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

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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
88 form of phyllosoma larvae is very different from that of early benthic juvenile and adult animals.
89 The phyllosoma larval stages are followed by metamorphosis into the free-swimming, non-feeding

90 puerulus stage, whose body form is much like early benthic juvenile and adult stages though
91 without coloration. Pueruli move into nearshore settlement habitat that includes seagrass,
92 hardbottom, or mangrove habitats [35,36]. Once settled, these early benthic juvenile lobsters begin
93 to feed and are initially solitary. When they reach about 15 mm carapace length, they move from
94 macroalgae to crevice shelters and live there communally for the remainder of their lives. In the
95 Florida Keys, they become adults of legal size at > 76.2 mm carapace length (CL) at ca. 18 months
96 of age since settlement. They have diurnal activity patterns in which they leave shelters at dusk to
97 forage and return to shelters at dawn. Early benthic juvenile spiny lobsters have a body form and
98 olfactory organ that is generally similar form to adults [37,38], though nothing is known about the
99 physiology of their ORNs. Responses of *P. argus* pueruli to chemical cues from red algae
100 associated with settlement and cues from conspecifics have been described [32,39,40]. Responses
101 of early benthic juveniles of other species of spiny lobsters to chemical cues have been described
102 for conspecific cues by *Jasus edwardsii* [41] and habitat cues by *Panulirus cygnus* [42].
103 Our study examines chemoreception in early benthic juvenile animals. We examined appetitive
104 behaviors to food related cues, antennule grooming behavior to L-glutamate, and alarm responses
105 to body fluids of conspecifics. We also characterized basic properties of the physiology of ORNs.
106 Our results indicate that early benthic juvenile animals are very similar to adults in many of their
107 chemosensory properties.
108

109 Materials and Methods

110 Experimental animals

111 Post-larvae of Caribbean spiny lobsters *Panulirus argus* were collected from floating artificial
112 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
122 made 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
154 equally spaced for clarity. Two examples of ORNs characterized by oscillatory activity are
155 highlighted in red. Calcium oscillations are likely associated with bursting ORNs. (B) Chemical
156 evoked activity from a cluster of ORNs associated with a single aesthetasc sensillum different than
157 in A (*left panel*). The *right panel* shows the increase in calcium concentration in ORNs in response
158 to an 800-ms pulse of a chemical stimulus, which was a fish food extract (TetraMarine 0.5 mg/ml).

159 Timing of stimulus presentation is marked by the gray line. Both resting calcium signal level and
160 slow 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
165 fish 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

182 ambient blue led light (Blue LED Light Panel: 225 psc, λ_em~465, dimension (cm): 30.5 x 30.5 x
183 3.2, 1080 Lux, 14W; HQRP, Harrison, NJ) were used to visualize the chemical plume. The
184 animal's navigation through the chemical plume was recorded on video and analyzed. This set-up
185 is shown in Fig 3.

186

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

198

199 Arena type 2 was smaller and was used for observing and analyzing smaller-scale behaviors
200 triggered by chemical stimuli. This arena was a glass cubic cuvette with dimension (in mm) of
201 either 42.5 x 45 x 45, with optical path length 40; wavelength range: VIS (360-2500 nm), or
202 dimension (in mm) of 52.5 x 55 x 55, with optical path length 50; wavelength range: VIS (360-
203 2500 nm), Helma USA Inc., Plainview, NY. In combination with an aluminized hypotenuse prism,
204 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
239 in 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
258 and their sensitivities to alarm cues and other pheromones will require further additional future
259 studies.

260 In a first set of behavioral experiments, we evaluated whether early benthic juvenile lobsters
261 exhibit chemically driven search behavior to food related chemicals, a behavior that in adults is
262 mediated by either ORNs or chemosensory neurons on the antennules [23]. For this set of
263 experiments, we put individual diet conditioned early benthic juvenile spiny lobsters in a large
264 (type 1) arena filled with sea water. After a 10-20 min acclimation period, we introduced chemical
265 stimuli into the arena and manually created random artificial plumes visualized with fluorophore.
266 In control experiments, when sea water + fluorophore was used as the stimulus, animals showed
267 no or very little searching for the stimulus source (n = 16, Fig 3). This result is consistent with the
268 conclusion that fluorophore itself as well as both excitation (blue ambient light, λ_em~465) and
269 fluorophore emission light (λ_em~518) does not trigger searching behavior in early benthic
270 juvenile lobsters. When a food-related stimulus (shrimp extract) was added to the fluorophore, in
271 a majority cases (14 out of 16 experiments), the animals performed search behavior as soon as they
272 detected the stimulus (Fig 3 and S3 Video). This study sets the stage for future studies that combine

273 hydrodynamics, sensory biology, behavior, and locomotion to help understand mechanisms
274 underlying chemically evoking orientation to chemical cues.

275 In a second set of behavioral experiments, we tested whether early benthic juvenile lobsters
276 show a diversity of chemosensory behaviors beyond food-evoked searching as has been observed
277 in adult animals. For these experiments, we used the smaller (type 2) arena (Fig 4). We put
278 individual early benthic juvenile lobsters into glass cubic cuvettes with inside dimensions of 40 or
279 50 mm and imaged simultaneously from the top and lateral views providing possibilities for quasi-
280 3D reconstruction (Fig 4). After 3-5 min acclimation to the cuvette conditions and blue ambient
281 illumination/light provided by blue LED strip light, the animals were video recorded for 2 min in
282 control conditions and then for at least 2 min before, during and after stimulus application. We
283 focused on a limited number of relatively well-described and well-defined behavioral phenomena
284 to avoid ambiguous interpretation. These included a series of food related behavioral patterns,
285 reaction to alarm cues, chemically evoked production of currents, and antennular grooming
286 behavior.

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

296

297 **Fig 5.** Early juvenile spiny lobster in type 2 arena (small cubic glass cuvette) showing localized
298 searching behavior to a food related chemical stimulus (shrimp extract). Left panel: control (sea
299 water). Right panel: after application of shrimp extract. Activity of juvenile lobster was visualized
300 using maximum intensity projection technique (Fiji/Image J). Note that more articulated body
301 counter suggests minimal movement, compare left and right panels (before and after stimulus
302 application, respectively). The same time interval was used in both cases.

303

304 Another behavioral pattern linked to stimulation of chemosensory receptors has been
305 demonstrated in adults: generation of directional water currents. These currents have been
306 previously described for many crustaceans [4–8], including adult spiny lobsters [9], but this has
307 not been described in early benthic juvenile spiny lobsters. These currents can be generated by
308 gills, fan organs (exopodites of mouthparts: crayfish [6,7,43], clawed lobsters [4], snapping shrimp
309 [44], and hermit crabs [45]), abdominal swimmerets (clawed lobsters [4,5]), and paddles on last
310 pair of legs (blue crabs [8]). In crayfish, gill currents are produced by drawing water into the gills
311 from around the animal and expelling it from the frontal opening of the gill chambers using the
312 gill bailers within the gills. It is always directed forward. Fan currents are generated by fan organs
313 associated with the mouthparts (maxillae and maxillipeds) at the anterior end of the animal. These
314 can be directed forward, laterally, upward, and backward [6,7]. In some cases, these currents are
315 described in the context of intraspecific sexual or social interactions that are not related to food.
316 These currents can direct urine released from the nephropores of one animal, the sender, towards
317 another animal, the receiver. They can be used in the context of intraspecific interactions, either
318 reproduction (*e.g.*, courtship, mating) or social interactions (*e.g.*, aggression) [4]. Information

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

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

354

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

359

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

365 adult spiny lobsters [47]. Behavioral responses to hemolymph-based alarm cues in early benthic
366 stage juvenile spiny lobsters have not been reported. To test whether this stage spiny lobsters
367 exhibit aversive responses to blood-borne conspecific alarm cues, we used the following
368 experimental paradigm: The animals were first recorded in control conditions (Fig 8, S9 Video)
369 and then exposed to food related chemicals (Fig 8, S10 Video). After showing stimulus evoked
370 behavioral excitation, animals were exposed to 5 µl of hemolymph and other body fluids squeezed
371 from conspecifics diluted in SW (Fig 8, S11 Video). At least in 3 out of 4 cases, we observed
372 robust cessation of movement (Fig 8, S11 Video). During this freezing response, the animals
373 lowered their body, stopped walking, and stopped moving their appendages. Aversive responses
374 in general might be characterized by a variety of context dependent behavioral patterns including
375 escape, hiding, aggressive responses and overall hyperactivity. However, the freezing response is
376 one of the most conserved and universal behavioral patterns inherent to a variety of species across
377 different phyla [48,49]. Experimentally, the significant advantage of the freezing response is that
378 it is often a slow event that can be unambiguously detected, especially in space-limited
379 experimental cuvettes.

380

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

386

387 ## Conclusions

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

399

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

411 **S11 Video.** Supporting Fig 8: Response to hemolymph presented after shrimp extract in small
412 arena
413

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419

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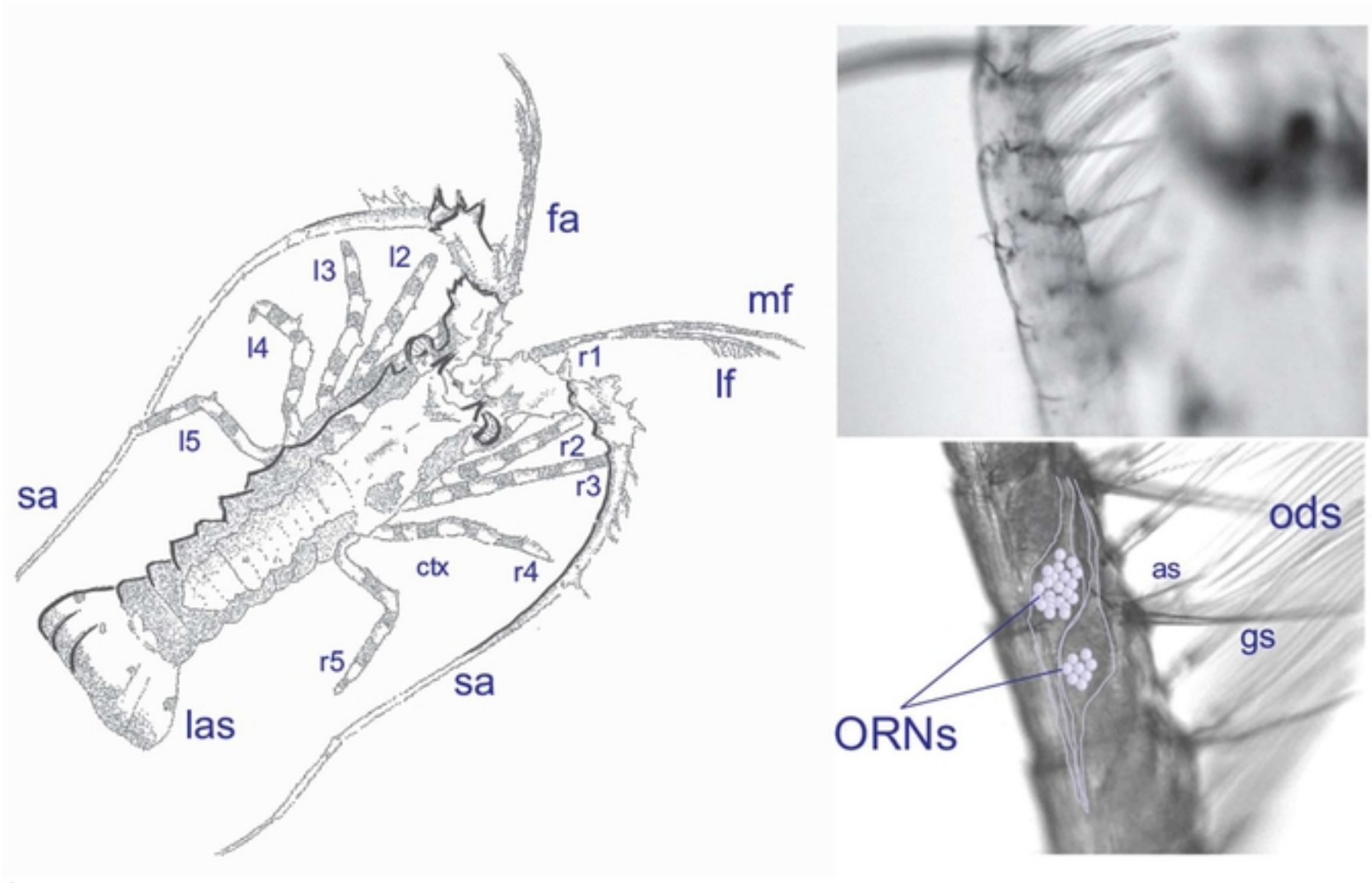

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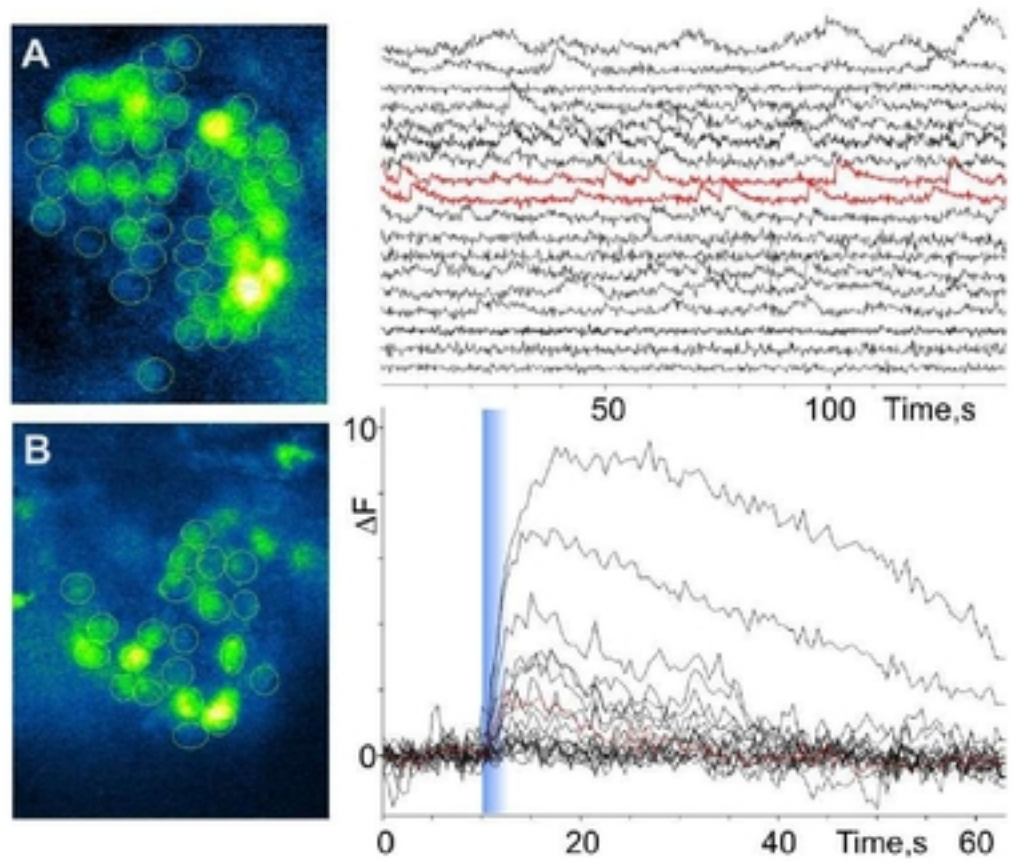

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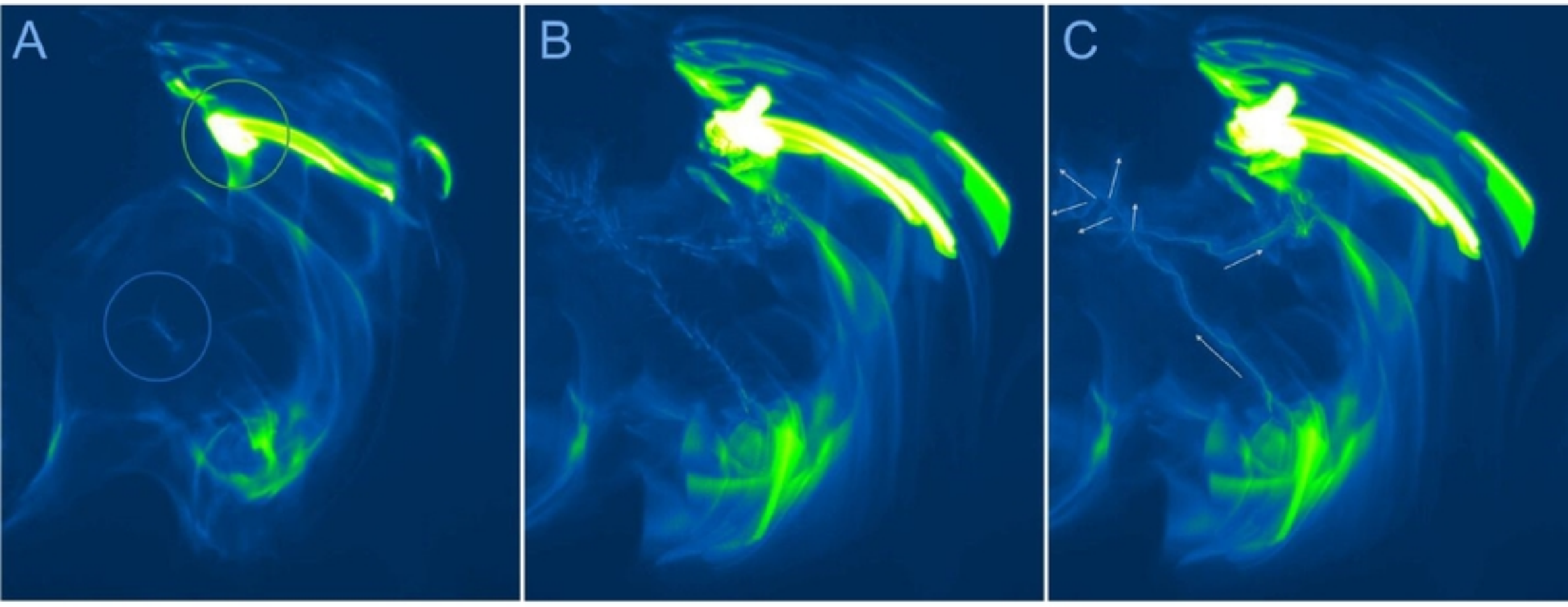

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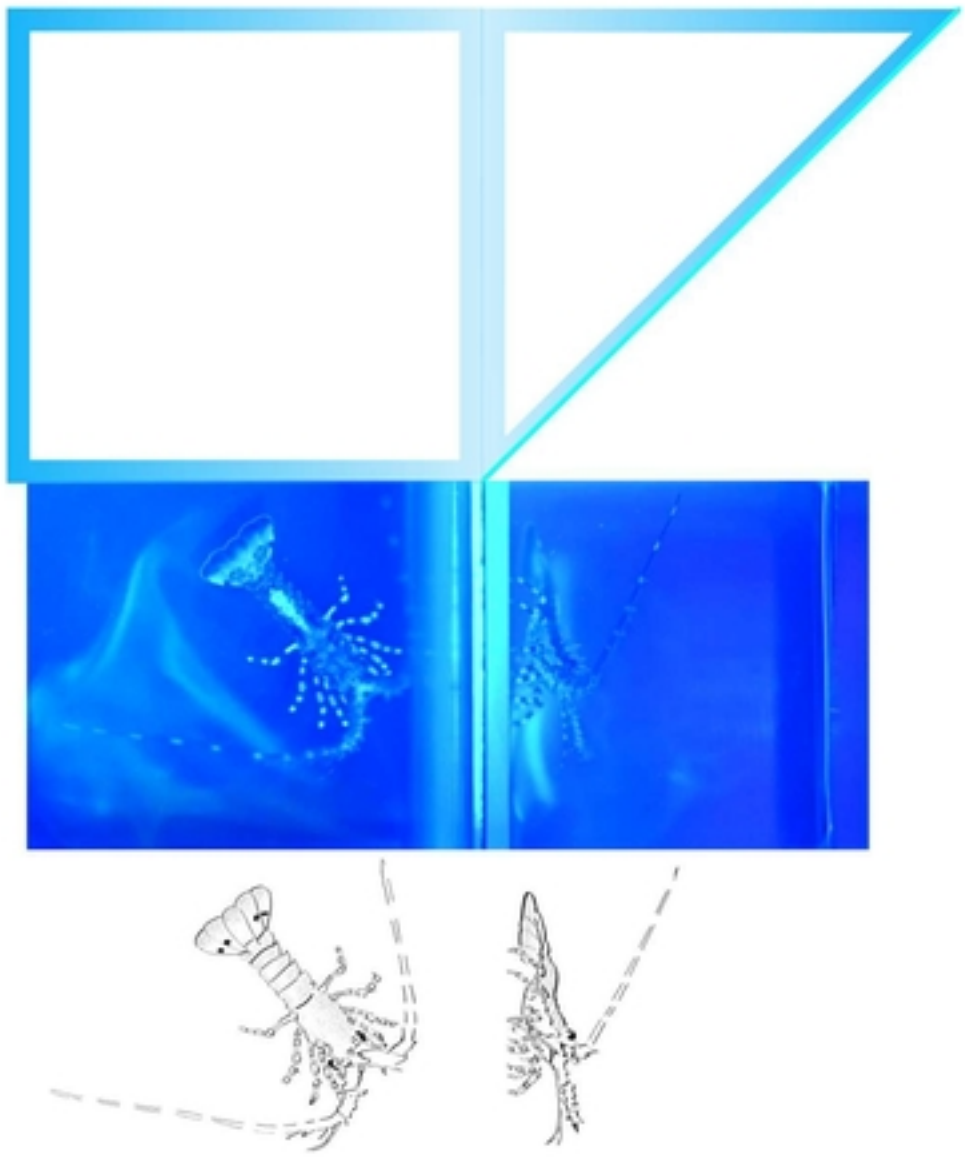

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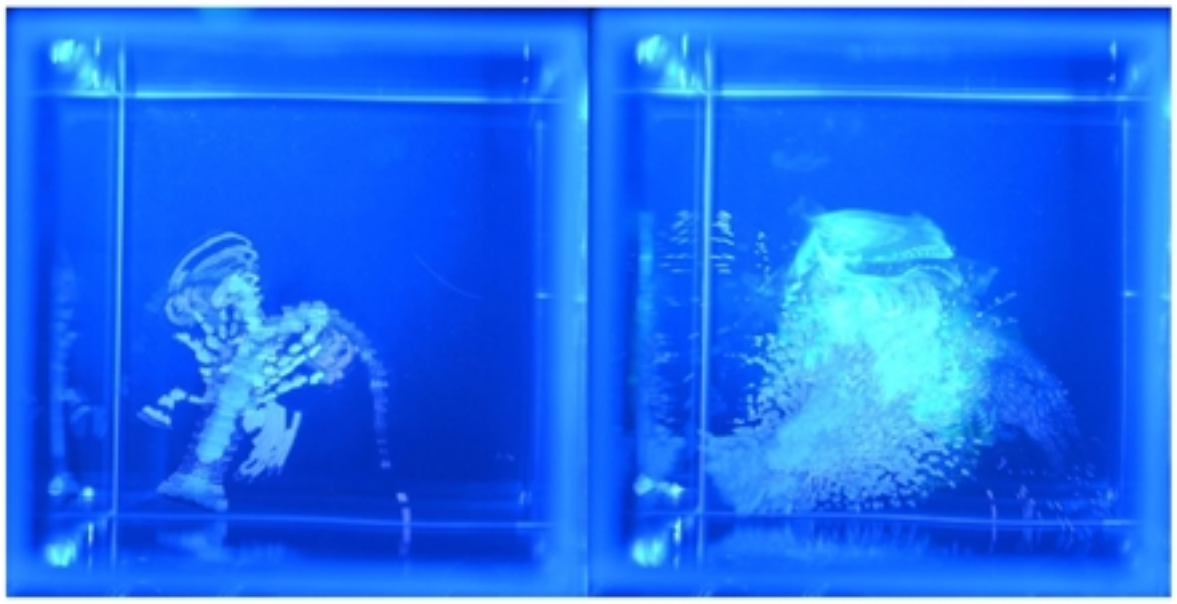

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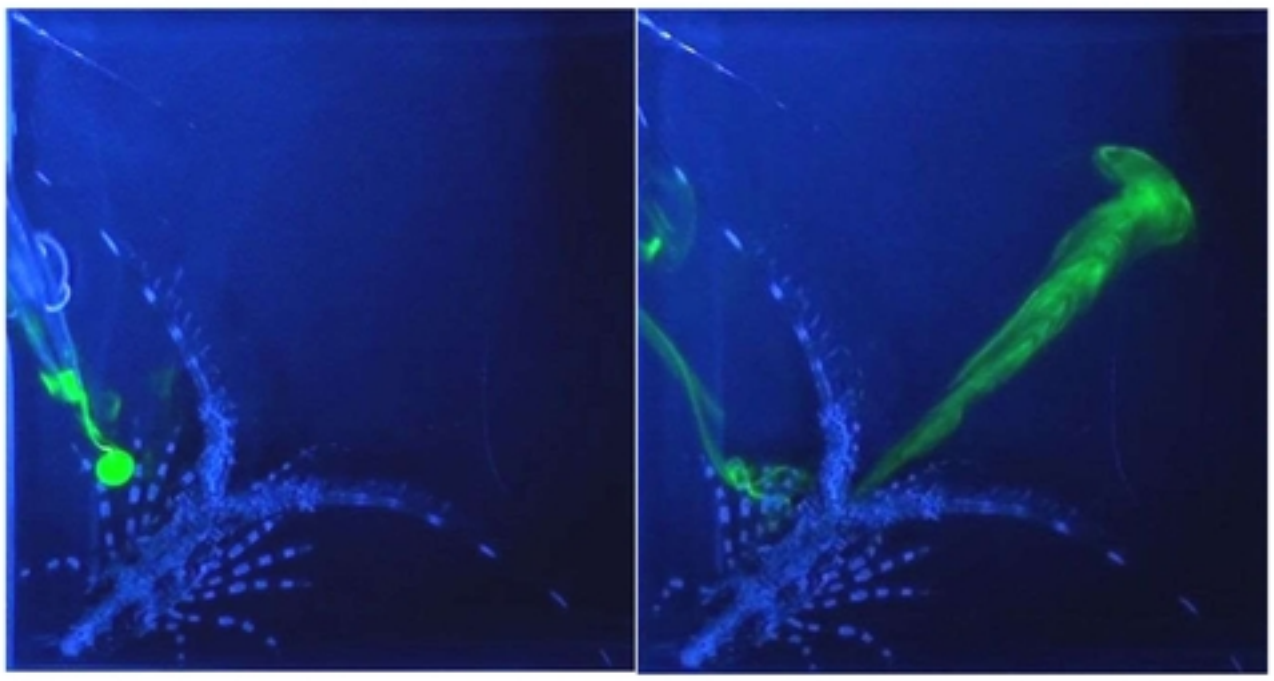

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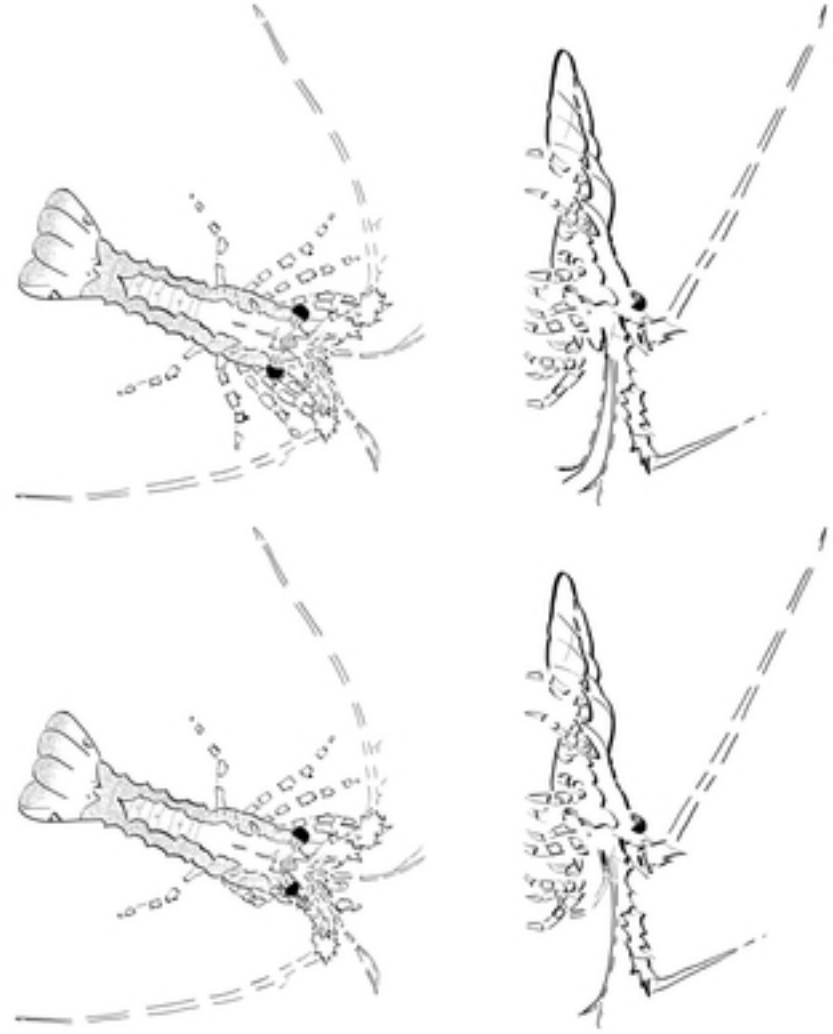

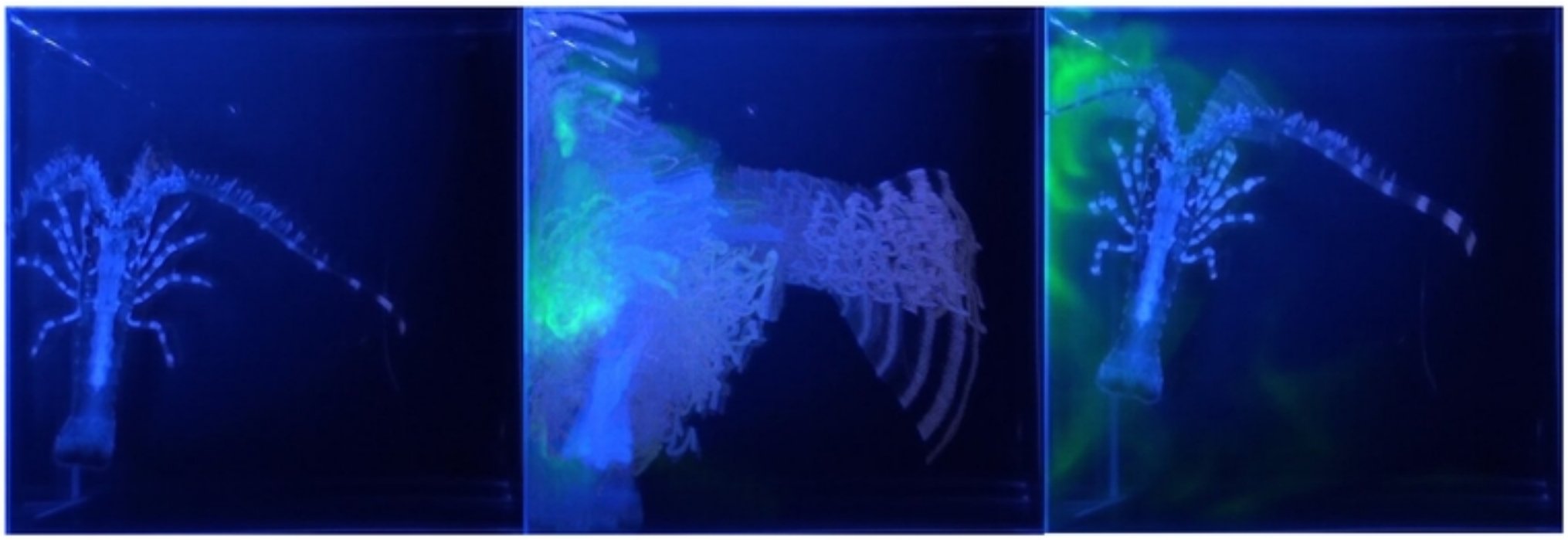

Fig8