Title: Chemically mediated neural and behavioral responses in early benthic juvenile Caribbean spiny lobsters, *Panulirus argus*

Authors: Yuriy V. Bobkov [1], J. Rudi Strickler [2,3], Charles D. Derby [4 *]

*Corresponding author: cderby@gsu.edu, https://orcid.org/0000-0002-5599-4976

Affiliations:

[1] Whitney Laboratory for Marine Bioscience, University of Florida, St Augustine, FL, USA

[2] University of Texas Marine Science Institute, Port Aransas, TX, USA

[3] Department of Biological Sciences, University of Wisconsin Milwaukee, Milwaukee, WI, USA

[4] Neuroscience Institute, Georgia State University, Atlanta, GA, USA

Short title: Chemically mediated responses in early benthic juvenile spiny lobsters

Running head: Chemoreception in spiny lobsters

Key words: behavior, calcium imaging, chemoreception, Crustacea, lobster, olfaction, olfactory receptor neurons

Abstract

Spiny lobsters use their chemical senses to acquire resources such as shelter and food, avoid predators, and interact with conspecifics. However, little is known about if and how these responses change over developmental stages. Here, we used early benthic juvenile stage Caribbean spiny lobsters, *Panulirus argus*, in calcium imaging studies to investigate physiological properties of olfactory receptor neurons in the olfactory organ, *i.e.*, the antennules, and in behavioral studies to characterize chemically triggered responses. The basic structural organization of the antennules is similar in early benthic juvenile, older juvenile, and adult lobsters. Our calcium imaging studies show that the olfactory receptor neurons of both life stages have generally similar patterns of spontaneous activity, tuning characteristics, sensitivity, and kinetic parameters of responses to chemicals. Our behavioral studies show that early benthic juvenile spiny lobsters have similar behaviors to adults in that they produce currents following stimulation with food-related chemicals, navigate through the chemical plumes to locate the source of food-related chemicals, show alarm responses to conspecific hemolymph, and groom their antennules following stimulation with L-glutamate. Our findings suggest that features of the olfactory organ and its sensory neurons and the behavioral patterns are generally similar across developmental stages, making early benthic juvenile lobsters a favorable model for studying chemosensory transduction, coding mechanisms, and chemical-driven behaviors. The smaller scale of early benthic juvenile lobsters allows the use of compact, miniature benchtop laboratory setups, offering significant flexibility for medium-throughput basic and applied studies.

Introduction

Spiny lobsters and other decapod crustaceans have well developed chemical senses that they use to detect and identify various chemicals in diverse contexts. Plumes of food related chemicals cause spiny lobsters to alert, search, and find the source [1–3]. Crustaceans stimulated with food related chemicals can also produce currents of different types and functions [4–9]. Besides food related compounds, adult spiny lobsters can detect alarm cues in the hemolymph of conspecifics [10–14]. Certain chemicals can also evoke antennular grooming behavior in spiny lobsters and other decapod crustaceans [15–19].

The chemical senses underlying these behaviors have been well studied in adult spiny lobsters. Crustaceans have several specialized chemical senses that can be categorized as either olfaction or distributed chemoreception, which differ in the location of sensors on the body, organization of sensilla, central projection patterns of the axons of their chemoreceptor neurons, organization of the central neuropil, and function in chemically mediated behaviors [14,20,21]. The spiny lobster's sensory organs, including the antennules, antennae, mouthparts, and legs, are shown in Fig 1.

Fig 1. Early benthic juvenile spiny lobsters. Left panel: lf, lateral flagella of the antennules; mf, medial flagella of the antennules; l1-l5, left walking legs; r1-r5, right walking legs; ctx, cephalothorax; sa, second antenna; abdomen; las, last abdominal segment. Note: pleopods are not visible, as they are on the ventral side of the animal. Right panel: Sensilla on the antennular lateral flagellum. *Top*: Distal region of the lf; *Bottom*: Medial region of the lf. a, aesthetasc sensilla; as, asymmetric sensilla, gs, guard setae, lf, lateral flagellum; ORNs, olfactory receptor neurons; ods, outer dendritic segment of the ORNs.

67 The olfactory pathway consists of aesthetasc sensilla on the lateral flagella of the antennules,
68 which are innervated by olfactory receptor neurons (ORNs) [20,22]. The olfactory pathway detects
69 both food-related chemicals and pheromones, the latter including hemolymph-based alarm cues,
70 aggregation cues, social cues, and sex pheromones. It mediates orientation toward the source of
71 the cues [23,24]. The second major chemical sense of crustaceans, distributed chemoreception, has
72 a diversity of chemical sensors with properties similar to each other but different from the olfactory
73 pathway in that their sensilla contain both chemosensory neurons and mechanosensory neurons
74 [20]. These bimodal sensilla are located across all body surfaces and appendages. Distributed
75 chemoreception has several subtypes, differing in location of sensilla, central connections, and
76 behaviors that they elicit. For example, distributed chemosensilla on the antennules are like
77 aesthetascs in being sufficient to drive orientation in a chemical plume to find the source of food
78 chemicals. Distributed chemosensory neurons in the asymmetric sensilla, located among other
79 bimodal sensilla on the antennules, uniquely mediate L-glutamate activated antennular grooming
80 behavior [18]. Distributed chemosensory neurons do not mediate responses to pheromones such
81 as alarm cues, aggregation and other social cues, or sex pheromones. Response properties of ORNs
82 and the chemosensory neurons of distributed chemoreception have been examined in adult spiny
83 lobsters, though detailed mechanisms of sensory transduction have been characterized only in
84 ORNs [25–31].

85 Little is known about the chemical senses and chemosensory behaviors in early stages of the
86 spiny lobster's life cycle, which is complex [32,33]. It begins with a long planktonic larval phase
87 consisting of phyllosoma larvae with ten stages typically lasting up to 5 to 9 months [34]. The form
88 of phyllosoma larvae is very different from that of early benthic juvenile and adult animals. The
89 phyllosoma larval stages are followed by metamorphosis into the free-swimming, non-feeding

puerulus stage, whose body form is much like early benthic juvenile and adult stages though without coloration. Pueruli move into nearshore settlement habitat that includes seagrass, hardbottom, or mangrove habitats [35,36]. Once settled, these early benthic juvenile lobsters begin to feed and are initially solitary. When they reach about 15 mm carapace length, they move from macroalgae to crevice shelters and live there communally for the remainder of their lives. In the Florida Keys, they become adults of legal size at > 76.2 mm carapace length (CL) at ca. 18 months of age since settlement. They have diurnal activity patterns in which they leave shelters at dusk to forage and return to shelters at dawn. Early benthic juvenile spiny lobsters have a body form and olfactory organ that is generally similar form to adults [37,38], though nothing is known about the physiology of their ORNs. Responses of *P. argus* pueruli to chemical cues from red algae associated with settlement and cues from conspecifics have been described [32,39,40]. Responses of early benthic juveniles of other species of spiny lobsters to chemical cues have been described for conspecific cues by *Jasus edwardsii* [41] and habitat cues by *Panulirus cygnus* [42].

Our study examines chemoreception in early benthic juvenile animals. We examined appetitive behaviors to food related cues, antennule grooming behavior to L-glutamate, and alarm responses to body fluids of conspecifics. We also characterized basic properties of the physiology of ORNs. Our results indicate that early benthic juvenile animals are very similar to adults in many of their chemosensory properties.

Materials and Methods

Experimental animals

Post-larvae of Caribbean spiny lobsters *Panulirus argus* were collected from floating artificial habitats in the Florida Keys by colleagues at the Florida Fish & Wildlife Conservation Commission

113 in Marathon, Florida Keys [33]. These animals were shipped to our laboratories in Florida and
114 Texas, where they were held in 10 L open glass aquaria (31.8 x 16.5 x 21 cm, TopFin®, Phoenix,
115 AZ, or similar) in groups of 7 to 10 animals. The animals were diet conditioned (food deprived for
116 at least 48 h) before experiments. Aquaria were supplied with flowing natural sea water under local
117 conditions (SW). The early benthic juvenile lobsters used in our study had carapace length (CL)
118 of 6.5 to 9.5 mm (7.8 ± 0.2, n = 20). Some animals were involved in multiple types of experiments.
119 This study was carried out in strict accordance with the recommendations in the Guide for the Care
120 and Use of Laboratory Animals of the National Institutes of Health and according to guidelines at
121 our universities. Animals were chilled on ice for anesthesia or euthanasia, and all efforts were made
122 to minimize suffering.

123 **Calcium Imaging**

124 Preparation. Olfactory receptor neurons (ORNs) were studied using an *in situ* preparation
125 developed earlier [27,29]. Briefly, a single annulus was excised from the lateral flagellum of the
126 antennule, and the cuticle on the side opposite the aesthetasc sensilla was removed to provide better
127 access to the somata of the approximately 200 ORNs associated with each of the approximately
128 10 aesthetasc sensilla per annulus. Following treatment with trypsin, papain, or collagenase (1
129 mg/ml, 20 min), the ensheathing tissue covering the clusters of ORNs was gently removed to allow
130 access to somata. All dissections were made in *Panulirus* saline (PS) containing (in mM): 486
131 NaCl, 5 KCl, 13.6 $CaCl_2$, 9.8 $MgCl_2$, and 10 HEPES, pH 7.9. The protease cocktail was prepared
132 using divalent cation free PS containing (in mM): 486 NaCl, 5 KCl, 0-10 Glucose, 0.1 EGTA, and
133 10 HEPES, pH 7.9.

134 Calcium imaging and data analysis. After enzymatic treatment and cleaning, annuli from the
135 antennular lateral flagellum were placed in an Eppendorf tube in PS containing the fluorescent

136 calcium indicator (Fluo-4AM, Invitrogen, Waltham, MA) at 5–10 μM prepared with 0.2–0.06%
137 Pluronic F-127 (Invitrogen, Waltham, MA). The tube was shaken for about 1 h on an orbital shaker
138 (70 rpm). The specimens were transferred into fresh PS, mounted on a glass-bottom 35 mm Petri
139 dishes, and placed on the stage of inverted microscope. Fluorescence imaging was performed on
140 an inverted microscope (Olympus IX-71) equipped with a cooled CCD camera (ORCA R2,
141 Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan) under the control of Imaging Workbench 6
142 software (INDEC Systems, Santa Clara, CA). FITC filter set (excitation at 510 nm, emission at
143 530 nm) was used for single-wavelength measurements. Recorded data were stored as image
144 stacks and were analyzed off-line using Imaging Workbench 6 or ImageJ 1.42
145 (http://rsbweb.nih.gov/ij/index.html). See Fig 2 for an example of fluorescent ORNs and S1 Video
146 and S2 Video for example of spontaneous activity and chemical evoked activity of ORNs.
147 Numerical analyses including event analysis and kinetic parameter estimates were performed using
148 either ClampFit 10 (Molecular Devices, San Jose, CA) or SigmaPlot 10 (Grafiti LLC, Palo Alto,
149 CA). All recordings were performed at room temperature.
150
151 **Fig 2.** Activity of olfactory receptor neurons (ORNs) from early benthic juvenile spiny lobsters.
152 (A) Spontaneous activity from a cluster of ORNs associated with a single aesthetasc sensillum (*left*
153 *panel*). The raw calcium signal time series (*right panel*) were background compensated and equally
154 spaced for clarity. Two examples of ORNs characterized by oscillatory activity are highlighted in
155 red. Calcium oscillations are likely associated with bursting ORNs. (B) Chemical evoked activity
156 from a cluster of ORNs associated with a single aesthetasc sensillum different than in A (*left panel*).
157 The *right panel* shows the increase in calcium concentration in ORNs in response to an 800-ms
158 pulse of a chemical stimulus, which was a fish food extract (TetraMarine 0.5 mg/ml). Timing of

159 stimulus presentation is marked by the gray line. Both resting calcium signal level and slow
160 intensity of fluorescence changes caused by bleaching were manually subtracted from each
161 individual trace.
162
163 Chemical stimuli and their delivery. Unless otherwise noted, the chemical stimulus was an aqueous
164 extract of TetraMarine (TET, Tetra Werke, Melle, Germany), a commercially available marine fish
165 food. Flakes of TET were powdered, dissolved in water (0.1 g/ml), filtered through a 0.2 µm
166 syringe filter, and diluted 1:200 in PS for experiments. The final maximum concentration was 0.5
167 mg/ml. The stream containing the chemical stimulus was switched with the flow of PS that
168 otherwise continuously superfused the sensilla (both 250 µl/min) using a multi-channel rapid
169 solution changer (RSC-160, BioLogic, France) under software control (Clampex 10, Molecular
170 Devices, San Jose, CA).
171
172 **Behavioral experiments**
173 For observing chemically elicited behaviors, we used two different experimental arenas. Arena
174 type 1 was a relatively large arena that allowed an animal to move over distances and thus allowed
175 us to visualize its chemically activated searching behavior. This arena was either a rectangular
176 glass aquarium with base dimensions of 31.8 cm x 16.5 cm (area of 525 cm^2) or a Carolina™
177 culture dish with diameter of 20 cm (area of 314 cm^2). Both were filled with SW to a depth of 4-5
178 cm. Animals were introduced into the arena and allowed to adapt for 5-20 min before being
179 exposed to food-related stimuli. In this type of arena, we created a chemical landscape using
180 fragments of shrimp tissue that had been incubated in sea water containing either fluorescein or 6-
181 carboxyfluorescein, 6-CF, (both at 250 µM initial concentration) for at least 2 h. Fluorophores and

ambient blue led light (Blue LED Light Panel: 225 psc, λ_em~465, dimension (cm): 30.5 x 30.5 x 3.2, 1080 Lux, 14W; HQRP, Harrison, NJ) were used to visualize the chemical plume. The animal's navigation through the chemical plume was recorded on video and analyzed. This set-up is shown in Fig 3.

Fig 3. Chemically mediated behaviors of early benthic juvenile spiny lobsters in large (type 1) arena. This arena was a rectangular glass aquarium with base dimensions of 31.8 cm x 16.5 cm (area of 525 cm^2). This example shows an animal exhibiting search behavior in a food-related (shrimp extract) chemical plume. (A) Single frame of the search trajectory showing the chemical source (*green circle*) and the lobster (marked with blue circle) crossing relatively low intensity portion of the chemical plume. (B and C) The search trajectory is visualized using maximum intensity projection (Fiji/Image J). (B) To better visualize the position of the lobster's body during search, the number of frames was reduced by a factor of 10. (C) Result of maximum intensity projection of all frames (n = 560). Arrows depict the direction of movement of the animal and vectors of typical exploratory "scent" tracking pattern, cluster of brief error correction moves. See S3 Video for details.

Arena type 2 was smaller and was used for observing and analyzing smaller-scale behaviors triggered by chemical stimuli. This arena was a glass cubic cuvette with dimension (in mm) of either 42.5 x 45 x 45, with optical path length 40; wavelength range: VIS (360-2500 nm), or dimension (in mm) of 52.5 x 55 x 55, with optical path length 50; wavelength range: VIS (360-2500 nm), Helma USA Inc., Plainview, NY. In combination with an aluminized hypotenuse prism, we were able to record simultaneously the top and side views of animals. (The prism was a right-

205 angle prism, N-BK7, 50 mm, λ/8, aluminum hypotenuse, supporting a 425 to 675 nm wavelength
206 range (Newport Corporation, Irvine, CA). This set-up is shown in Fig 4. Animals were video
207 recorded for approximately 2 min in control conditions and then for at least 2 min before, during,
208 and after stimulus presentation. Stimuli were applied as a 5 µl volume delivered using a handheld
209 micropipette to specific body regions such as the antennules or legs. To visualize the plume of the
210 chemical stimulus, we used fluorescein and ambient blue LED light (Blue LED Light Panel: 225
211 psc, λ_em~465, Dimension (in mm): 30.5 x 30.5 x 3.2, 1080 Lux, 14W; HQRP, Harrison, NJ).

212

213 **Fig 4.** Chemically mediated behaviors of early benthic juvenile spiny lobsters in small (type 2)
214 arena. This arena is a cubic glass cuvette with dimensions (in mm) 42.5 x 45 x 45. A glass prism
215 with an aluminized hypotenuse enabled simultaneous two-side imaging, thus allowing quasi three-
216 dimensional visualization. The animal was exposed to a chemical stimulus (shrimp extract), which
217 was visualized by fluorescein and blue ambient light. This configuration facilitates observing the
218 animal's sensory appendages including the antennules, antennae, mouthparts, and legs, and how
219 each appendage responds when contacting the chemical plume. Left panel: top view of an animal
220 inside the cuvette. Right panel: side view, prism optical path.

221

222 Chemical stimuli. Chemical stimuli were mixed in SW that contained fluorescein (250µM initial
223 concentration). The concentration of each stimulus as experienced by the animals was less than
224 that injected since there was some dilution. We estimated a 10-20 times dilution based on intensity
225 of fluorophore assuming linear dependence. We used three chemical stimuli, in addition to SW as
226 the control stimulus. 1) An aqueous extract of shrimp muscle was used as a food-related chemical
227 stimulus. This shrimp extract was prepared by crushing the abdomen of fresh, local penaeid shrimp

228 in SW, incubating it for ca. 2 h, then filtering it through a 0.2 µm filter and diluting it to a final
229 concentration of 5 µl/ml. 2) L-Glutamate at 5 mM was used to determine if animals show
230 chemically activated antennular grooming behavior. 3) A conspecific alarm cue was generated by
231 squeezing the hemolymph and other body fluids from cold anaesthetized live spiny lobsters into
232 SW, then filtering through a 0.2 µm filter for a final concentration of 5 µl/ml.

233 Video recordings. Two cameras were used in behavioral experiments. 1) Nikon Z7 camera, Nikon
234 FX-format, backside illumination CMOS sensor, full-frame 4K UHD/30p video footage, frame
235 size used: 1920 x 1080; at ~30Hz acquisition rate; lens: AF-S Micro NIKKOR 60mm f/2.8G ED
236 with autofocusing (Nikon Corporation, Tokyo, Japan). 2) Sony HDR-XR500, High Definition,
237 Handycam 'PAL' Camcorder, CMOS Sensor with Back-Illumination captures 1920 x 1080 HD
238 6MP video at 30 Hz frequency (Sony Corporation, Tokyo, Japan). Video recordings were saved in
239 MOV or MTS video formats, converted to AVI using a FFmpeg video converter (free and open-
240 source software, https://ffmpeg.org), and analyzed with ImageJ (free public domain software
241 supported by NIH, USA. http://imagej.org).

242

243 Results and Discussion

244 We characterized the activity of ORNs in early benthic juvenile spiny lobsters using a preparation
245 developed previously for adult animals in which ORNs were loaded with calcium sensitive
246 fluorescent probe, Fluo-4, and stimulated with odorants delivered to the aesthetasc sensilla
247 containing the outer dendrites of the ORNs. The time course of fluorescent intensity of ORNs of
248 early benthic juvenile spiny lobsters suggests that the spontaneous activity is generally
249 characterized by activity patterns similar to those of adult animals. Some ORNs are tonically
250 active, while other ORNs exhibit pronounced calcium oscillations suggesting bursting activity (Fig

251 2A and S2 Video) [27,29]. Similar to adult spiny lobsters, the majority of ORNs in early benthic
252 juvenile animals are sensitive to a complex food-related stimulus (Fig 2B and S1 Video, compare
253 with an adult ORN activity from Bobkov et al. [27,29]), while a smaller subpopulation of ORNs
254 was sensitive to monomolecular odorants (data not shown). While ORNs of both early benthic
255 juvenile and adult lobsters are characterized by similar patterns of spontaneous activity and kinetic
256 parameters of the responses, their tuning characteristics and sensitivity to either monomolecular
257 or complex chemical stimuli may differ, though a detailed characterization of these differences and
258 their sensitivities to alarm cues and other pheromones will require further additional future studies.
259 In a first set of behavioral experiments, we evaluated whether early benthic juvenile lobsters
260 exhibit chemically driven search behavior to food related chemicals, a behavior that in adults is
261 mediated by either ORNs or chemosensory neurons on the antennules [23]. For this set of
262 experiments, we put individual diet conditioned early benthic juvenile spiny lobsters in a large
263 (type 1) arena filled with sea water. After a 10-20 min acclimation period, we introduced chemical
264 stimuli into the arena and manually created random artificial plumes visualized with fluorophore.
265 In control experiments, when sea water + fluorophore was used as the stimulus, animals showed
266 no or very little searching for the stimulus source (n = 16, Fig 3). This result is consistent with the
267 conclusion that fluorophore itself as well as both excitation (blue ambient light, λ_em~465) and
268 fluorophore emission light (λ_em~518) does not trigger searching behavior in early benthic
269 juvenile lobsters. When a food-related stimulus (shrimp extract) was added to the fluorophore, in
270 a majority cases (14 out of 16 experiments), the animals performed search behavior as soon as they
271 detected the stimulus (Fig 3 and S3 Video). This study sets the stage for future studies that combine
272 hydrodynamics, sensory biology, behavior, and locomotion to help understand mechanisms
273 underlying chemically evoking orientation to chemical cues.

In a second set of behavioral experiments, we tested whether early benthic juvenile lobsters show a diversity of chemosensory behaviors beyond food-evoked searching as has been observed in adult animals. For these experiments, we used the smaller (type 2) arena (Fig 4). We put individual early benthic juvenile lobsters into glass cubic cuvettes with inside dimensions of 40 or 50 mm and imaged simultaneously from the top and lateral views providing possibilities for quasi-3D reconstruction (Fig 4). After 3-5 min acclimation to the cuvette conditions and blue ambient illumination/light provided by blue LED strip light, the animals were video recorded for 2 min in control conditions and then for at least 2 min before, during and after stimulus application. We focused on a limited number of relatively well-described and well-defined behavioral phenomena to avoid ambiguous interpretation. These included a series of food related behavioral patterns, reaction to alarm cues, chemically evoked production of currents, and antennular grooming behavior.

As a food related stimulus, we added 5 µl of shrimp extract in fluorescein into the cuvette using a hand-held pipetter in close proximity to the animal's head. Fluorescein helped to visualize the plume structure and detection and reaction timings. In all cases (n = 11), the early benthic juveniles were relatively calm in control conditions, exhibited relatively low frequency of antennule flicking and little movement such as walking (Fig 5 left panel, and S4 Video). When food stimulus was presented to the antennules, animals typically increased waving and flicking of antennules and probing with their legs (10 out of 11 trials, Fig 5 right panel and S5 Video). Presentation of the food stimulus to their legs typically resulted in extensive probing with legs, digging, and grabbing at the stimulus.

296 **Fig 5.** Early juvenile spiny lobster in type 2 arena (small cubic glass cuvette) showing localized
297 searching behavior to a food related chemical stimulus (shrimp extract). Left panel: control (sea
298 water). Right panel: after application of shrimp extract. Activity of juvenile lobster was visualized
299 using maximum intensity projection technique (Fiji/Image J). Note that more articulated body
300 counter suggests minimal movement, compare left and right panels (before and after stimulus
301 application, respectively). The same time interval was used in both cases.
302
303 Another behavioral pattern linked to stimulation of chemosensory receptors has been
304 demonstrated in adults: generation of directional water currents. These currents have been
305 previously described for many crustaceans [4–8], including adult spiny lobsters [9], but this has
306 not been described in early benthic juvenile spiny lobsters. These currents can be generated by
307 gills, fan organs (exopodites of mouthparts: crayfish [6,7,43], clawed lobsters [4], snapping shrimp
308 [44], and hermit crabs [45]), abdominal swimmerets (clawed lobsters [4,5]), and paddles on last
309 pair of legs (blue crabs [8]). In crayfish, gill currents are produced by drawing water into the gills
310 from around the animal and expelling it from the frontal opening of the gill chambers using the
311 gill bailers within the gills. It is always directed forward. Fan currents are generated by fan organs
312 associated with the mouthparts (maxillae and maxillipeds) at the anterior end of the animal. These
313 can be directed forward, laterally, upward, and backward [6,7]. In some cases, these currents are
314 described in the context of intraspecific sexual or social interactions that are not related to food.
315 These currents can direct urine released from the nephropores of one animal, the sender, towards
316 another animal, the receiver. They can be used in the context of intraspecific interactions, either
317 reproduction (*e.g.*, courtship, mating) or social interactions (*e.g.*, aggression) [4]. Information
318 currents are another current type, being used for both dispersal and reception of chemicals. These

319 currents have been previously described in adult crustaceans. The results of our study show that
320 early benthic stage juvenile spiny lobsters also produce chemically evoked water currents in
321 response to shrimp extract (Fig 6 and S6 Video). In some experiments, when shrimp extract applied
322 directly to the legs, the animals generated currents in a manner similar to the adult animals. While
323 detailed mechanisms triggering these behavioral patterns or the types of currents are not yet
324 described in these early benthic juvenile animals, it is clear that the production of these currents is
325 chemically evoked and conserved across different ontogenetic stages.
326
327 **Fig 6.** Early benthic juvenile spiny lobster in type 2 arena (small cubic glass cuvette) generating a
328 water current in response to a food-related stimulus. (A) A food related chemical stimulus (5 µl of
329 shrimp extract) was applied to the lobster's legs using a micropipette. (B) This triggered the animal
330 to generate a current. (B) The animal's reaction was to generate of current blowing the stimulus
331 away from the anterior end of the animals.
332
333 Antennular grooming behavior is one of the most extensively studied and well-understood
334 behavioral patterns, having been described in many decapod crustaceans. It can occur
335 spontaneously (*i.e.*, without any apparent sensory input), but it occurs much more often after an
336 animal has been feeding [15–18]. Antennular grooming behavior consists of two major
337 components [18]. In the first, called 'antennule wiping', both antennular flagella are repeatedly
338 brought down towards the last pair of mouthpart appendages (third maxillipeds) and are grabbed
339 by their endopodites equipped with pad-like structures consisting of densely packed specialized
340 setae and are repeatedly pulled through these pads. In the second component of antennular
341 grooming behavior, called auto grooming, the two third maxillipeds are rubbed against each other;

342 this usually occurs after a bout of wipes. Antennular grooming behavior can be evoked by
343 relatively high concentration of L-glutamate [17,19,46]. This chemically evoked antennular
344 grooming behavior has been shown only in adult spiny lobsters, and in them it is mediated
345 exclusively by chemosensory neurons in the asymmetric sensilla on the antennules [18]. We
346 therefore examined whether glutamate is capable of evoking grooming behavior in early benthic
347 juvenile lobsters. The response to a 5 µl solution of 5 mM glutamate and fluorescein was initially
348 somewhat similar to reaction to shrimp extract including increased frequency of antennule flicking,
349 antennule waving, and leg probing, but in 8 out of 8 cases, this initial arousal was followed by
350 and/or interrupted by pronounced antennular grooming (Fig 7 and S7 Video). The side view
351 demonstrates this behavioral pattern particularly well. Stimulation with L-glutamate also evoked
352 eye grooming in some cases (S8 Video).

353

354 **Fig 7.** Early benthic juvenile spiny lobster in type 2 arena (small cubic glass cuvette) producing
355 antennular grooming behavior in response to L-glutamate (5 mM, initial concentration). Left: control
356 before glutamate application. Right: after application of L-glutamate. Top: antennular grooming.
357 Bottom: eye grooming.

358

359 Another chemically evoked behavior is the response to chemicals released by injured
360 conspecifics, called alarm cues. This has been shown in older juvenile and adult spiny lobsters
361 [10–12]. These alarm cues are present in hemolymph and are detected by ORNs in the antennules
362 of conspecific animals [13]. The molecular nature of the alarm cues in hemolymph has been
363 identified for some adult crustacean species [14] and candidate molecules have been identified in
364 adult spiny lobsters [47]. Behavioral responses to hemolymph-based alarm cues in early benthic

stage juvenile spiny lobsters have not been reported. To test whether this stage spiny lobsters exhibit aversive responses to blood-borne conspecific alarm cues, we used the following experimental paradigm: The animals were first recorded in control conditions (Fig 8, S9 Video) and then exposed to food related chemicals (Fig 8, S10 Video). After showing stimulus evoked behavioral excitation, animals were exposed to 5 µl of hemolymph and other body fluids squeezed from conspecifics diluted in SW (Fig 8, S11 Video). At least in 3 out of 4 cases, we observed robust cessation of movement (Fig 8, S11 Video). During this freezing response, the animals lowered their body, stopped walking, and stopped moving their appendages. Aversive responses in general might be characterized by a variety of context dependent behavioral patterns including escape, hiding, aggressive responses and overall hyperactivity. However, the freezing response is one of the most conserved and universal behavioral patterns inherent to a variety of species across different phyla [48,49]. Experimentally, the significant advantage of the freezing response is that it is often a slow event that can be unambiguously detected, especially in space-limited experimental cuvettes.

Fig 8. Early juvenile spiny lobster in type 2 arena (small cubic glass cuvette) responding to an alarm cue. Left: negative control (sea water). Middle: food control (shrimp extract). Right: alarm cue (body fluid from a crushed conspecific). Activity of juvenile lobster was visualized using maximum intensity projection technique (Fiji/Image J). Note that more articulated body counter suggests minimal movement. The same time interval was used in all cases.

Conclusions

387 Our findings show that the properties of the chemical senses and behavioral patterns of early
388 benthic juvenile spiny lobsters are generally similar to those of adult animals. This makes early
389 benthic juveniles a useful model for studying mechanisms underlying neural activation (*e.g.*,
390 chemosensory transduction and coding) and behavioral responses. The smaller size of early
391 benthic juvenile lobsters confers advantages including the ability to use of a compact, miniature
392 benchtop laboratory setup to rapidly screen candidate compounds from metabolomics and/or
393 bioassay-guided fractionation to identify ecologically relevant chemicals such as alarm cues, sex
394 pheromones, aggregation cues, and food-related cues. In addition, the ability to reliably control
395 conditions within miniature experimental cuvettes and arenas makes it practical to study various
396 environmental factors (including light, visual stimuli, temperature, pH, toxic plankton metabolites,
397 and other favorable or stress conditions) on lobster chemosensory abilities.

398

399 **S1 Video.** Supporting Fig 2: Calcium imaging of food stimulus evoked activity of ORNs
400 **S2 Video.** Supporting Fig 2: Calcium imaging of spontaneous activity of ORNs
401 **S3 Video.** Supporting Fig 3: Searching behavior to food stimulus in large arena
402 **S4 Video.** Supporting Fig 5: Behavior to control stimulus in small arena
403 **S5 Video.** Supporting Fig 5: Behavioral responses to food stimulus in small arena
404 **S6 Video.** Supporting Fig 6: Generation of water current to food stimulus in small arena
405 **S7 Video.** Supporting Fig 7: Antennular grooming in response to L-glutamate in small arena
406 **S8 Video.** Supporting Fig 7: Eye grooming in response to L-glutamate in small arena
407 **S9 Video.** Supporting Fig 8: Response to control stimulus in small arena
408 **S10 Video.** Supporting Fig 8: Response to shrimp extract presented after control stimulus in small
409 arena

S11 Video. Supporting Fig 8: Response to hemolymph presented after shrimp extract in small arena

Acknowledgments. We thank Dr. Edward Buskey for providing laboratory space at the University of Texas Marine Science Institute, Casey Butler and Thomas Matthews of the Florida Fish & Wildlife Conservation Commission, Marathon, Florida Keys, for providing early benthic juvenile spiny lobsters, and Dr. Joseph Ryan of the Whitney Laboratory for Marine Bioscience, University of Florida, for his valuable discussions and general support.

References

1 Derby CD, Weissburg MJ. The chemical senses and chemosensory ecology of crustaceans. In: Derby C, Thiel M, editors. The natural history of the Crustacea, Vol. 3, Nervous systems & control of behavior. New York: Oxford University Press; 2014. pp. 263–92

2 Kamio M, Derby CD. Finding food: how marine invertebrates use chemical cues to track and select food. Nat Prod Rep. 2017; 34:514–28. doi:10.1039/c6np00121a

3 Kamio M, Yambe H, Fusetani N. Chemical cues for intraspecific chemical communication and interspecific interactions in aquatic environments: applications for fisheries and aquaculture. Fish Sci. 2022; 88:203–39. doi.org/10.1007/s12562-021-01563-0

4 Atema J. Opening the chemosensory world of the lobster, *Homarus americanus.* Bull Mar Sci. 2018; 94:479–516. doi.org/10.5343/bms.2018.0030

5 Atema J, Steinbach MA. Chemical communication and social behavior of the lobster *Homarus americanus* and other decapod Crustacea. In: Duffy JE, Thiel M, editors. Evolutionary ecology

432 of social and sexual systems: Crustaceans as model organisms. Oxford UK, Oxford University
433 Press; 2007. pp. 115–44. doi/10.1086/590592
434 6 Breithaupt T. Fan organs of crayfish enhance chemical information flow. Biol Bull. 2001;
435 200:150–4. doi:10.2307/1543308
436 7 Breithaupt, T. Chemical communication in crayfish. In: Breithaupt T, Thiel M, editors.
437 Chemical communication in crustaceans. New York: Springer; 2011. pp. 257–76.
438 doi.org/10.1007/978-0-387-77101-4
439 8 Kamio M, Reidenbach MA, Derby CD. To paddle or not: context dependent courtship display
440 by male blue crabs, *Callinectes sapidus*. J Exp Biol. 2008; 211:1243–8.
441 doi:10.1242/jeb.014977
442 9 Shabani S, Kamio M, Derby CD. Spiny lobsters use urine-borne olfactory signaling and
443 physical aggressive behaviors to influence social status of conspecifics. J Exp Biol. 2009;
444 212:2464–74. doi:10.1242/jeb.026492
445 10 Briones-Fourzán P, Pérez-Ortiz M, Lozano-Álvarez E. Defense mechanisms and antipredator
446 behavior in two sympatric species of spiny lobster, *Panulirus argus* and *P. guttatus*. Mar Biol.
447 2006; 149:227–39. doi:10.1007/S00227-005-0191-2
448 11 Briones-Fourzán P, Ramírez-Zaldívar E, Lozano-Álvarez E. Influence of conspecific and
449 heterospecific aggregation cues and alarm odors on shelter choice by syntopic spiny lobsters.
450 Biol Bull. 2008; 215:182–90. doi:10.2307/25470699
451 12 Parsons DM, Eggleston DB. Human and natural predators combine to alter behavior and
452 reduce survival of Caribbean spiny lobster. J Exp Mar Biol Ecol. 2006; 334:196–205.
453 doi:10.1016/j.jembe.2006.01.020

454 13 Shabani S, Kamio M, Derby CD. Spiny lobsters detect conspecific blood-borne alarm
455 pheromones exclusively through olfactory sensilla. J Exp Biol. 2008; 211:2600–8.
456 doi.org/10.1242/jeb.016667
457 14 Poulin RX, Lavoie S, Siegel K, Gaul DA, Weissburg MJ, Kubanek J. Chemical encoding of
458 risk perception and predator detection among estuarine invertebrates. Proc Natl Acad Sci USA.
459 2018; 115:662–7. doi.org/10.1073/pnas.1713901115
460 15 Bauer RT. Grooming behavior and morphology in the decapod Crustacea. J. Crust. Biol. 1981;
461 1:153–73. doi.org/10.2307/1548154
462 16 Bauer RT. Adaptive modification of appendages for grooming (cleaning; antifouling) and
463 reproduction in the Crustacea. In: Thiel M, Watling L, editors. The natural history of Crustacea.
464 Vol. 1: Functional morphology and diversity. New York: Oxford University Press; 2013. pp.
465 337–75. doi:10.1093/acprof:osobl/9780195398038.003.0013
466 17 Daniel PC, Shineman M, Fischetti M. Comparison of chemosensory activation of antennular
467 grooming behaviour in five species of decapods. Mar Freshwater Res. 2001; 52:1333–7.
468 doi:10.1071/MF01013
469 18 Schmidt M, Derby CD. Non-olfactory chemoreceptors in asymmetric setae activate antennular
470 grooming behavior in the Caribbean spiny lobster, *Panulirus argus*. J Exp Biol. 2005; 208:233–
471 48. doi:10.1242/jeb.01357
472 19 Cericola VJ, Daniel PC. Chemically-mediated antennular grooming behavior and associated
473 asymmetric setae: toward a hypothesis on their evolution in reptantian decapods. J Crust Biol.
474 2010; 30:557–70. doi.org/10.1651/09-3204.1

475 20 Schmidt M, Mellon D. Neuronal processing of chemical information in crustaceans. In:
476 Breithaupt T, Thiel M, editors. Chemical communication in crustaceans. New York: Springer;
477 2011. pp. 123–47. doi.org/10.1007/978-0-387-77101-4
478 21 Derby CD, Caprio J. What are olfaction and gustation, and do all animals have them? Chem
479 Senses. 2024; 49:bjae009. doi.org/10.1093/chemse/bjae009
480 22 Derby CD. The crustacean antennule: a complex organ adapted for lifelong function in diverse
481 environments and lifestyles. Biol Bull. 2021; 240:67–81. doi: 10.1086/713537.
482 23 Horner AJ, Weissburg MJ, Derby CD. Dual antennular chemosensory pathways can mediate
483 orientation by Caribbean spiny lobsters in naturalistic flow conditions. J Exp Biol. 2004;
484 207:3785–96. doi.org/10.1242/jeb.01200
485 24 Horner AJ, Weissburg MJ, Derby CD. The olfactory pathway mediates sheltering behavior of
486 Caribbean spiny lobsters, *Panulirus argus*, to conspecific urine signals. J Comp Physiol A.
487 2008; 194:243–53. doi: 10.1007/s00359-007-0302-2
488 25 Bobkov YV, Ache BW. Calcium sensitivity of a sodium-activated nonselective cation channel
489 in lobster olfactory receptor neurons. J Neurophysiol. 2003; 90:2928–40.
490 doi:10.1152/jn.00181.2003
491 26 Bobkov YV, Ache BW. Pharmacological properties and functional role of a TRP-related ion
492 channel in lobster olfactory receptor neurons. J Neurophysiol. 2005; 93:1372–80.
493 doi:10.1152/jn.00990.2004
494 27 Bobkov YV, Ache BW. Intrinsically bursting olfactory receptor neurons. J Neurophysiol. 2007;
495 97:1052–7. doi: 10.1152/jn.01111.2006

496 28 Bobkov YV, Pezier A, Corey EA, Ache BW. Phosphatidylinositol 4,5-bisphosphate-dependent
497 regulation of the output in lobster olfactory receptor neurons. J Exp Biol. 2010; 213:1417–24.
498 doi: 10.1242/jeb.037234
499 29 Bobkov Y, Park I, Ukhanov K, Principe J, Ache B. Cellular basis for response diversity in the
500 olfactory periphery. PLoS One. 2012; 7:e34843. doi: 10.1371/journal.pone.0034843
501 30 Kozma MT, Ngo-Vu H, Rump MT, Bobkov YV, Ache BW, Derby CD. Single cell
502 transcriptomes reveal expression patterns of chemoreceptor genes in olfactory sensory neurons
503 of the Caribbean spiny lobster, *Panulirus argus.* BMC Genomics. 2020; 21:649.
504 doi.org/10.1186/s12864-020-07034-7
505 31 Ukhanov K, Bobkov Y, Ache BW. Imaging ensemble activity in arthropod olfactory receptor
506 neurons in situ. Cell Calcium. 2011; 49:100–7. doi: 10.1016/j.ceca.2010.10.009
507 32 Ambrosio LJ, Baeza JA. 2023. Chemical sensing and avoidance of PaV1-infected conspecifics
508 by pueruli post-larvae of the reef-dwelling Caribbean spiny lobster *Panulirus argus* Latreille,
509 1804 (Decapoda: Achelata: Palinuridae). J Crust Biol. 2023; 43:1–9.
510 doi.org/10.1093/jcbiol/ruad065
511 33 Hutchinson E, Matthews TR, Renchen GF. Relationships between postlarval settlement and
512 commercial landings of Caribbean spiny lobster (*Panulirus argus*) in Florida (USA). Fish Res.
513 2024; 279:107137. doi.org/10.1016/j.fishres.2024.107137
514 34 Goldstein JS, Matsuda H, Takenouchi T, Butler MJ IV. The complete development of larval
515 Caribbean spiny lobster *Panulirus Argus* (Latreille, 1804) in culture. J Crust Biol. 2008;
516 28:306–27. doi.org/10.1163/20021975-99990376
517 35 Childress MJ, Herrnkind WF. The ontogeny of social behaviour among juvenile Caribbean
518 spiny lobsters. Anim Behav. 1996; 51:675–87. doi:10.1006/anbe.1996.0071

36 Childress MJ, Heldt KA, Miller SD. Are juvenile Caribbean spiny lobsters (*Panulirus argus*) becoming less social? ICES J Mar Sci. 2015;72 (Suppl. 1): i170–6. doi:10.1093/icesjms/fsv045

37 Derby CD, Cate HS, Steullet P, Harrison PJH. Comparison of turnover in the olfactory organ of early juvenile stage and adult Caribbean spiny lobsters. Arthrop Struct Develop. 2003; 31:297–311. doi.org/10.1016/S1467-8039(02)00050-6

38 Steullet P, Cate HS, Derby CD. A spatiotemporal wave of turnover and functional maturation of olfactory receptor neurons in the spiny lobster *Panulirus argus*. J Neurosci. 2000; 20:3282–94. doi:10.1523/JNEUROSCI.20-09-03282.2000

39 Goldstein JS, Butler, MJ. Behavioral enhancement of onshore transport by postlarval Caribbean spiny lobster (*Panulirus argus*). Limol Oceanogr 2009; 54:1669–78. doi 10.4319/lo.2009.54.5.1669

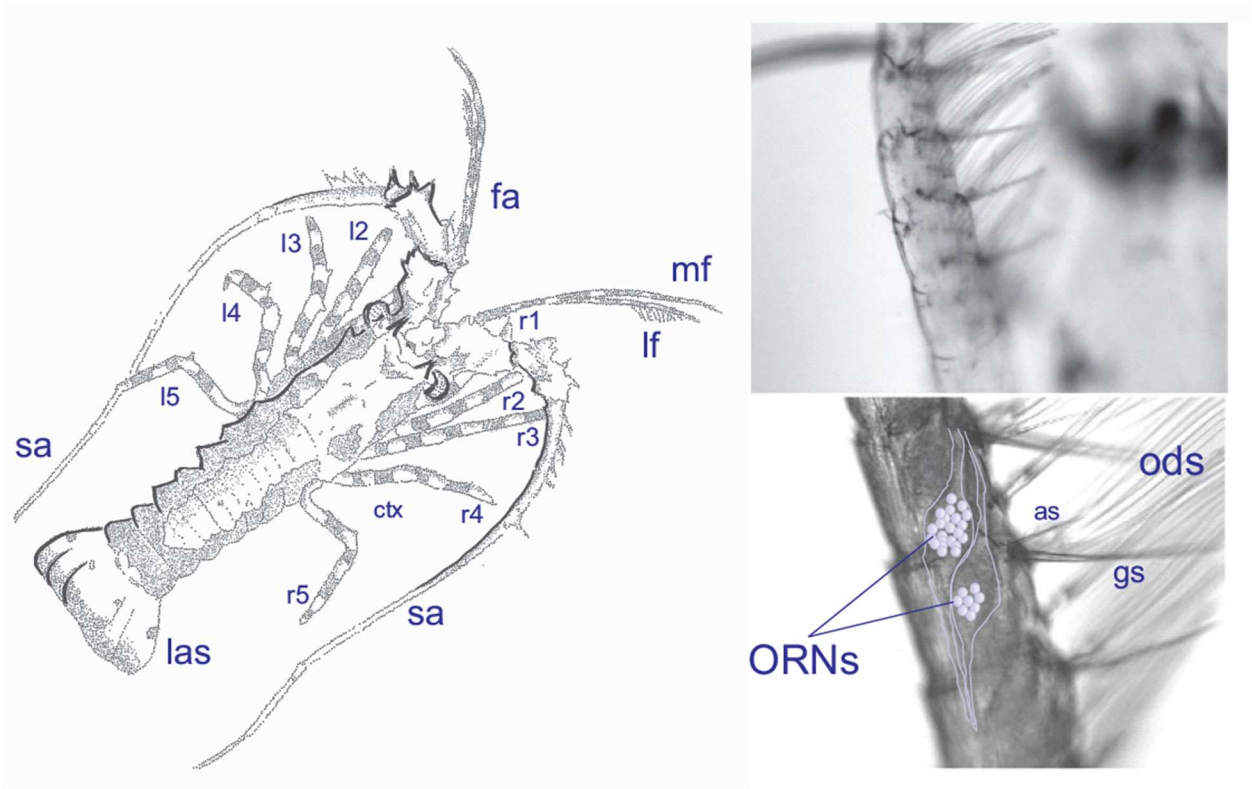
40 Baeza JA, Childress MJ, Ambrosio LJ. Chemical sensing of microhabitat by pueruli of the reef-dwelling Caribbean spiny lobster *Panulirus argus*: testing the importance of red algae, juveniles, and their interactive effect. Bull Mar Sci. 2018; 94:603–18. doi.org/10.5343/bms.2017.1132

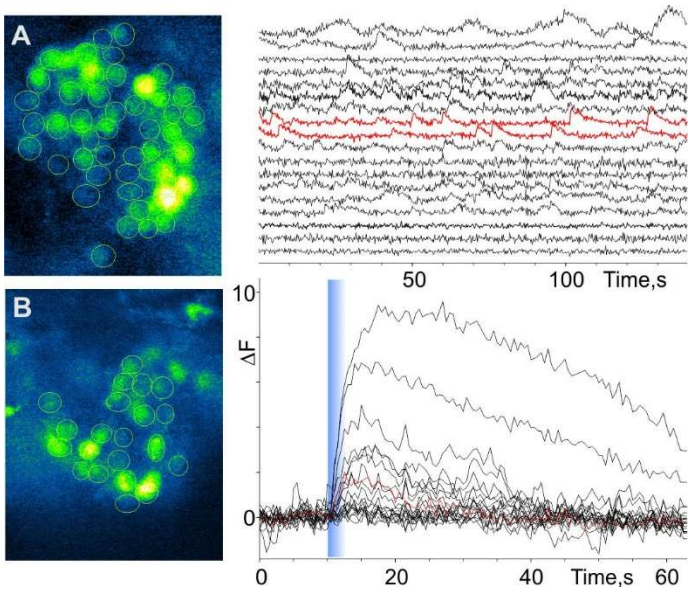
41 Butler MJ, MacDiarmid AB, Booth JD. The cause and consequence of ontogenetic changes in social aggregation in New Zealand spiny lobsters. Mar Ecol Prog Ser. 1999; 188:179–91. doi 10.3354/meps188179

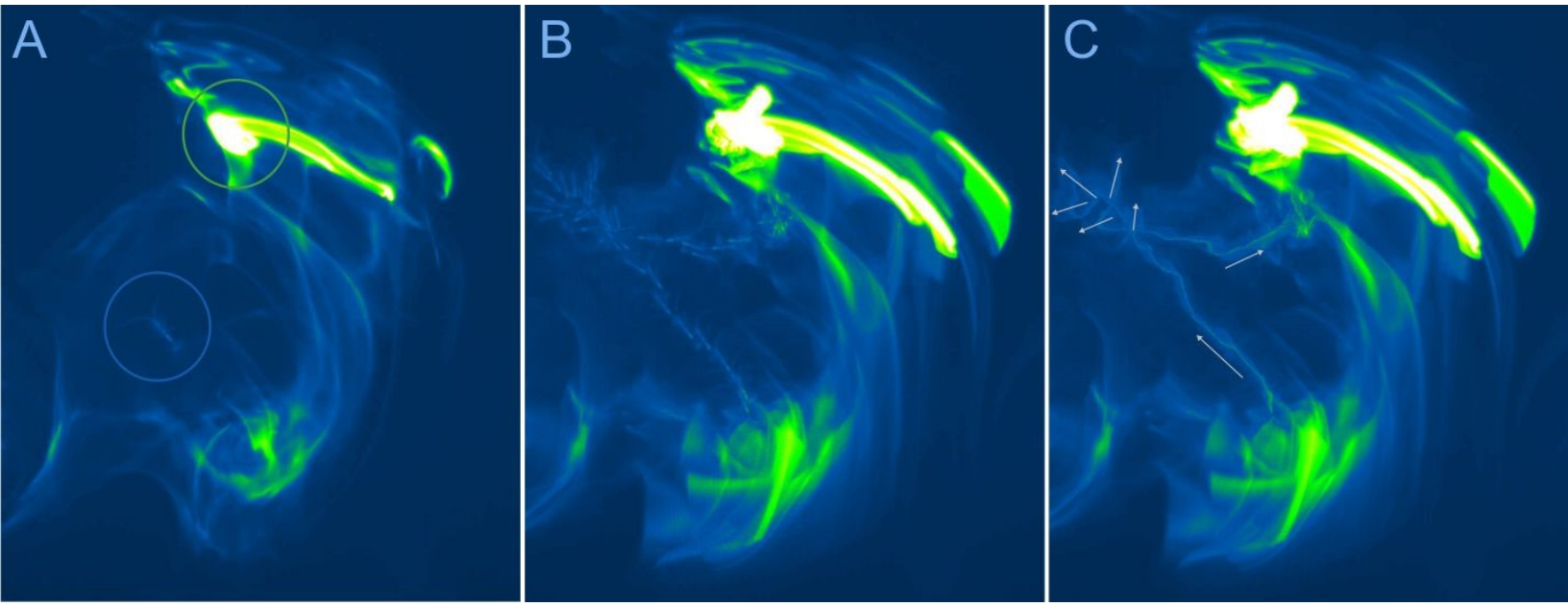
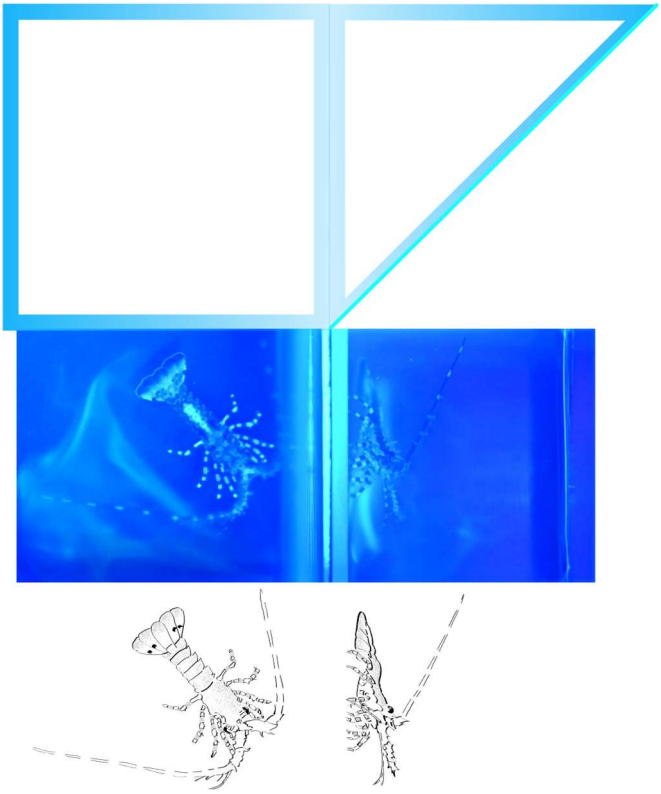
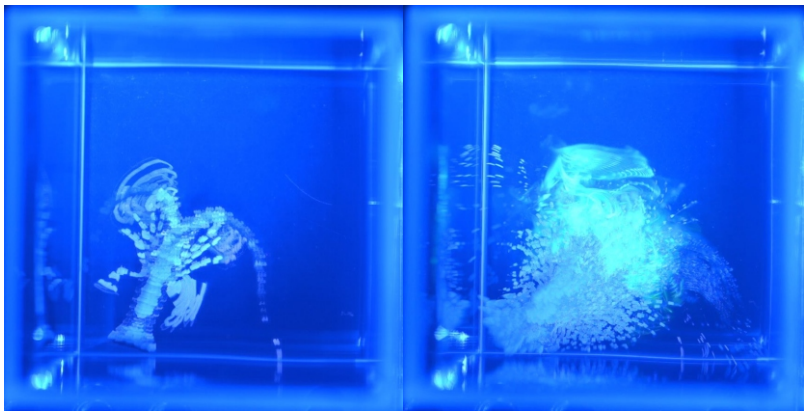
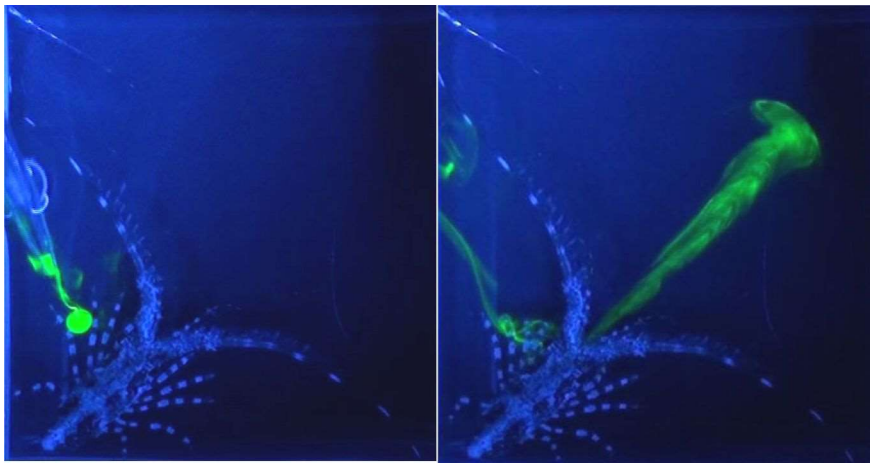
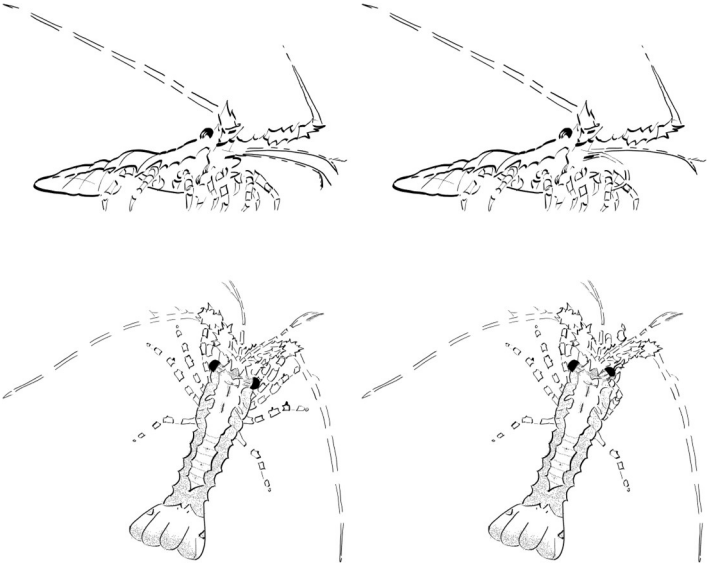
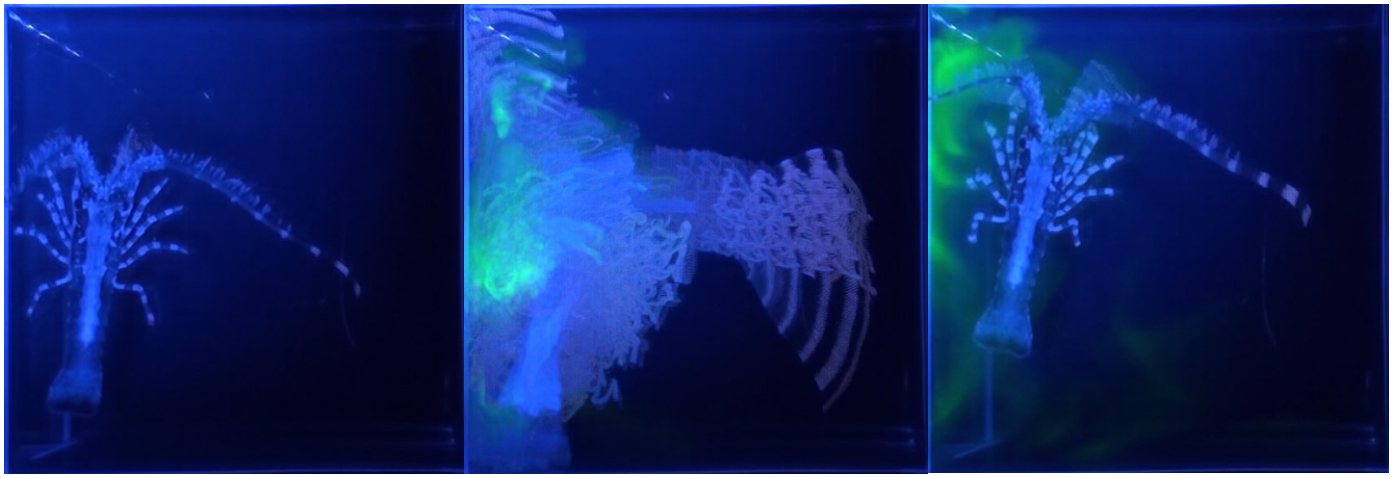
42 Brooker MA, de Lestang SN, How JR, Langlois TJ. Chemotaxis is important for fine scale habitat selection of early juvenile *Panulirus cygnus.* J Exp Mar Biol Ecol. 2022; 553:151753. doi 10.1016/j.jembe.2022.151753

43 Denissenko P, Lukaschuk S, Breithaupt T. Flow generated by an active olfactory system of the red swamp crayfish. J Exp Biol. 2007; 210:4083–91. doi.org/10.1242/jeb.008664

542 44 Herberholz J, Schmitz B. Signaling via water currents in behavioral interactions of snapping
543 shrimp (*Alpheus heterochaelis*). Biol Bull. 2001; 201:6–16. doi:10.2307/1543520
544 45 Barron LC, Hazlett BA. Directed currents: a hydrodynamic display in hermit crabs. Mar Behav
545 Physiol. 1989; 15:83–7. doi.org/10.1080/10236248909378720
546 46 Schmidt M, Haulk J, Derby CD. A chemosensory pathway detecting amino acid efflux from
547 olfactory sensilla mediates grooming of the olfactory organ in the spiny lobster, *Panulirus*
548 *argus*. Soc Neurosci Meeting 2012; New Orleans. Abstract 174.21
549 47 Qin B, Behringer D, Scro AK, Ross E, Uchida H, Yoshimura S, Tebben J, Harder T, Derby C,
550 Kamio M. The smell of Panulirus argus Virus 1 (PaV1) infection: disease-induced changes in
551 metabolites in urine and hemolymph of Caribbean spiny lobsters *Panulirus argus*. J Invert
552 Pathol. 2026; 214:108446. doi 10.1016/j.jip.2025.108446
553 48 Verheggen FJ, Haubruge E, Mescher MC. Alarm pheromones-chemical signaling in response
554 to danger. Vitam Horm. 2010; 83:215-39. doi: 10.1016/S0083-6729(10)83009-2
555 49 Wyatt TD. Pheromones and animal behavior: chemical signals and signature*s*. 2nd edition.
556 Cambridge: Cambridge University Press; 2014. doi 10.1017/CBO9781139030748


fa
l3
l2
mf
l4
r1
lf
l5
r2
sa
r3
ctx
r4
r5
sa
las
ods
as
gs
ORNs


A
B
50
100
Time,s
10
ΔF
0
0
20
40
Time,s
60


A
B
C