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4 1 Enzymes manage biofilms on crab surfaces aiding in feeding and antifouling

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15 7 **Keywords**

16 8 Biofilm; microfouling; decapod crustaceans; enzymes; chemical ecology; fouling
17 9 management
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22 12 **Introduction**

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25 13 In aqueous environments, biofilms are ubiquitous on all inert surfaces.

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27 14 Microfouling biofilms are composed of a consortia of microorganisms embedded in

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29 15 an exopolymer matrix (Reviews Costerton et al., 1987, 1995; Whitfield, 1988;

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32 16 Wingender et al., 1999; Review Sutherland, 2001; Review Flemming & Wingender,

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35 17 2010). Understanding mechanisms used by marine organisms to remove biofilms

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37 18 from surfaces and prevent microfouling is of great interest to understand animal-

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40 19 microbe interactions at the interfaces of which they meet (Wahl, 1989, 2008; Dale &

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42 20 Moran, 2006; Wahl et al., 2012; Mcfall-Ngai et al., 2013), and for bioinspired

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45 21 environmental and medical applications (Essock-Burns & Rittschof, In Press).

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50 23 The present study examines macrofouler use of biochemical mechanisms to manage

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52 24 biofilms in two contexts, direct removal from another surface for feeding and

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55 25 preventative measures on the macroorganism itself to protect egg masses. To do

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57 26 this, fiddler crabs *Uca pugilator* were used to assess biochemical measures

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60 27 employed to detect and remove biofilms from sand particles and blue crabs

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4 28 *Callinectes sapidus* were used to assess biochemical measures to manage biofilms on
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6 29 brooded egg masses.
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11 31 Sand fiddler crabs, *Uca pugilator*, are semi-terrestrial deposit feeders, removing
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13 32 organic content from sand particles. *U. pugilator* rely on chemoreceptors on their
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15 33 chelae, or claws, and dactyls, or legs to detect phagostimulants, or food cues, in their
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17 34 habitat (Robertson et al., 1981; Rittschof & Buswell, 1989; Reinsel & Rittschof,
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19 35 1995). Phagostimulants include diatoms, benthic algae, blue-green algae, ciliates
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21 36 and bacteria (Robertson et al., 1981) and carbohydrate components and amino
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23 37 acids of diatoms (Robertson et al., 1981; Rittschof & Buswell, 1989; Weissburg &
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25 38 Zimmer-Faust, 1991), stimulating a feeding response in *Uca* species. Robertson et al.,
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27 39 1981 suggested that continued feeding after initial chemoreception may require
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29 40 additional input from the buccal region.
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33 42 Once stimulated to feed, fiddler crabs are proficient at separating organic content
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35 43 from sand (Miller, 1961; Robertson et al., 1980; Reinsel & Rittschof, 1995). *U.*
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37 44 *pugilator* feed by using their minor cheale, to deliver pinches of damp sand to their
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39 45 buccal cavity, removing biofilms containing microorganisms and proteinaceous
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41 46 matter and leaving behind balls of sand (Miller, 1961; Reinsel & Rittschof, 1995).
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43 47 *Uca* feeding efficiency is attributed to their specialized appendages used for
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45 48 mechanical removal of organic material (Miller, 1961; Robertson & Newell, 1982;
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47 49 Lim, 2004) and biochemical contributions to feeding have been unexplored. The
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49 50 present study investigates the enzymology of buccal and dactyl secretions through
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4 51 saliva samples and leg washings to address the role of exoenzymes in *U. pugilator*
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6 52 food detection and processing.
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11 54 In addition to the role of biochemical mechanisms to remove biofilms from separate
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13 55 surfaces, the role of hydrolytic enzymes in prevention of microfouling on a
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15 56 macroorganism surface is of interest. How do crustaceans keep themselves clean
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17 57 where they cannot scratch? To investigate this, ovigerous female blue crabs were
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19 58 used with attention to their externally brooded egg masses. As in most crustaceans,
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21 59 blue crabs (*Callinectes sapidus*) molt frequently, groom appendages and bury, all
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23 60 mechanisms to control microfouling. The egg mass of an ovigerous female is a large
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25 61 area too sensitive to use mechanical means of biofilm removal. Each egg mass
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27 62 averages 2 million to over 6 million eggs of approximately 250-275 µm diameter
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29 63 (Dickinson et al., 2006; Darnell et al., 2009).
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31 64
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33 65 Brooded crustacean eggs are glued in strings to abdominal pleopod filaments
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35 66 (Rogers-Talbert, 1948; Saigusa, 1994). Embryo development is generally
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37 67 synchronous and can be tracked by observing color changes and embryo details in
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39 68 the egg clutch, starting with yellow eggs, transitioning to orange and brown, and
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41 69 finally black, prior to larval release (DeVries et al., 1983). For embryo survival, eggs
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43 70 must be minimally fouled to facilitate oxygen delivery to embryos and waste
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45 71 removal (Crisp, 1959; Baeza & Fernández, 2002; Fernandez & Brante, 2003; Reinsel
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47 72 et al., 2014). If crustacean embryos become detached from the female, they become
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49 73 fouled with biofilm and die (Fisher, 1976, 1983; Fisher et al., 1976; Forward &
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4 74 Lohmann, 1983; Saigusa, 1992, 1993). Due to the detrimental outcomes of such
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7 75 microfouling, other crustaceans have even developed symbiotic relationships with
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9 76 bacteria to protect egg masses from biofilms leading to fungal infections for shrimp
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11 77 (Gil-Turnes et al., 1989) and lobster (Gil-Turnes & Fenical, 1992). The present study
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13
14 78 examined the enzymology of blue crab egg masses to gain insight into biochemical
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17 79 mechanisms to prevent biofilm formation on eggs.
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21 81 Enzymology on externally brooded egg masses is also relevant to understanding the
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24 82 role of chemical cues during development that elicit various behaviors of the female.
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27 83 Decapod crustaceans are well studied with respect to chemical and physical cues
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29 84 and synchrony of hatching (Forward, 1987; Review Rittschof, 1993; Tankersley et
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31 85 al., 2002; Review Rittschof & Cohen, 2004; Reinsel et al., 2014). Specific chemical
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34 86 cues trigger larval release behavior (DeVries et al., 1989), in which females
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37 87 physically assist in egg hatching and propel the embryos into the water column
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39 88 (Forward & Lohmann, 1983; Rittschof et al., 1985; Forward, 1987; Saigusa, 1992).
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42 89 Due to the types of peptide pheromones detected from ovigerous females, trypsin-
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44 90 like proteases are implicated in pheromone production and egg hatching (Rittschof
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47 91 et al., 1985; Forward, 1987; DeVries & Forward, 1991; Saigusa, 1992; Darnell &
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49 92 Rittschof, 2010).
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54 94 Fouling management is defined by any biochemical or physical measures that are
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57 95 preventative or responses to microfouling (Essock-Burns and Rittschof, In Press).
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59 96 The present study aims to understand biochemical measures, specifically hydrolytic
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enzymes, to prevent and remove products of microfouling. The context for studying preventative measures of microfouling was blue crab egg masses and the context for response to microfouling was fiddler crab feeding. Fiddler crab feeding exemplifies responses to microfouling in its habitat, rather than on the macroorganism itself. The hydrolytic enzymes examined were amylase, trypsin and lysozyme as they hydrolyze chemical bonds in carbohydrates, proteins and bacterial cell walls, respectively. These three enzymes were hypothesized to have roles in degrading biofilm components including the extracellular matrix made up of glycoproteins and proteoglycans, as well as bacterial cell walls and cells embedded in the matrix. The data support the roles of enzymes in both contexts through the removal of biofilms and provide insight into biochemical measures to manage fouling.

Methods

1. Crab Collection

Sand fiddler crabs (*Uca pugilator*) were collected by hand at night in the Rachel Carson National Estuarine Research Reserve near the Duke University Marine Laboratory in Beaufort, North Carolina (34°42.65' N, 76°40.40'W). Large crabs were used to facilitate the collection of test fluids. Fiddler crabs used in feeding experiments were maintained in groups of 100-200 in a large water table (1.5 m diameter) maintained at ambient temperature with constant dripping seawater and sand for burrowing. Crabs were exposed to the natural diurnal light cycle and fed pulverized dry "Ocean Flavored" Friskies cat food. Ambient temperature was 25°C and animals used for collection of fluids for studies were kept individually in 40 mL

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4 124 glass finger bowls with approximately 0.5 cm seawater and fasted overnight prior to
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6 125 feeding experiments. Sexes were tested separately and each test group ranged from
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9 126 10-30 crabs.

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14 128 Blue crabs (*Callinectes sapidus*) were collected in the Rachel Carson Research
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16 129 Reserve on foot using dipnets at night. Ovigerous females with egg clutches at each
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18 130 stage of development as indicated by clutch color (Rogers-Talbert, 1948) were used.
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20 131 Ovigerous females in each stage were as follows: 5 yellow, 11 orange, 8 brown, and
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22 132 12 black. Crabs were kept in tanks with flowing seawater at 33 to 36 salinity and
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24 133 ambient temperature. For experiments, crab fluids were sampled 1-3 hours after
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26 134 placement in a damp bucket at 25°C.

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34 137 **2. Fluid Sample Collection**

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37 138 Fiddler crab saliva and leg washings were collected from the crabs using a 200 µL
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39 139 mechanical micropipette. Saliva was extracted from the buccal cavity by gentle
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41 140 pipetting of the fluid at the entrance to the mouth cavity. Leg washings were
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43 141 obtained by repeatedly pipetting 50-80 µL aliquots of 100,000 kDa-filtered aged
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45 142 seawater over all 10 legs in a 1.5 mL Eppendorf tube. Samples were kept on ice and
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47 143 used in assays immediately following collection.

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4 145 Blue crab egg masses were sampled by pipetting 100 μ L of fluid from each of the 36
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6 146 egg clutches. Control samples were taken from the tanks in which the crabs were
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9 147 kept. The color of the egg mass was recorded for each crab at the time of sampling.
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13 14 149 **3. Enzyme Activity Measures** 15

16 17 150 18 19 151 **3a. Overview** 20

21 152 Enzyme activities were measured using enzyme specific substrates for all sample
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23 153 types including: *U. pugilator* saliva and leg washings, and *C. sapidus* egg mass fluid.
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25 154 The substrates become absorptive or fluorescent when cleaved by an amylase,
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27 155 trypsin or lysozyme. Enzyme activity was quantified as a function of change in
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29 156 absorbance or fluorescence over time, using a spectrophotometer Spectramax M2
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31 157 Microplate Reader (Molecular Devices). The substrates and enzymes for standards
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33 158 are described below in detail. Using the unit definition for each enzyme the optical
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35 159 density readings from the spectrophotometer were converted into moles of product
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37 160 produced per milliliter fluid per unit time.
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46 162 Filtered seawater was used as the solvent and buffer to maximize the similarities
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48 163 between the lab standards and the natural crab environment. The maximum
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50 164 velocity ($V_{\max}$) was determined for the assays and was used to generate a ratio of
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52 165 units of enzyme activity (units/mL) to $V_{\max}$ (mOD/min or RFU/min). Standard
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54 166 curves were produced by combining 150 μ L substrate solution and 100 μ L of varying
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58 167 combinations of seawater and enzyme solution. There were five replicates for each
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4 168 enzyme used, giving 25 points for each standard curve. Negative controls were
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6 169 filtered seawater and positive controls included addition of known units of pure
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9 170 enzyme. To assay the samples for each sample type, each 100 μ L crab fluid sample
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11 171 was combined with 150 μ L of substrate solution in a 250 μ L microwell of a clear
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14 172 bottom 96 well microtiter plate. For egg mass washings, controls were seawater
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16 173 from the tank where crabs were kept and those values were subtracted from egg
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19 174 mass activity measures. All samples and reagents were kept on ice prior to the start
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22 175 of spectrophotometer readings.
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26 177 **3b. α -Amylase**

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29 178 The enzyme standard for α -Amylase was from *Bacillus licheniformis*, Type XII-A
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31 179 (Sigma, A-3403, LOT 67H1563, 30 mg protein/mL, 870 units/mg protein). The
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34 180 enzyme was first diluted to 26 units/mL with deionized water. Amylase activity
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36 181 was measured using an α -amylase kit (Stanbio, REF 0970-101, LOT 083291CE). The
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39 182 substrate reagent was reconstituted with 10 mL of DI water. When a protein with α -
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42 183 amylase-like activity is present, para-nitrophenol-D-maltaheptaoside (PNPG7) is
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44 184 converted to para-nitrophenol, a yellow product. Absorbance was measured at 405
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47 185 nm at 37°C over one hour, at one-minute intervals. One unit of amylase cleaves 1.0
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49 186 mg of maltose every 3 min at 20°C. Units per liter were calculated as the change in
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52 187 absorbance times total reaction volume times 1000, divided by the extinction
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54 188 coefficient times the light path times the sample volume, following the kit
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57 189 instructions. The units per liter were divided by 3.66 according to the Q10 factor for
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59 190 a 17°C change as our measurements were done at 37°C not 20°C. Then they were
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multiplied by 0.33 mg to get mg product per liter per minute and divided by the formula weight of the product and converted to get μmol product per minute (Table 1). The standard curve produced a slope of 0.049, a y-intercept of -0.790, and a correlation coefficient of 0.980

3c. Trypsin

The enzyme standard for trypsin was from bovine pancreas, Type 1 (Sigma, Pcode 1000575748, LOT 049K7002, 13964 units/mg). Trypsin stock was 3212 units/mL in 1.0 mM HCl. Trypsin activity was measured using the substrate *N* α -Benzoyl-L-arginine 4-nitroanilide hydrochloride BAPNA (Sigma) (Erlanger et al., 1961). The concentration of the stock solution was 1.48 mM in deionized water, with 100 μL DMSO per 10 mL water to dissolve the solid BAPNA. Final concentration of substrate was 0.89 mM in each 250 μL well. Absorbance was then measured at 405 nm for one hour at one-minute intervals at 37°C. Using BAPNA as a substrate; one trypsin activity unit is defined as the amount of trypsin that produces 1 μmol of p-nitroanilide, in one minute.

3d. Lysozyme

The enzyme standard for lysozyme was from chicken egg white (Sigma, 088K1358, 46900 units/mg solid, 49900 units/mg protein). The stock solution was initially diluted to 1600 units/mL in deionized water. The fluorogenic substrate 4-methylumbelliferyl β -D-N,N',N''-triacetylchitotrioside (MUF 3-NAG) (Sigma, 029K4024, CAS 53643-13-3, 785.8 g/mol) was used to assess lysozyme activity

(Jacks & Kircher, 1967). A 1 mL 1:1 deionized water and pyridine solution dissolved 1 mg of MUF 3-NAG solid before the concentration of the stock solution was diluted to 26 μ M. Final concentration of substrate was 15.6 μ M in each 250 μ L well. Activity was detected at an excitation wavelength of 360 nm and an emission wavelength of 450 nm, and each plate was run at 37°C for two hours at one-minute intervals (Table 1).

4. Fiddler Crab Feeding Experiments

Experiments were conducted on *Uca pugilator* to investigate the role of exoenzymes in feeding. Feeding experiments were performed on eyestalk ablated female crabs as described previously (Rittschof & Buswell, 1989). To test the feeding efficiency on diatoms from sediments, autofluorescent diatom cells were counted using an epifluorescent microscope. In the test, 10 ablated female crabs were placed on field sediments. A chemical spatula was used to collect undisturbed sediment and to collect sediments after the crabs had processed them and were returning feeding pellets to the surface of the sediment. In the later instance, the spatula was placed under the pellet as it was being deposited. To visualize the autofluorescence, chlorophyll in diatoms was stimulated to fluoresce by excitation with ultraviolet light (480 nm). The number of diatoms per microscope ocular field was determined for 10 unprocessed and 10 processed sediment samples.

To test functionality of amylase in the *U. pugilator* feeding response, thirty eyestalk-ablated crabs were tested in the presence of dextrose or starch, with or without

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4 237 amylase inhibitor. Treatments included seawater as a negative control, 0.125 g/mL
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6 238 dextrose in seawater as a test monomer, and 0.045 g/mL corn starch in seawater as
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9 239 a test polysaccharide. Crabs were tested in groups of two in a solution for thirty
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11 240 seconds, then rinsed for 30 seconds in seawater and tested in the next solution.
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14 241 Crabs were then incubated in a solution of 300 units of human amylase inhibitor
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16 242 (Sigma, chemical company, St Louis Mo, #A1520) for 5 minutes and the tested again
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19 243 in each solution with rinses in amylase inhibitor in seawater between tests.
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24 246 ***5. Detached Blue Crab Egg Experiments***

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26 248 To determine the contribution of exoenzymes to antifouling on *C. sapidus* egg

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29 249 masses, eggs were detached and developed separately from the female. Clusters of

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32 250 10 to 20 eggs from late stage (black) egg masses were removed from a female and

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34 251 put immediately in either 5 mL of aged 100 kDa filtered seawater or put into 5 mL

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36 252 aged filtered seawater containing 0.5 U/mL of amylase, 4 U/mL of lysozyme and 2

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39 253 U/mL of trypsin. Eggs were incubated for 1.5 hours. After 1.5 hours, individual eggs

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41 254 were discarded and eggs in cluster were transferred to different treatments. From

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44 255 the eggs incubated in control seawater 5 clusters of 10 to 20 eggs were transferred

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46 256 into 5 mL fresh aged 100kDa seawater in 5 separate Falcon 1006 sterile lidded

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49 257 polystyrene petri dishes. From the enzyme treated eggs, 20 egg clusters were

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51 258 transferred, 5 clusters into each of 4 separate enzyme treatments. The treatments

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54 259 are given as a ratio of amylase, trypsin and lysozyme units, including: 2A:2T:4L,

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56 260 20A:10T:4L, 40A:20T:8L, and 80A:40T:24L. Remaining eggs in clusters from the

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59 261 original treatments were incubated until eggs hatched or died. The 25 new groups

of eggs were incubated without shaking at 25°C and the seawater plus enzyme treatments and seawater controls were changed every two days until Day 6 when larvae and dead embryos in eggs were counted.

6. Statistical Analyses

Enzyme activity levels are presented as means with 95% confidence intervals (Fig. 1). Mean values were from five replicates with 30 crabs per test unless otherwise indicated. A Z-test of proportions was used for the *U. pugilator* feeding response with amylase inhibitor experiment (Fig. 3) and the effect of enzyme treatments on detached egg clusters on mortality and hatching response (Fig 5). Egg mass enzyme activity data were analyzed by one-way ANOVA (Fig. 4).

Results

Enzyme Activities – feeding

All three hydrolytic enzymes, amylase, trypsin, and lysozyme, had activity detected on leg washings and saliva for both males and females. Amylase activity was the lowest of all three enzymes and not different between saliva or leg washings or between males and females (Fig. 1). Both males and females had similarly high trypsin activity in both saliva samples and leg washings. Trypsin activity in female saliva (95% CI [143.52, 1958]) and leg samples (95% CI [347.42, 1084]) was significantly higher than all amylase activity (saliva 95% CI [2.37, 4.30] and legs 95% CI [2.95, 5.11]), and significantly higher than lysozyme activity in female saliva

(95% CI [1.54, 5.24]) (Fig. 1). Although trypsin activity was an order of magnitude higher in females than males, the difference was not significant. Lysozyme activity in male leg washings (95% CI [348.03, 964.34]) was nearly two orders of magnitude higher than female lysozyme activity in saliva (95% CI [1.54, 5.24]) and all amylase activity for males and females including: male saliva (95% CI [5.38, 7.8]), male leg washings (95% CI [5.46, 10.90]), female saliva (95% CI [2.37, 4.30]), and female leg washings (95% CI [2.95, 5.11]) (Fig. 1).

Fiddler Crab Feeding Experiments

Diatom extraction efficiency was determined by counting autofluorescent cells in epifluorescent images of sediment grains before and after *Uca* feeding. Diatom removal was between 60-90% and achieved in approximately 9 seconds (Fig. 2).

Feeding responses to dextrose and starch were probed with and without human amylase inhibitor (Fig 3). Dextrose, a monosaccharide, and starch, a polysaccharide, both elicited a feeding response of 100%, which was significantly different from the response to the seawater control ($z=8.211$, $p<0.0001$) (Fig. 3). Exposure to the amylase inhibitor prior to the test significantly reduced feeding responses from 100% in the absence of the inhibitor to 80% for dextrose and 30% for starch (Dextrose $z=2.738$, $p<0.05$; Starch $z=-2.190$, $p<0.05$) (Fig. 3). Treatment with the inhibitor significantly reduced feeding responses to starch more than it reduced feeding response to dextrose ($z=-6.846$, $p<0.01$).

Enzyme Activity Measures – fouling management

Enzyme activities in *C. sapidus* egg masses were measured at each of four stages of development. All enzyme measures were significantly different from the control of seawater from their tank: lysozyme (ANOVA; $F(4,39)=7.53$, $p=0.0001$), amylase (ANOVA; $F(4,36)=4.57$, $p=0.004$) and trypsin (ANOVA; $F(4,38)=5.58$, $p=0.001$). The enzyme activity in the seawater controls was subtracted from that of the egg masses. As embryos in blue crab egg clutches develop, lysozyme activity remained low, trypsin activity remained elevated and amylase activity was highest throughout development and increased in late stage eggs (Fig. 4). Lysozyme levels were not significantly different between groups (ANOVA; $F(3,32)=1.68$, $p=0.19$) (Fig. 4). The amylase activity detected in black eggs was an order of magnitude higher than earlier stage eggs and significantly different from all other clutch colors (ANOVA; $F(3,29)=4.15$, $p=0.015$) (Fig. 4). Trypsin activity in black clutches was 1.5 fold higher than other clutch colors and groups were significantly different (ANOVA; $F(3,31)=4.43$, $p=0.01$) (Fig. 4).

Blue Crab Egg Fouling Experiments

To test effectiveness of exoenzymes in an antifouling context, egg masses were incubated separately from females in several seawater conditions. Conditions included seawater alone and various ratios of amylase, trypsin and lysozyme, modeled from enzyme mixtures that were found in egg mass fluid from intact clutches on females (Fig. 5). The added enzymes greatly improved egg hatching and embryo survival. Aged filtered seawater with no enzyme added resulted in 90-100%

mortality rates and 0-10% hatching. All enzyme treatments with amylase, trypsin, and lysozyme improved mortality from the seawater controls ($z=8.366$, $p<0.0001$)(Fig. 5). The most successful ratio of enzymes was 20U Amylase: 4U Lysozyme: 10U Trypsin, per milliliter seawater gave 80% hatching and >90% survival ($z=6.283$, $p<0.0001$)(Fig. 5).

Discussion

The present study investigates biochemical management of biofilms by crabs. Three hydrolytic enzymes, amylase, trypsin, and lysozyme, were present on areas of interest and had clear roles in removal of biofilms and prevention of microfouling in ecologically relevant contexts. This group of enzymes collectively targets exopolysaccharides including glycoproteins and proteoglycans relevant to microbial glues within biofilms, as well as proteins and bacterial cell walls. The two contexts where enzyme use was ecologically relevant was for food detection and food extraction in fiddler crabs, *Uca pugilator* and prevention of microfouling on sensitive parts of a macroorganism, externally brooded *Callinectes sapidus* egg masses, where biofilms pose threats to embryo survival.

Enzymes in Feeding

Amylase, trypsin and lysozyme were present on fiddler crab legs, where reception of phagostimulants is critical for food detection, and in saliva where separation of organic content from sand particles occurs. Hydrolytic enzymes on legs may prevent microfouling on the sensory appendages themselves, maintaining chemoreception

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4 362 efficiency. Additionally, the enzymes generate phagostimulants by cleaving
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6 363 polysaccharide, protein and peptidoglycan polymers in the sediment.
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11 365 While the detected levels of amylase were lower than trypsin and lysozyme, amylase
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14 366 is clearly important for the *U. pugilator* feeding response. Feeding response to
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16 367 starch was significantly decreased in the presence of human amylase inhibitor. As
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19 368 expected, the observed decrease in feeding behavior was greater when starch was
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22 369 the stimulant than when dextrose was the stimulant. While less dramatic than the
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24 370 reduction to feeding on starch, amylase inhibitor reduced feeding to dextrose as
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26 371 well. One explanation is that the amylase inhibitor may interact directly with
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29 372 chemoreceptors in a way that reduces the feeding response. Other enzyme
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32 373 inhibitors are known to interact with crustacean chemoreceptors (Review Rittschof
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34 374 and Cohen 2004). Inhibition of the amylase response with human amylase inhibitor
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36 375 supports the hypothesis that the amylase enzyme observed on crab legs is similar
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39 376 human amylase.
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43 378 The hydrolytic enzymes measured in saliva seem to play a direct role in feeding.
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45 379 Diatom removal efficiency was particularly striking. It is unlikely that mechanical
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48 380 mechanisms alone could achieve 60-90% diatom removal in less than 9 seconds.
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51 381 Given the behavior of fiddler crabs during feeding, the enzymes present in saliva
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54 382 would be useful to digest biofilm components including glues during the incubation
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56 383 of sand in the buccal cavity. Future work exploring the direct contribution of
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59 384 salivary enzymes to feeding efficiency may be determined using enzyme inhibitors
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4 385 similar to the feeding response experiment conducted with human amylase
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6 386 inhibitor.
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11 388 Amylase was of particular interest for antifouling applications because it cleaves
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13 389 glycosidic linkages in polysaccharides, a major component of biofilm glues
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15 390 (Sutherland, 1999, 2001; Flemming & Wingender, 2001, 2010). Polysaccharides, or
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17 391 carbohydrates are important phagostimulants for *U. pugilator* feeding (Robertson et
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19 392 al., 1981; Rittschof & Buswell, 1989; Weissburg & Zimmer-Faust, 1991) and are
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21 393 approximately as potent as total organic content (Reinsel & Rittschof, 1995).
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23 394 Carbohydrates are important components of the extracellular sheaths of diatoms
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25 395 and blue-green algae (Weissburg, 1993). Previous work in fiddler crab feeding
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27 396 shows that disaccharides and insoluble macromolecules stimulated feeding more
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29 397 than monosaccharides and amino acids at similar molar concentrations (Robertson
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31 398 et al., 1981). Amylase is a good candidate for the degradation of disaccharides and
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33 399 macromolecules, which would increase the concentrations of phagostimulants,
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35 400 explaining the increased feeding response.
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46 402 Sex differences were compared because dimorphic feeding behavior has been
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48 403 observed previously (Weissburg, 1992, 1993; Weissburg & Derby, 1995). Weissburg
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50 404 and Derby (1995) reported that female chemoreceptor pathways are more sensitive
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52 405 than males and that increased sensitivity allows females to detect phagostimulants
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54 406 that do not stimulate male feeding. Generally, there were few differences in enzyme
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56 407 levels between male and female fiddler crabs. Measured female trypsin levels for
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4 408 both saliva and legs were higher than males by about an order of magnitude, but
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7 409 due to high levels of variation the difference was not significant. Male lysozyme
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9 410 levels in leg washings were two orders of magnitude higher than lysozyme in female
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11 411 saliva. The different patterns of exoenzymes may indicate differences in feeding
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14 412 outcomes. Females may have a higher protein demand, as reflected by the pattern of
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16 413 higher trypsin activity and males may feed more on microbial content as reflected
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19 414 by the high lysozyme activity on legs. The patterns of sexual differences identified
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21 415 here expand this previous work on sexual dimorphism in feeding behaviors.
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26 417 ***Enzymes manage fouling in blue crab egg masses.***
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29 418 All three of the hydrolytic enzymes amylase, trypsin and lysozyme, were prevalent
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31 419 in egg masses throughout embryo development and shown to directly contribute to
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33 420 embryo survival and hatching success. Trypsin and amylase were particularly high
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35 421 in egg mass fluid and increased significantly just prior to hatching. Surprisingly,
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37 422 amylase was even higher than trypsin activity throughout development and over an
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39 423 order of magnitude higher in black eggs as compared to trypsin. Trypsin was
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41 424 expected to be most prevalent because of the clear role of trypsin-generated peptide
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43 425 pheromones in coordinating multiple types of crustacean behavior (Rittschof &
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45 426 Cohen, 2004). It was expected that lysozyme levels would be higher than the
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47 427 observed levels because of how clean egg masses remained on the female, however
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49 428 the observed levels do not necessarily correlate to bacteriocidal effectiveness.
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4 430 Amylase activity in egg masses was higher than the activity of the other enzymes
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7 431 during embryo development and peaked dramatically prior to egg hatching. As
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9 432 amylase targets exopolysaccharides, which are a large component of microbial glues,
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11 433 it appears that this is an effective mechanism to manage microfouling on eggs.
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14 434 Additionally, the saccharides generated by amylase may act as another signal or
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16 435 ventilation pheromone, similar to signals reported from fish mucus which alter
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19 436 crustacean larval behavior (McKelvey & Forward, 1995; Forward & Rittschof, 1999,
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21 437 2000; Rittschof & Cohen, 2004).
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26 439 As expected, trypsin levels were high throughout development and increased in
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29 440 black egg masses, prior to hatching. Trypsin-like serine proteases are
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31 441 endopeptidases known to generate important peptide signaling molecules
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33 442 (Reviews: Rittschof, 1993b; Rittschof & Cohen, 2004; Reinsel et al., 2014), including
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35 443 larval release pheromones (Rittschof et al., 1985; Rittschof, 1990; DeVries et al.,
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37 444 1991). The constant production of trypsin by the female with eggs may generate
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39 445 signaling molecules that cue egg mass ventilation (Saigusa, 1996; Reinsel et al.,
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41 446 2014). This explains why all stages of brooded eggs generate larval release
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43 447 pheromones (Forward & Lohmann, 1983; Rittschof et al., 1985; Forward, 1987).
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51 449 The lysozyme activity observed on egg masses is potentially a direct mechanism for
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53 450 fouling management by crabs, as lysozyme functions by lysing bacterial cell walls.
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55 451 Previous work has shown lysozyme-like activity in hemolymph extracts and
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58 452 exoskeleton fluid of four marine decapod crustaceans (Haug et al., 2002). Lysozyme
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4 453 levels were constant across all egg mass stages, indicating a continuous demand for
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7 454 biochemical management of microfouling.
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11 456 As shown previously, eggs fouled and embryos died when separated from females
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14 457 without enzyme treatment, underscoring the importance of enzymes to manage
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16 458 microfouling (Forward & Lohmann, 1983; Saigusa, 1992). Treating removed eggs
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19 459 with amylase, trypsin and lysozyme allowed eggs to hatch and embryos to survive.
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21 460 While there is clear evidence of enzymes present in egg clutches, the source is not
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23 461 known. There are several potential sources of enzymes in egg clutches (Rittschof et
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25 462 al., 1990; DeVries & Forward, 1991; Saigusa, 1994, 1996). Three obvious sources
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28 463 are microorganisms, the brooding female and the embryos, and all may have roles in
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30 464 controlling female behavior. If the source of enzymes was the microorganisms on
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33 465 eggs or the embryos themselves, activity should have continued after removal from
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35 466 the female. This suggests the source of enzymes is the brooding female. If not
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38 467 originating directly from the female, the embryos need to be attached to the female
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40 468 for enzyme production. These experiments show that eggs hatch in the absence of
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43 469 shaking, which mimics female larval release behavior (Naylor et al., 1999). Because
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45 470 the timing of hatching was unaffected by separation from the female, this suggests
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48 471 the enzymes involved in egg hatching are from embryos and the enzymes to prevent
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50 472 fouling are from the female.
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55 474 While the primary focus in this study of the role of exoenzymes on externally
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58 475 brooded eggs was prevention of microfouling, their activity may contribute to other
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important aspects female behavior and interactions with conspecifics. Enzymes have three roles in management of brooded eggs: 1) manage fouling on brooding eggs, 2) synchronize egg hatching and 3) generate species-specific body odors. Preventing microfouling on egg clutches is which is critical for adequate gas and waste transport (Crisp, 1959; Strathmann & Strathmann, 1995), thus preventing embryo mortality, which was very high in treatments without enzymes. Experimental antimicrobial treatment lowers embryo mortality in the Dungeness crab, *Metacarcinus magister* (Fisher, 1976; Fisher & Nelson, 1977), American Lobster, *Homarus americanus* (Fisher et al., 1976) and the mud crab, *Rhithropanopeus harrisi* (Forward & Lohmann, 1983), further showing that active removal and prevention of microfouling is needed for high embryo survival.

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488 **Body odor chemical cues**

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Body odors serve as settlement pheromones (Review Rittschof, 1993; Clare & Matsumura, 2000) prey cues (Rittschof et al., 1983, 1984; Rittschof, 1985, 1993; Review Rittschof & Cohen, 2004) and shell availability cues (Reviews: Rittschof, 1990, 1993; Rittschof & Cohen, 2004). The data presented here support the hypothesis that in addition to enzymes in hatching eggs from late stage embryos (DeVries & Forward, 1991) larval release pheromones are generated by enzymes from females and opens up questions of egg mass structure and additional cues to trigger larval release. The findings suggest that amylase, trypsin and lysozyme

contribute to fouling management and likely generate biochemical cues and
pheromones.

Externally secreted enzymes are a well-established and generalized phenomenon in
microorganisms (Reviews: Glenn, 1976; Miller & Bassler, 2001; Diggle et al., 2008)
and they are observed in secretions of macroorganisms in saliva, stomach, and eye,
including humans (Ridley, 1928; Blandamer & Beechey, 1966; Wiesner & Vilcinskis,
2010). The hydrolysis of structural polymers and glues are the origin of many
kairomones and body odors in marine communities (Review Rittschof & Cohen,
2004). Products of enzyme hydrolysis conveying habitat information are critical for
larval behavior (Rittschof, 1993; Forward et al., 1996), including settlement cues for
a variety of marine larvae (Burke, 1986) including sand dollars (Burke, 1984),
abalone (Morse & Morse, 1984), barnacles (Rittschof, 1985), polychaete worms
(Jensen & Morse, 1990), oysters (Zimmer-Faust & Tamburri, 1994), mud crabs
Rhithropanopeus harrisi (Rittschof et al., 1990), and *Panopeus herbstii* (Rodriguez &
Epifanio, 2000). Exoenzymes, especially the hydrolytic ones shown here, may target
biofilms for a variety of purposes, enabling marine organisms to sense their
environment, feed, coordinate development, manage fouling, cue behaviors and
induce metamorphosis.

Conclusion

Enzymes are used by decapod crustaceans for feeding and fouling management.
Three central functions of enzymes are generation of phagostimulants, removal and

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4 521 degradation of food, and preventing microfouling on eggs. Fiddler crabs use
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6 522 enzymes on their legs for removing biofilms from substrates generating detectable
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9 523 phagostimulants, through chemoreception. Fiddler crabs use enzymes in their saliva
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12 524 to degrade polymers, freeing organics from biofilms on sediment and aiding food
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14 525 processing. Blue crabs use enzymes in their egg mass fluid to prevent fouling by
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16 526 microorganisms on the clutches. Enzymes in egg masses also generate pheromones
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19 527 that coordinate female behaviors that improve hatching success and survival. The
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22 528 absence of these enzymes has detrimental consequences for egg hatching and
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24 529 embryo survival, while replacement of enzymes rescues this consequence and leads
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26 530 to low mortality and high hatching success. This study exemplifies how marine
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29 531 macroorganisms use biochemical mechanisms to manage of biofilms on and around
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32 532 them. These are two examples of marine organisms using the same suite of enzymes
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34 533 to remove biofilms in different contexts. Biofilm removal achieves different goals in
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36 534 the two systems, facilitating both feeding and fouling management on eggs.
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40 41 536 Acknowledgements

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43
44 537 The authors would like to thank the many undergraduate researchers (especially
45
46 538 Julie Jarrard) who contributed to this work in D. Rittschof's laboratory over the
47
48
49 539 course of 25 years. We would like to acknowledge our funding sources from the
50
51
52 540 Office of Naval Research and grants that have supported the Rittschof laboratory,
53
54 541 most recently, N00014-11-1-0180 and N00014-12-1-0365.
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Figures Captions

Figure 1. Enzyme product (μmol) measured in washings of male (A) and female (B) *U. pugilator* body parts. Bars indicate the 95% confidence interval and letters indicate significant differences in enzyme activity. Substrates used are for detecting activity resembling amylase, trypsin and lysozyme. For A, male amylase measures (N=56), trypsin (N=10), and lysozyme (N=10). For B, female amylase measures (N=24), trypsin (N=10), and lysozyme (N=10).

Figure 2. The number of diatoms counted in one field of vision of an epifluorescent microscope at the 100x power before and after feeding by *Uca pugilator* (N=30). The cleaning process took place over an average interval of 8.6 seconds. Bars indicate the standard error of the mean.

Figure 3. *U. pugilator* feeding responses with and without human amylase inhibitor treatment (N=30). For feeding stimulants, seawater was used as a negative control, dextrose as a positive control, and starch as the test polysaccharide. Different letters represent significantly different feeding responses as determined by a Z-test of proportions.

Figure 4. The difference in lysozyme, trypsin, and amylase activity from the surrounding seawater as compared to samples from blue crab egg clutches. Egg clutch color approximates the stage of embryo development: yellow (newly deposited and mostly yolk), orange (less yolk and developing), brown (developing eyes), and black (from fully developed eyes) (Rogers-Talbert, 1948; Devries et al., 1983). Sample sizes ranged from 5 to 12 crabs per egg clutch color. Error bars indicate the standard error of the mean.

Figure 5. Mortality and hatching success of embryos in eggs detached from female blue crabs *C. sapidus* and incubated in seawater-enzyme treatments (5 replicates clusters or ~ 75 eggs per treatment). Ratios indicate units/mL of amylase, lysozyme and trypsin.

Tables

Enzyme Activity	Substrate	Units Definition	Wavelength	Other Notes
α-Amylase	PNPG7 Para-nitrophenol-D-maltaheptaoside	1 U enzyme cleaves 2.39 μmol product per min	405 nm	Units were divided by 3.66 as the Q10 factor for a 17°C difference in measurement temperature and definition
Trypsin	BAPNA Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride	1 U enzyme cleaves 1 μmol product per min	405 nm	NA
Lysozyme	MUF 3-NAG 4-methylumbelliferyl β-D-N,N',"-triacetylchitotrioside	1 U enzyme cleaves 1 mmol product per min	350 nm	Fluorescence measured, Emission 450 nm

Table 1. Enzyme-specific substrates and formulas to calculate activity from spectrophotometer readings.