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New electroantennography method on a marine shrimp in water

RUNNING TITLE: Electroantennography on a marine shrimp

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SUMMARY STATEMENT

Electroantennography allows the recording of global olfactory responses from the antennal appendages, and is developed here for studies on marine shrimp in aqueous condition.

ABSTRACT

Olfaction in aquatic decapods is well-studied via the recording of single olfactory receptor neuron activity in the main olfactory organ, the antennule, but global responses of the antennule (or antennae in insects) by electroantennography (EAG) was so far mainly restricted to aerial conditions. We present here a well-established EAG method to record the global antennule activity in the marine shrimp *Palaemon elegans* in natural (aqueous) conditions. EAG responses to food extracts, recorded as net positive deviations of the baseline, are reproducible, dose-dependent and exhibit sensory adaptation. This new EAG method opens a large field of possibilities for studying *in vivo* olfaction in aquatic decapods, in a global approach to supplement current more specific techniques.

34 INTRODUCTION

35

36 Crustaceans, in particular lobsters and crayfishes, have emerged as excellent
37 models for research in chemoreception (Ache, 2002). They rely on olfaction for
38 diverse behaviors such as food detection and social interactions (Derby and
39 Sorensen, 2008). Their main olfactory organ is the antennule (Barber, 1961), the
40 lateral flagellum of which bears presumably unimodal sensilla specialized in olfaction,
41 the aesthetascs, innervated only by olfactory receptor neurons (ORNs) (Ghiradella et
42 al., 1968). Antennular flagella also bear bimodal sensilla, innervated by mechano-
43 and chemoreceptor neurons (Schmidt and Derby, 2005). Responses of crustacean
44 chemoreceptor neurons to odor stimuli were classically recorded either extracellularly
45 from their axons (Derby, 1989; Kamio et al., 2005) or intracellularly after the
46 introduction of the patch-clamp method (Ache, 2002; Anderson and Ache, 1985;
47 Bobkov et al., 2012).

48 Study of olfaction is essential for understanding the biology of our model
49 species, the blind Alvinocarididae deep-sea hydrothermal shrimp. Despite the fact
50 that olfaction may play a major role in the detection of their environment
51 (Chamberlain, 2000; Jinks et al., 1998; Renninger et al., 1995), there is still little
52 information about their chemosensory sensitivity. Specimens of these species are
53 extremely difficult to collect and maintain alive and therefore are available in low
54 numbers for experiments. The objective of this work was to develop a method with a
55 high rate of success, allowing the recording of global responses of the olfactory
56 organ, the antennule, in marine decapods. In insects, such a technique called
57 electroantennography (EAG; Schneider, 1957) is commonly used to measure the
58 global response profile of antennal ORNs to odor stimuli. This technique is widely
59 used in screening pheromones, particularly in moths (Roelofs, 1984). In crustaceans,
60 EAG measurements were performed in aerial conditions on two terrestrial crabs: the
61 giant robber crab *Birgus latro* (Stensmyr et al., 2005) and the hermit crab *Coenobita*
62 *clypeatus* (Krång et al., 2012), and in the marine hermit crab *Pagurus bernhardus*
63 (Stensmyr et al., 2005). Two papers reported very briefly EAG recordings from fresh
64 water crustaceans: the branchiopoda *Daphnia* spp. (Simbeya et al., 2012) and the
65 crayfish *Procambarus clarkii* (Ameyaw-Akumfi and Hazlett, 1975).

66 We present here a new EAG method established for shrimp in their aquatic
67 environment. It was developed on the coastal marine species *Palaemon elegans*
68 (Rathke, 1837) but it can easily be adapted to other decapods.

69
70

71 **MATERIALS AND METHODS**

72

73 **Animals**

74 Caridean shrimp *Palaemon elegans* (Rathke, 1837) were collected in January,
75 October and December 2015 from Saint-Malo bay (France), housed communally in
76 100-L aquaria with oxygenated artificial sea water (Red Sea Salt, Red Sea, Houston,
77 USA) at $15.5 \pm 0.5^\circ\text{C}$ under a 12 h:12 h light:dark cycle, and fed twice a week with
78 shrimp food pellets (Novo Prawn (NP), JBL, Neuhofen, Germany). The specimens for
79 experiments were transferred to a 25-L aquarium maintained at room temperature
80 ($21 \pm 1^\circ\text{C}$) for at least 48 hours of starvation to prevent any potential adaptation of
81 their ORNs to food odors. The sex and age of animals were not determined.

82

83 **Biological preparation**

84 The animal was restrained in a 1-mL or 5-mL pipette tip cut according to the
85 shrimp's size with the antennal appendages and the posterior part of the abdomen
86 out at each extremity of the cone (Fig. 1). The shrimp was placed ventral face to the
87 top. The preparation was attached to a UM-3C micromanipulator (Narishige, London,
88 UK) and angled at approximately 45° so that the anterior part of the animal (i.e.
89 antennal appendages) was submerged in a Petri dish filled with *Panulirus* saline (PS)
90 and the posterior part (i.e. telson and abdomen) remained in the air. Composition of
91 PS was (in mM): 486 NaCl, 5 KCl, 13.6 CaCl₂, 9.8 MgCl₂ and 10 HEPES, pH 7.8-7.9
92 (Hamilton and Ache, 1983). A gravity fed PS perfusion was inserted in the pipette tip
93 just over the cephalothorax, to irrigate the branchial cavity and keep the animal alive,
94 and to renew the PS bath solution. The antennules were immobilized with U shape
95 tungsten hooks on a piece of Styrofoam stuck on the bottom of the Petri dish. The
96 preparation was visualized under a dissecting microscope (M165C, Leica, Nanterre,
97 France). Experiments were done at room temperature ($21 \pm 1^\circ\text{C}$).

98

99

100 **Electrophysiological recordings and data analysis**

101 Electrodes were pulled from GB150F-8P glass capillaries (Science Products,
102 Hofheim, Germany) using a P-97 puller (Sutter Instrument, USA). They had a tip
103 diameter of 1 to 2.5 μm and were filled with PS. The reference electrode was
104 introduced through the soft articular membrane between the telson and the
105 abdomen. The recording electrode was inserted with a NMM-25 micromanipulator
106 (Narishige, London, UK) in the base, middle or apex region of the flagellum area
107 bearing the aesthetascs, between two aesthetasc rows (Fig. 1B). Signals were
108 amplified (x100) and filtered (0.1-1000 Hz) using an EX1 amplifier with a 4002
109 headstage (Dagan, Minneapolis, USA), and digitized at 2 kHz by a 16-bit acquisition
110 board (Digidata 1440A) under Clampex 10.3 (Molecular Devices, Union City, USA).
111 Data were analyzed using Clampfit (Molecular Devices). Signals were filtered offline
112 at 20 Hz with a Gaussian low-pass filter. Data are given as mean \pm SEM.

113

114 **Chemical stimuli preparation and delivery**

115 An aqueous extract of shrimp food NP was used as odorant in most experiments.
116 NP pellets were dissolved for 48 h at room temperature at 0.2 $\text{g}\cdot\text{mL}^{-1}$ in PS. The
117 extract was then centrifuged at 7500 rpm for 10, 15 and 20 min and the supernatant
118 was collected after each centrifugation and filtered (0.45 μm), aliquoted and stored at
119 -20°C until use. For dose-response experiments, NP extracts were diluted from 1:2 to
120 1:200 with PS. Aqueous extracts of green crab *Carcinus maenas*, blue mussel
121 *Mytilus edulis* and dead *P. elegans* individuals were prepared from fresh material at
122 approximately 0.1 $\text{g}\cdot\text{mL}^{-1}$, 0.5 $\text{g}\cdot\text{mL}^{-1}$ and 0.6 $\text{g}\cdot\text{mL}^{-1}$ in PS, respectively, using the
123 same protocol as for NP extract. Osmolarity (1050 $\text{mOsm}\cdot\text{l}^{-1}$ with mannitol) and pH
124 (7.85 with NaOH) were adjusted for all solutions.

125 To deliver chemical stimuli, we used a pressurized perfusion system with 8
126 channels (AutoMate Scientific, Berkeley, CA). Each reservoir was connected to one
127 entry of a MPP-8 multi-barrel manifold (Harvard Apparatus, Les Ulis, France). A
128 segment (60 mm) of deactivated gas chromatography (GC) column (0.25 mm internal
129 diameter) was glued to the manifold exit. The manifold was mounted onto a UM-3C
130 micromanipulator and the tip of the stimulus GC column was positioned
131 approximately 1.5 mm from the recorded flagellum in its longitudinal axis. Stimuli
132 were applied for 1 s at 5 psi (1.1 $\text{mL}\cdot\text{min}^{-1}$). Consecutive stimuli were delivered with

133 at least 2 min intervals to prevent olfactory adaptation. To establish the dose-
134 response relationship for NP extract, stimuli were applied in increasing
135 concentrations. To analyze how global responses are, glass capillaries with two
136 diameters were used to stimulate wide and narrow portions of the antennule (0.86
137 mm and 100 μm internal diameter, respectively) perpendicular to the antennule axis.
138 Responses to 0.1 or 0.2 $\text{g}\cdot\text{mL}^{-1}$ of NP extract (positive control) were measured at the
139 beginning and at the end of each experiment to ensure that the quality of recording
140 remained constant throughout the experiment. PS was used as a negative control.

141

142

143 **RESULTS AND DISCUSSION**

144

145 We implemented a new EAG technique on a marine decapod while keeping both
146 its olfactory organ and the stimulus in their natural (marine) environment. This
147 technique is derived from the EAG on insects. Insect antennae have different shapes
148 which impacts the positioning of the recording electrode. In Lepidoptera, EAG is
149 typically performed from whole insect preparations by cutting the tip of the antenna
150 and inserting it in a glass electrode filled with electrolyte, with the reference electrode
151 inserted in the insect body. Excised antenna can also be used but they have a
152 shorter lifetime (Martinez et al., 2014). In Coleoptera, the recording electrode is
153 inserted in the antenna (Roelofs, 1984). In aquatic species, having the two electrodes
154 in water would result in a short circuit between them. We used the air-water interface
155 to prevent this short circuit, putting the anterior part of the shrimp (i.e. antennular
156 appendages, one lateral flagellum being impaled by the recording electrode) in the
157 bath solution and the posterior part (i.e. telson and abdomen, connected to the
158 reference electrode) in the air. Because cutting the extremity of the antennule
159 induced a prominent outflow of fluid, we inserted the recording electrode tip between
160 aesthetascs.

161

162 **Determination of recording parameters**

163 To establish the best conditions to record EAG responses, NP extract stimuli
164 were used. Responses to NP were positive deviations of the baseline while control
165 stimuli (PS) induced negative deviations of the baseline.

166 To estimate if the recording electrode sample the responses from a large or small
167 portion of the antennule (i.e. global vs local response), different portions were
168 stimulated with 0.2 g.mL^{-1} of NP extract. When narrow stimuli were applied along the
169 antennule while recording from the base region (Fig. 1C), the electrode recorded
170 responses from zones stimulated far from the electrode location. Then, increasing
171 lengths of the antennule were stimulated by moving a larger stimulation capillary
172 along the antennule while recording from the apex (Fig. 1D) or the base (Fig. 1E)
173 regions. For both electrode placements, the EAG amplitude increased with the size
174 of the stimulated area. Thus, there is a good propagation of the olfactory response
175 within the antennule, so a large fraction of receptor neurons can be recorded. To
176 maximize the amplitude of recorded responses (i.e. to record the largest number of
177 receptor neurons on both sides of the electrode), following experiments were done by
178 placing the recording electrode in the middle region of the antennule (Fig. 1B).

179 In decapods, the antennule is not only equipped with unimodal (chemoreceptive)
180 aesthetascs housing ORNs. It also carries bimodal (chemo- and mechanoreceptive)
181 non-aesthetasc sensilla, however in much lower density than aesthetascs (Cate and
182 Derby, 2001; Hallberg et al., 1997; Obermeier and Schmitz, 2004; Steullet et al.,
183 2002). Hence, to verify whether control responses were mechanical, PS flows were
184 applied at increasing pressures (2 to 10 psi). PS responses were pressure-
185 dependent, with no significant response for the lowest pressure (2 psi) and significant
186 response for pressure values of 4 psi and more (Fig. 2A, B) indicating that PS
187 responses were likely mechanical. The response increase from 4 ($-0.17 \pm 0.04 \text{ mV}$, n
188 $= 24$) to 10 psi ($-0.32 \pm 0.09 \text{ mV}$, $n = 25$) was not significant due to a high variability
189 of the amplitude of PS responses across recordings. We thus decided to adjust the
190 pressure of all stimuli to 5 psi to facilitate stimulus access to aesthetascs through
191 their dense packing and to distributed chemosensilla without eliciting strong
192 mechanical responses that would have impeded measuring correctly olfactory
193 responses.

194 Responses of ORNs not only depend on the stimulus characteristics (quality,
195 quantity) but also on previous olfactory experience via the process of adaptation
196 (Kaissling et al., 1987). When ORNs are adapted by a stimulus, responses to
197 subsequent stimuli are reduced. The recovery from adaptation is time-dependent. To
198 define a time interval between consecutive stimuli that could prevent measuring

199 responses from adapted sensory neurons, we measured EAG responses to pairs of
200 identical stimuli (0.1 g.mL^{-1} of NP extract) with increasing inter-stimulus intervals (4 to
201 90 s) (Fig. 2C, D). As the inter-stimulus interval was increased, the average
202 amplitude of the response to the second stimulus increased and eventually did not
203 differ significantly from the amplitude of the first response when the interval was at
204 least 90 s. For safety, in the following we kept an interval of at least 2 min between
205 consecutive stimuli.

206 **EAG responses according to defined recording parameters**

207 All the responses to NP extracts were positive deviations of the baseline (Fig.
208 3A). Increasing concentrations of NP extract (0.001 g.mL^{-1} to 0.2 g.mL^{-1}) elicited
209 dose-dependent responses with a threshold between 0.001 and 0.003 g.mL^{-1} (Fig.
210 3B) and amplitudes reaching 2.6 mV for the highest concentration (0.2 g.mL^{-1}). The
211 delay between the electrovalve opening command and the beginning of the EAG
212 response to 0.1 or 0.2 g.mL^{-1} is $62 \pm 3 \text{ ms}$ ($n = 20$). Antennules were also stimulated
213 with other food extracts made from green crabs, blue mussels and dead *P. elegans*
214 individuals, and responses had the same polarity as those for NP extracts (Fig. 3C).
215 A similar response profile to dead shrimp extract was obtained for the deep
216 hydrothermal shrimp *Mirocaris fortunata* (Fig. 3D).

217 EAG responses to food related odor in *P. elegans* are reproducible, dose-
218 dependent and exhibit sensory adaptation, confirming we indeed recorded olfactory
219 responses. We could not reach the saturation level in the dose-response curve
220 because we reached the saturating concentration of the NP extract. All stimuli we
221 tested (extracts of NP, crab, mussel and dead shrimp) elicited positive EAGs in
222 *P. elegans* whereas insect EAGs are usually negative (Roelofs, 1984), with some
223 exceptions related to the chemical structure of single odorants (e.g. Pavis and
224 Renou, 1990). The EAG response is assumed to represent the summation of
225 receptor potentials of many synchronously activated chemoreceptor neurons
226 (Schneider, 1957; Schneider, 1999; Nagai, 1983; Mayer et al., 1984). As slow
227 electrical events (e.g. receptor potentials) travel better than action potentials due to
228 low-pass filtering of the extracellular space (Bedard et al., 2004), the fact that odor-
229 evoked signals travelled far within the antennule suggests that EAGs in shrimp are
230 also summed receptor potentials. In the terrestrial hermit crab *Coenobita clypeatus*,
231 compounds with different chemical properties elicited EAG responses of opposite

232 polarity (Krång et al., 2012), which has been proposed to result from activation of
233 different signal transduction pathways. In marine lobsters, odors may depolarize or
234 hyperpolarize ORNs, and different pathways are directly linked to opposing outputs:
235 excitatory and inhibitory receptor potentials coexist in the same ORN and the
236 response type (excitation or inhibition) is not a property of the stimulant but it
237 depends on the ORN (Doolin et al., 2001; McClintock and Ache, 1989; Michel et al.,
238 1991). Positive EAG responses were obtained in robber crab, hermit crab and
239 daphnia (Krång et al., 2012; Simbeya et al., 2012; Stensmyr et al., 2005). In the
240 marine hermit crab *P. bernhardus*, EAG responses to amines were positive, and
241 responses to acids were negative (Krång et al., 2012). Here we worked with complex
242 mixtures. Comparison of EAG responses to single odorants will allow determining if
243 different chemical groups also elicit different response polarities in *P. elegans*.
244 Mechanical stimuli elicited negative EAGs. Recordings from single chemoreceptor
245 neurons and single mechanoreceptor neurons could help clarifying why olfactory and
246 mechanical responses have opposite polarities.

247 The development of the EAG method in shrimp opens a large field of possibilities
248 for studying olfaction in decapods and may be other crustaceans. This method is
249 relatively easy to use on species larger than 1 cm, and it allows to work on intact
250 animals. EAG is complementary to other existing electrophysiological methods. It
251 gives a more general idea of the animal sensitivity than single ORN recordings, and
252 is thus relevant in an ecological approach. EAG allows the screening of compounds
253 eliciting electrophysiological response, before testing potential odor-gated behavioral
254 responses.

255

256

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260

261

262 **COMPETING INTERESTS**

263 The authors declare no competing or financial interests.

264

265

266 **AUTHOR CONTRIBUTIONS**

267 P.L., J.R. and M.Z. conceived the study and J.M. drafted the paper. J.M. performed
268 all the experiments, with the help of P.L. All authors participated in data analysis and
269 in writing the manuscript.

270

271

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- 386

387 **FIGURE LEGENDS**

388

389 **Figure 1. Setup, electrode placements and EAG responses from the marine**
390 **shrimp *Palaemon elegans*.**

391 (A) Sketch of the setup used to record EAG responses. The shrimp, restrained in a
392 cut pipette cone, is fixed head down in the bath solution, ventral face to the top, and
393 is perfused with *Panulirus* saline solution near the branchial cavity. The recording
394 electrode is inserted in the antennular lateral flagellum that bears the olfactory
395 aesthetasc sensilla, and the reference electrode is inserted between the telson and
396 the abdomen. The stimulus delivery system is placed approximately 1.5 mm from the
397 lateral flagellum. Shrimp size (from telson tip to eyes) is 2.7 ± 0.4 cm; antennae,
398 antennules and materials are not to scale (B) Sketch of *P. elegans* aesthetascs
399 bearing antennular flagellum with the different electrode placements at the base,
400 middle and apex regions, respectively. Not to scale. (C) EAG amplitudes recorded
401 from the base region to narrow stimuli at different zones along the antennule (Z1, Z2,
402 Z5, $n = 4$; Z3, Z4, $n = 5$). (D) EAG amplitudes recorded from the apex region to wide
403 stimuli at different zones along the antennule (Z1, Z4, Z5, $n = 5$; Z2, $n = 7$; Z3, $n = 6$).
404 (E) EAG amplitudes recorded from the base region with wide stimuli at different
405 zones along the antennule (Z1, Z2, Z3, $n = 7$; Z4, $n = 6$; Z5, $n = 4$). In C, D and E,
406 EAGs were recorded in response to NP stimuli (0.2 g.mL^{-1} of NP extract for 1 s in C,
407 0.5 s in D and E). Fast Green was used to visualize stimulated zones; it did not
408 modify responses to NP stimuli. The gray zone on the antennule sketch represents
409 the aesthetascs bearing area. Means \pm SEM were compared with -Kruskal Wallis test
410 (C, $p = 0.002$; D, $p = 0.0005$; E, $p = 0.02$) and with Student T-test to each stimulated
411 zone. Means with different letters are significantly different ($p < 0.05$).

412

413 **Figure 2. Research of recording parameters.**

414 (A) Superimposed traces of EAG responses to *Panulirus* saline with increasing
415 pressure, the upper trace corresponding to 2 psi, and the followings to 4, 6, 8 and 10
416 psi. (B) EAG pressure-response curve to *Panulirus* saline ($n = 21$ for 2 psi; $n = 24$ for
417 4 psi; $n = 25$ for 6, 8 and 10 psi). (C) Examples of EAG responses to two consecutive
418 NP stimuli (0.1 g.mL^{-1} of NP extract for 1 s at 5 psi) separated by 20, 60 and 90 s. (D)
419 Amplitude of EAG responses to NP stimuli (same as in C) relative to the amplitude of
420 EAG response to a previous identical stimulation, as a function of the time between

421 the two stimuli ($n = 7$ for 10, 60 and 90 s, $n = 9$ for 4 and 40 s; $n = 11$ for 20 s). In A
422 and C, bars indicate the stimulus delivery. Transient peaks are valve opening
423 artefacts. Means \pm SEM were compared with tStudent T-test to reference values (0 in
424 B, 1 in D) and to each condition (B). NS: not significant; *: $P < 0.05$; **: $P < 0.01$; ***:
425 $P < 0.001$.

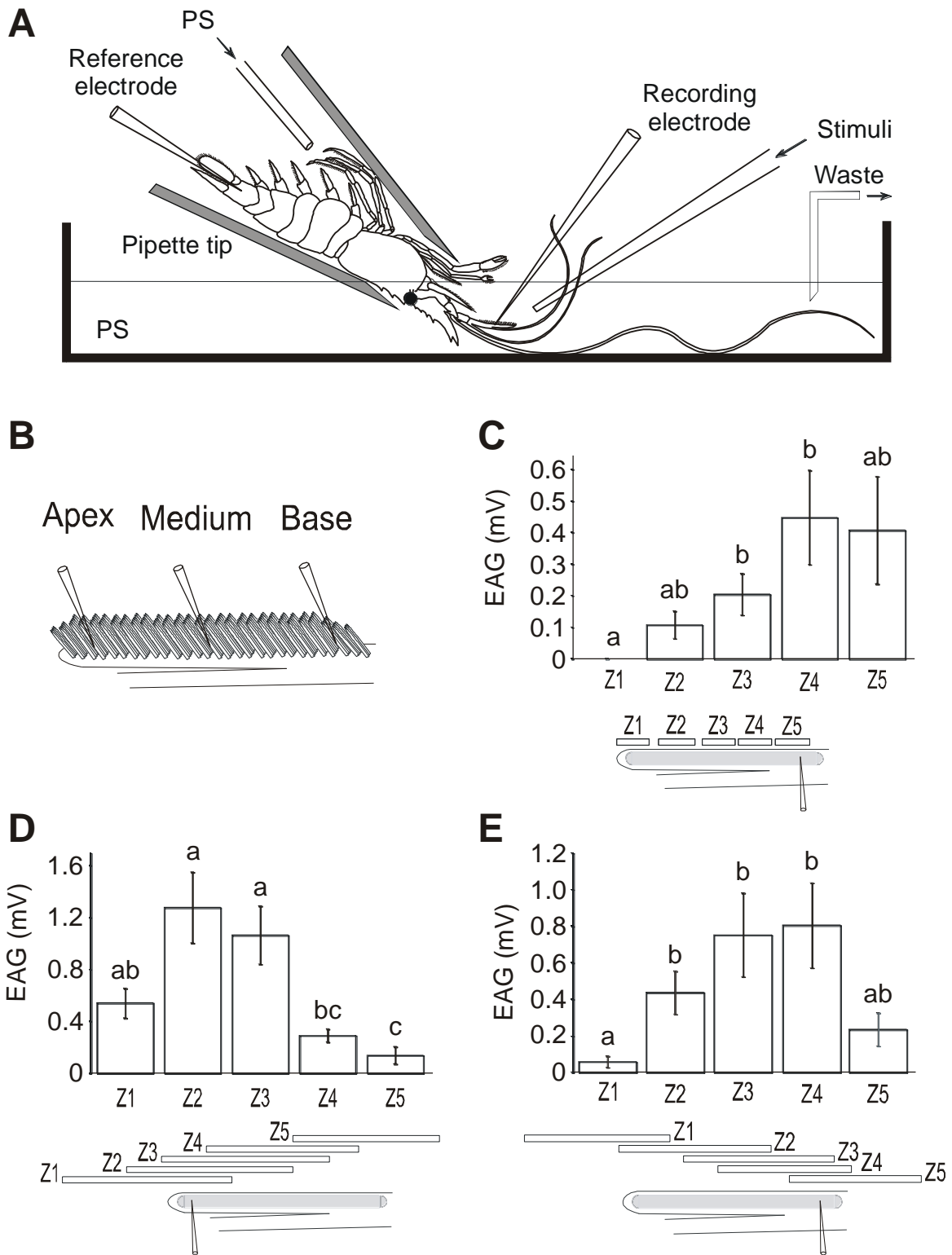
426

427 **Figure 3. EAG responses to odorant extracts in the marine shrimp *Palaemon***
428 ***elegans*.**

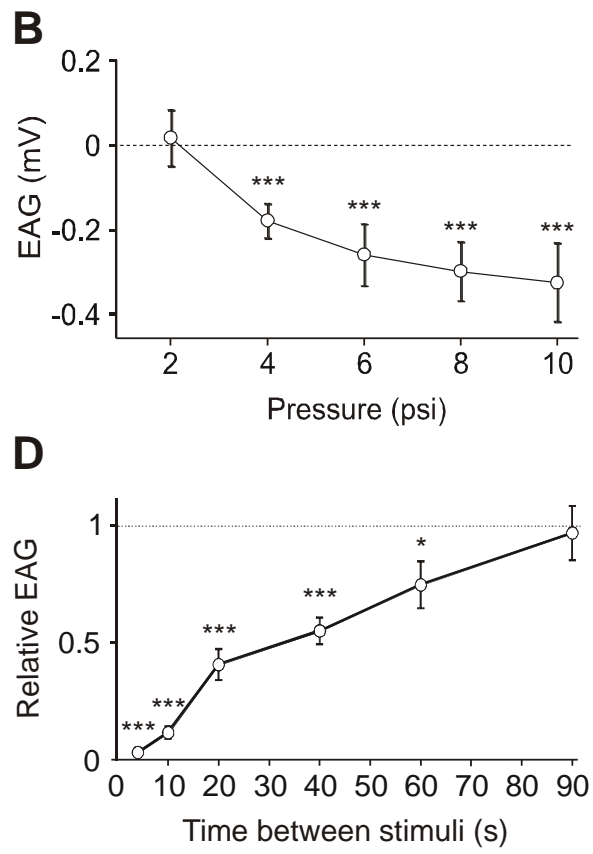
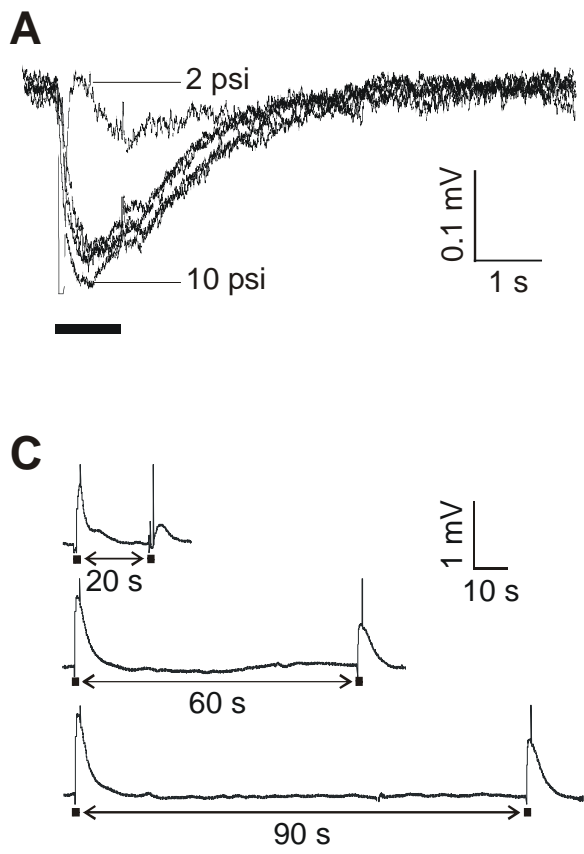
429 (A) Superimposed traces of EAG responses to dilutions of NP extract (0.001, 0.003,
430 0.01, 0.03, 0.05, 0.1 and 0.2 g.mL⁻¹). (B) EAG dose-response curve to dilutions of NP
431 extract ($n = 10$ for 0.05 g.mL⁻¹; $n = 11$ for 0.001 and 0.003 g.mL⁻¹; $n = 12$ for control; n
432 = 14 for 0.2 g.mL⁻¹; $n = 16$ for 0.01, 0.03 and 0.1 g.mL⁻¹). Means \pm SEM were
433 compared with Student Tt-test to control stimuli (PS). *: $P < 0.0$; **: $P < 0.01$; ***:
434 $P < 0.001$. (C) EAG responses to fresh mussel extract, extract of dead *P. elegans*
435 individuals, and fresh green crab extract. The order of stimuli (mussel, crab, dead
436 shrimp) was randomized. (D) EAG response to dead shrimp extract from the deep
437 hydrothermal shrimp *Mirocaris fortunata*. In A, C and D, bars indicate the stimulus
438 delivery. Transient peaks are valve opening artefacts.

439

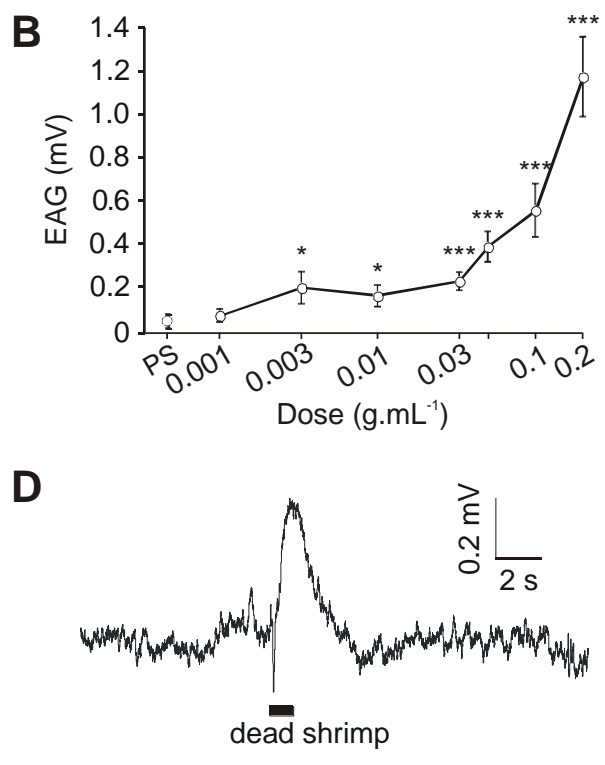
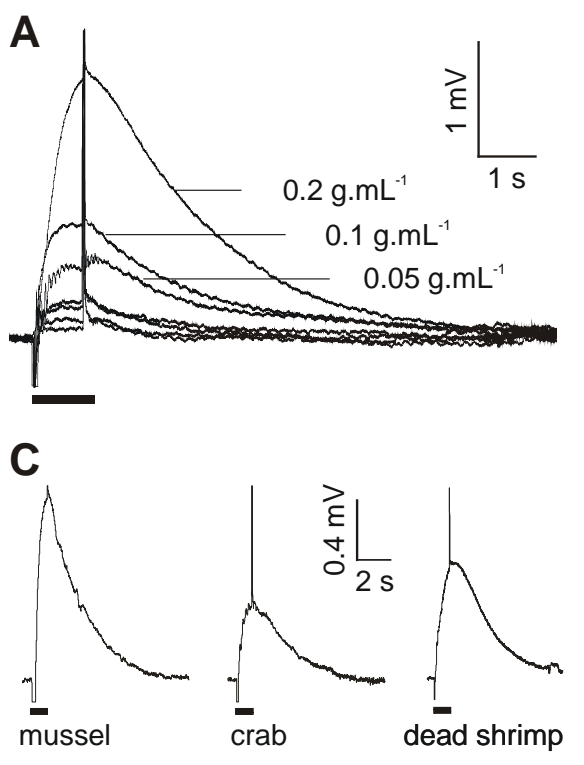
442 **Figure 1.**



444 **Figure 2.**



445
446
447 **Figure 3.**



448