} M bradykinin was used. This delivery volume maximized abdominal response in preliminary experiments using egg extract.

2.5. Data analysis

For each test solution, 10–25 crabs were tested in each assay during the times of ebb and flood tides at the collection site. Although crabs were held in constant conditions, their behavior is rhythmic and thus consistent with the times of each tidal phase in the field. The proportion of crabs responding with increased pumping or swimming to each test solution was compared to the control using a Z statistic for testing differences between two proportions (Walpole, 1968). If egg extract assays indicated that there was no significant difference in the proportion responding positively between the times of ebb and flood tide, then either ebb or flood tide was randomly chosen for each crab and those responses were used in analyses.

3. Results

3.1. Abdominal pumping assay

3.1.1. Egg extract

The percentage of crabs responding positively with increased pumping to the seawater control solution was 40% during the time of ebb tide in the field and 26% during the time of flood tide in the field. Percentages responding positively to the egg extract solution varied from 57–75% during the time of ebb tide and 41–72% during the time of flood tide (Fig. 2). Delivery of egg extract significantly increased abdominal pumping activity during the times of ebb and flood tides. During the time of ebb tide, a significant increase in the proportion responding positively above the seawater control was observed at delivery volumes above 250 μL (n = 16, Z = 2.1, $p<0.05$), corresponding to 4750 eggs hatching. During the time of flood tide, a significant increase was observed at delivery volumes above 100 μL (n = 15, Z = 1.86, $p<0.05$), corresponding to 1900 eggs hatching. Although the threshold volumes differed between ebb and flood tides,

Fig. 1. Ovigerous blue crab with tubing and support wire attached for abdominal pumping and swimming assays.
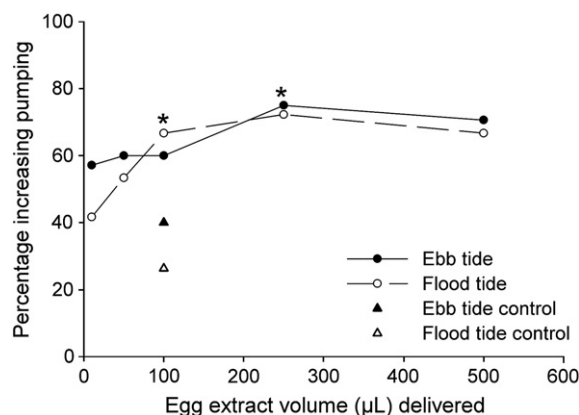

Fig. 2. Percentage of crabs increasing pumping following delivery of different volumes of egg extract when tested at the times of ebb and flood tide. Circles indicate responses to egg extract and triangles indicate the responses to seawater control solutions. Controls are plotted above their volume of filtered seawater. * indicates the minimum egg extract volume that elicited a significant ($p<0.05$) positive response compared to the seawater controls. The average sample size for each test concentration was 16.

the proportions of crabs responding positively at either threshold volume (100 μL or 250 μL) did not differ significantly between tidal phases (100 μL: n = 13, $Z = 0.13$, $p > 0.05$; 250 μL: n = 17, $Z = 0.18$, $p > 0.05$). Thus, in subsequent trials each crab was tested in the bradykinin and trypsin assays on both tidal phases, but only one response for each concentration from each crab, chosen at random, was used for analyses.

3.1.2. Trypsin

The percentage of crabs responding positively by pumping to the seawater control solution was 12.5% (Fig. 3). The percentage of crabs responding positively to 0.1 mM HCl in an NaCl solution isotonic to seawater was 39.1%, which was significantly higher than the response to the seawater control (n = 23, $Z = 1.82$, $p < 0.05$). The lower trypsin concentration (~17,000 BAEE units mL^{-1}) resulted in a 75.0% positive response, which was significantly higher than both the seawater (n = 16, $Z = 3.56$, $p < 0.001$) and the HCl controls (n = 16, $Z = 2.21$, $p < 0.05$). The higher trypsin concentration (~44,000 BAEE units mL^{-1}) resulted in 50.0% responding positively, which was significantly higher than the seawater control (n = 16, $Z = 2.28$, $p < 0.05$), but not statistically different from the HCl control (n = 16, $Z = 0.67$, $p > 0.05$).

3.1.3. Bradykinin

The percentage of crabs responding positively to the seawater control solution was 33.3%. The percentage responding positively to the bradykinin test solutions ranged from 44.4–89% (Fig. 4). Delivery of 10^{-7} M bradykinin significantly increased abdominal pumping activity (n = 17, $Z = 2.96$, $p < 0.01$). However, the proportion responding positively to the highest concentration tested (10^{-6} M) was not significantly different from the seawater control (n = 17, $Z = 1.26$, $p > 0.05$).

3.2. Swimming assay

3.2.1. Egg extract

Delivery of egg extract stimulated vertical swimming (Fig. 5). Following delivery of filtered seawater, 25% and 16.7% of females responded positively during the times of ebb and flood tide, respectively. Following delivery of egg extract, the percentages of positive responses ranged from 50–67% during the time of ebb tide and 46–66% during the time of flood tide. All volumes of egg extract
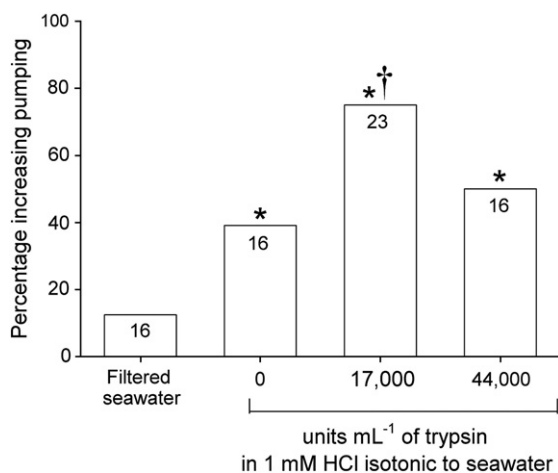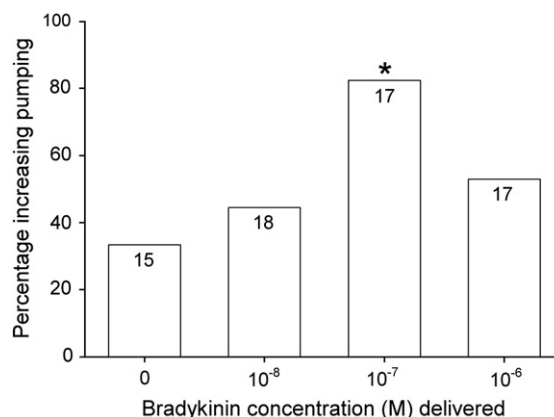
Fig. 4. Percentage of crabs increasing pumping following delivery of different concentrations of bradykinin. The number within each bar indicates sample size. * indicates proportions significantly different from seawater control at $p < 0.05$.

tested (250 μL–1500 μL, ≥4750 eggs hatching) stimulated swimming during both tidal phases, as the proportions of crabs responding positively were significantly greater for all egg extract treatments than for the seawater controls (n = 11–24, Z > 1.72, $p < 0.05$). The threshold volume of 250 μL corresponds to 4750 eggs. The proportion responding positively at the threshold volume (250 μL) was not statistically different between ebb and flood tides (n = 14, Z = 0.76, $p > 0.05$). Egg extract stimulated increased swimming in ovigerous crabs and responses did not differ between the times of flood and ebb tides. Thus, for subsequent bradykinin and trypsin assays, only responses at one tidal phase for each concentration from each crab were used for analyses. The tidal phase for responses was chosen at random.

3.2.2. Trypsin

The percentage of crabs responding positively by swimming in response to the seawater control solution was 21.4%, while the percentage of crabs responding positively to the HCl control solution was 16.7%. Delivery of 17,000 BAEE units mL^{-1} trypsin resulted in a slight increase, as 30.0% responded positively. Nevertheless, the proportions of crabs responding positively by swimming to the three solutions tested (filtered seawater control, HCl control, and trypsin) were not statistically different from each other (n = 14–20, Z < 1.33, $p > 0.05$).

Fig 3. Percentage of crabs increasing pumping following delivery of 250 μL of filtered seawater, 250 μL of 1 mM HCl made isotonic to seawater with NaCl, 250 μL of 17,000 and 44,000 U mL^{-1} trypsin in 1 mM HCl isotonic to seawater. The number within each bar indicates sample size. * indicates proportions significantly different from seawater control and † indicates proportions differently from HCl control at $p < 0.05$.
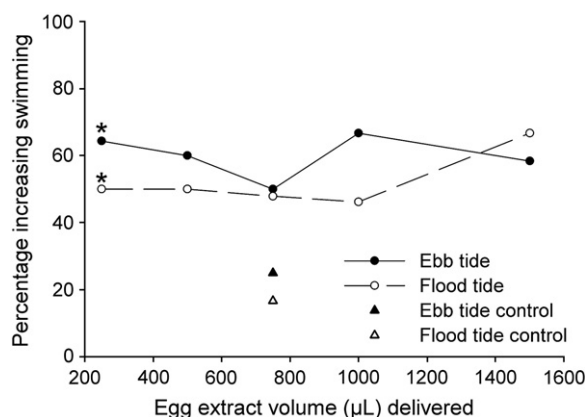
Fig. 5. Percentage of crabs increasing swimming following delivery of different volumes of egg extract when tested at the times of ebb and flood tide. Circles indicate responses to egg extract and triangles indicate the responses to seawater control solutions. Controls are plotted above their volume of filtered seawater. * indicates the minimum egg extract volume that elicited a significant ($p < 0.05$) positive response compared to the seawater controls. The average sample size for each test concentration was 16.

3.2.3. Bradykinin

Delivery of the filtered seawater control resulted in 18.18% responding whereas the delivery of 10^{-6} M bradykinin resulted in 35.3% responding positively. Though delivery of bradykinin resulted in increased swimming behavior, this increase was not statistically different from the control (n = 17, Z = 1.21, $p > 0.05$).

4. Discussion

We tested the hypothesis that larval release pheromones from eggs induce abdomen pumping and swimming behavior in ovigerous blue crabs. Egg extract containing pheromones, trypsin (an enzyme that generates peptide pheromones upon interaction with the egg mass), and bradykinin (a nanopeptide larval release pheromone mimic) were delivered into the egg mass of ovigerous crabs to assess the effects of these solutions on abdominal pumping and vertical swimming. All test solutions induced abdominal pumping; only egg extract induced vertical swimming.

Delivery of egg extract resulted in increased abdominal pumping (Fig. 2), indicating larval release pheromones are in eggs with late stage embryos. This result confirms that abdominal pumping in blue crabs is stimulated by egg extract (Tankersley et al., 2002) and shows delivery of the test solutions directly into the egg mass is a viable delivery method for the assays. Abdominal pumping in response to egg extract was similar during both tidal phases for all volumes tested. However, the threshold volumes inducing pumping differed between the two tidal phases, with a lower threshold volume during flood tide. Thus, female sensitivity to larval release pheromones is higher during flood tide. At higher delivery volumes, however, there was no difference in response between ebb and flood tides (Fig. 1).

Our results are consistent with Forward et al. (2003), who identified a circatidal rhythm in abdominal pumping, with maximum pumping occurring during the time of flood tide. Our data support the hypothesis that the abdominal pumping rhythm results from increased female sensitivity to larval release pheromones during the time flood tide. Female blue crabs also have a circatidal activity rhythm (Forward et al., 2003, 2005) and are more active during ebb tides. Increased activity might increase flow around the egg mass and dilute pheromones to levels below those necessary to induce abdominal pumping. During flood tides, when crabs are less active, flow around the egg mass would be decreased and higher concentrations of the pheromone would be present, possibly causing increased abdominal pumping. However, our experiments with direct delivery into the egg mass suggest that a rhythmic change in female sensitivity is a more likely mechanism.

Trypsin (Fig. 3) and bradykinin (Fig. 4) also stimulated abdominal pumping compared to control levels and the highest percentage response to each test chemical was similar to the highest response to egg extract. These data support the hypothesis that blue crab larval release pheromones are similar to those of other crustaceans (Forward et al., 1987; Rittschof et al., 1989; Rittschof and Cohen, 2004; Ziegler, 2007) and include peptides generated from the eggs with one or more neutral amino acids preceding arginine or lysine at the carboxyl terminus.

Proteolytic enzymes released around the time of hatching have been identified in several crab species. DeVries and Forward (1991) demonstrated that proteolytic enzymes are released from the eggs of *N. sayi* near the time of larval release. These enzymes, along with rapid osmotic swelling of the eggs, cause the egg membranes to rupture. Proteolytic enzymes including trypsin-like serine proteases are also released from the eggs of *Sesarma hematocheir* around the time of hatching (Saigusa, 1994, 1996; Saigusa and Iwasaki, 1999; Gusev et al., 2004). Partial hydrolysis of the egg membrane proteins by these enzymes would produce peptides with the neutral-basic amino acid sequence that induces abdominal pumping.

Delivery of 1 mM HCl resulted in increased abdominal pumping compared to filtered seawater, though the response was significantly lower than the response to trypsin (Fig. 3). Abdominal pumping serves multiple purposes for ovigerous blue crabs. During larval release, abdominal pumping aids in synchronizing egg hatching by mechanically rupturing the egg membranes (Davis, 1968, 1981; Forward and Lohmann, 1983; DeVries and Forward, 1991). Abdominal pumping occurs throughout embryo development (Tankersley et al., 2002) and serves to ventilate the egg mass, carrying away waste products and ensuring that embryos near the center of the egg mass receive adequate oxygen (Fernandez et al., 2002, 2006). In many species, abdominal pumping increases as the embryos develop (DeVries et al., 1991; Tankersley et al., 2002). Increased respiration results in decreased oxygen concentrations in the egg mass which stimulates abdominal pumping in crabs (Fernandez et al., 2002, 2006). Increased respiration also results in increased CO_2 production which, through the production of carbonic acid, would decrease pH in and around the egg mass. A pH decrease of the water in and around the egg mass may serve as a cue for abdominal pumping. Delivery of the HCl solution decreases the pH without altering the oxygen concentration. The increase in pumping we observed with 1 mM HCl suggests pumping can be in response to changes in pH. However, while delivery of HCl stimulated abdominal pumping, HCl was less potent than the combination of HCl and trypsin.

Although ovigerous blue crabs have a circatidal rhythm in abdominal pumping (Forward et al., 2003) and there were tidal differences in sensitivity to pumping pheromones, abdominal pumping actually takes places during both tidal phases. Additionally, crabs responded with increased abdominal pumping during both phases. Abdominal pumping serves multiple purposes for ovigerous crabs, including ventilation of the egg mass (Fernandez et al., 2002, 2006) and synchronizing hatching and larval release. It is thus advantageous for the female to respond, regardless of tidal phase, if the appropriate chemical cues are present, as failure to respond could result in anoxic conditions or the buildup of toxic waste products in the egg mass.

In the vertical swimming assay, egg extract stimulated swimming (Fig. 5), supporting the hypothesis that pheromones from the eggs stimulate swimming behavior associated with migration and larval release. Female swimming sensitivity to egg extract did not vary with tidal phase (Fig. 5). Delivery of trypsin or bradykinin resulted in slight increases in vertical swimming, though these increases were not significantly different from control levels. These results suggest that other pheromones in addition to the peptide pumping pheromones are necessary for a robust swimming response.

Although it is clear that pheromones from the egg mass stimulate swimming, egg extract is a very complex mixture and the nature of the additional molecules in the blend is unknown. Neutral-basic peptides alone did not stimulate increased vertical swimming as bradykinin and trypsin failed to induce a significant swimming response. We hypothesize that compounds in addition to neutral-basic peptides are necessary to induce vertical swimming. Crustaceans possess receptors for detecting numerous hydrolysis products in addition to peptides, including sulfated and acetylated amino disaccharides (Forward and Rittschof, 1999; Rittschof and Cohen, 2004). In addition to trypsin-like serine proteases, amylase and lysozyme are found in egg masses of ovigerous blue crabs (Hinshaw et al., Duke University, unpublished data). Molecules generated by enzymatic digestion of complex carbohydrates by these enzymes may play an important role in stimulating swimming. Because osmotic pressure in the eggs increases as embryos develop (Davis, 1965; DeVries and Forward, 1991) and dye studies indicate molecules as small as 400 Da cannot enter eggs (Rittschof et al., unpublished data), it is unlikely that molecules other than ammonium and CO_2 are released from the eggs. Thus, molecules stimulating swimming and abdominal pumping prior to larval release must be produced through

enzymatic digestion of compounds on the surface of the eggs or the glue that attaches the eggs to the pleopods.

Ovigerous blue crabs possess a circatidal rhythm in vertical swimming, with peak swimming occurring during ebb tides (Forward et al., 2003, 2005; Darnell et al., 2010). This behavior is the basis for ebb-tide transport and the seaward spawning migration. Our results indicate that compounds from the eggs stimulate swimming behavior. Swimming in response to egg extract provides information about the circatidal rhythm in female swimming behavior. As responses did not differ between the times of ebb and flood tides (Fig. 5), female sensitivity to molecules that stimulate swimming is constant throughout the tidal cycle. Thus, the circatidal swimming rhythm arises through a mechanism other than female sensitivity to stimulation. Candidates might be the rhythmic release of enzymes from the female or rhythmic changes in metabolic or other activity of the embryos.

We hypothesize that a blend of molecules produced through enzymatic digestion of the egg membranes and/or the glue that attaches the eggs to the pleopods stimulates vertical swimming in ovigerous *C. sapidus*. Upon production of the first clutch of eggs, enzymatic digestion of the egg membranes and glue begins to produce these compounds and stimulate vertical swimming behavior. Following release of a clutch of eggs, some of the glue that had been used to attach the eggs as well as remnants of the egg membranes remains on the pleopods for several days to several weeks. Continued hydrolysis of the residual glue and egg remnants continues to release the compounds, though signal production decreases over time. Thus, the swimming rhythm persists for many crabs between clutches of eggs, though not as strongly as while carrying a clutch.

Acknowledgements

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