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METHODS & TECHNIQUES

New electroantennography method on a marine shrimp in water

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ABSTRACT

Antennular chemoreception in aquatic decapods is well studied via the recording of single chemoreceptor neuron activity in the antennule, but global responses of the antennule (or antennae in insects) by electroantennography (EAG) has so far been mainly restricted to aerial conditions. We present here a well-established underwater 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* antennular chemoreception in aquatic decapods, in a global approach to supplement current, more specific techniques.

KEY WORDS: Electroantennography, Shrimp, Olfaction, Antennule

INTRODUCTION

Crustaceans, in particular lobsters and crayfishes, have emerged as excellent models for research in chemoreception (Ache, 2002). They rely on antennular chemoreception for diverse behaviors such as food detection and social interactions (Derby and Sorensen, 2008). Their main olfactory organ is the antennule (Derby and Weissburg, 2014), the lateral flagellum of which bears presumably unimodal sensilla specialized in olfaction, the aesthetascs, innervated only by olfactory receptor neurons (ORNs) (Ghiradella et al., 1968). Antennular flagella also bear bimodal sensilla, innervated by mechanoreceptor and chemoreceptor neurons (Schmidt and Derby, 2005). Responses of crustacean chemoreceptor neurons to odor stimuli were classically recorded either extracellularly from their axons (Derby, 1989; Kamio et al., 2005) or intracellularly after the introduction of the patch-clamp method (Ache, 2002; Anderson and Ache, 1985; Bobkov et al., 2012).

Study of antennular chemoreception is essential for understanding the biology of our model species, the blind Alvinocarididae deep-sea hydrothermal shrimp. Despite the fact that the antennal appendages may play a major role in the detection of their environment (Chamberlain, 2000; Jinks et al., 1998; Renninger et al., 1995), there is still little information about their chemosensory sensitivity. Specimens of these species are extremely difficult to collect and maintain alive, and therefore are available in low numbers for experiments. The objective of this work was to develop a method with a high rate of success, allowing the recording of global responses of the antennule, in marine decapods. In insects, such a technique called electroantennography (EAG) (Schneider, 1957) is commonly used to measure the global response profile of antennal ORNs to odor stimuli. This technique is widely used in screening moth pheromones (Roelofs, 1984). In crustaceans, EAG measurements were performed in aerial conditions on two terrestrial crabs, the giant robber crab *Birgus latro* (Stensmyr et al., 2005) and the hermit crab *Coenobita clypeatus* (Krång et al., 2012), and in the marine hermit crab *Pagurus berhnardus* (Stensmyr et al., 2005). Two papers reported very briefly EAG recordings, with no technical demonstration, from freshwater crustaceans: the branchiopoda *Daphnia* spp. (Simbeya et al., 2012) and the crayfish *Procambarus clarkii* (Ameyaw-Akumfi and Hazlett, 1975).

We present here the first demonstration of an underwater EAG method established for shrimp. It was developed on the coastal marine species *Palaemon elegans* (Rathke, 1837) but it can easily be adapted to other marine or freshwater decapods.

MATERIALS AND METHODS

Animals

Caridean shrimp *Palaemon elegans* (Rathke, 1837) were collected from Saint-Malo Bay (France), housed communally in 100 liter aquaria with oxygenated artificial seawater (Red Sea Salt, Red Sea, Houston, TX, USA) at 15.5±0.5°C under a 12 h:12 h light:dark cycle, and fed twice a week with shrimp food pellets [Novo Prawn (NP), JBL, Neuhofen, Germany]. The specimens for experiments were transferred to a 25 liter aquarium maintained at room temperature (21±1°C) for at least 48 h of starvation to prevent any potential adaptation of their chemoreceptor neurons to food odors. The sex and age of animals were not determined.

Biological preparation

The animal was restrained in a 1 or 5 ml pipette tip cut according to the shrimp's size with the antennal appendages and the posterior part of the abdomen out at each extremity of the cone (Fig. 1). The shrimp was placed ventral side up. The preparation was attached to a UM-3C micromanipulator (Narishige, London, UK) and angled at approximately 45 deg so that the anterior part of the animal (i.e. antennal appendages) was submerged in a Petri dish filled with *Panulirus* saline (PS) and the posterior part (i.e. telson and abdomen) remained in air. The composition of PS was (in mmol l^{-1}): 486 NaCl, 5 KCl, 13.6 $CaCl_2$, 9.8 $MgCl_2$ and 10 Hepes, pH 7.8–7.9 (Hamilton and Ache, 1983). A gravity-fed PS perfusion was inserted in the pipette tip just over the cephalothorax, to irrigate the branchial cavity and keep the animal alive, and to renew the PS bath solution. The antennules were immobilized with U-shape tungsten hooks on a piece of Styrofoam stuck to the bottom of the Petri dish. The preparation was visualized under a dissecting microscope (M165C, Leica, Nanterre, France). Experiments were performed at room temperature (21±1°C).

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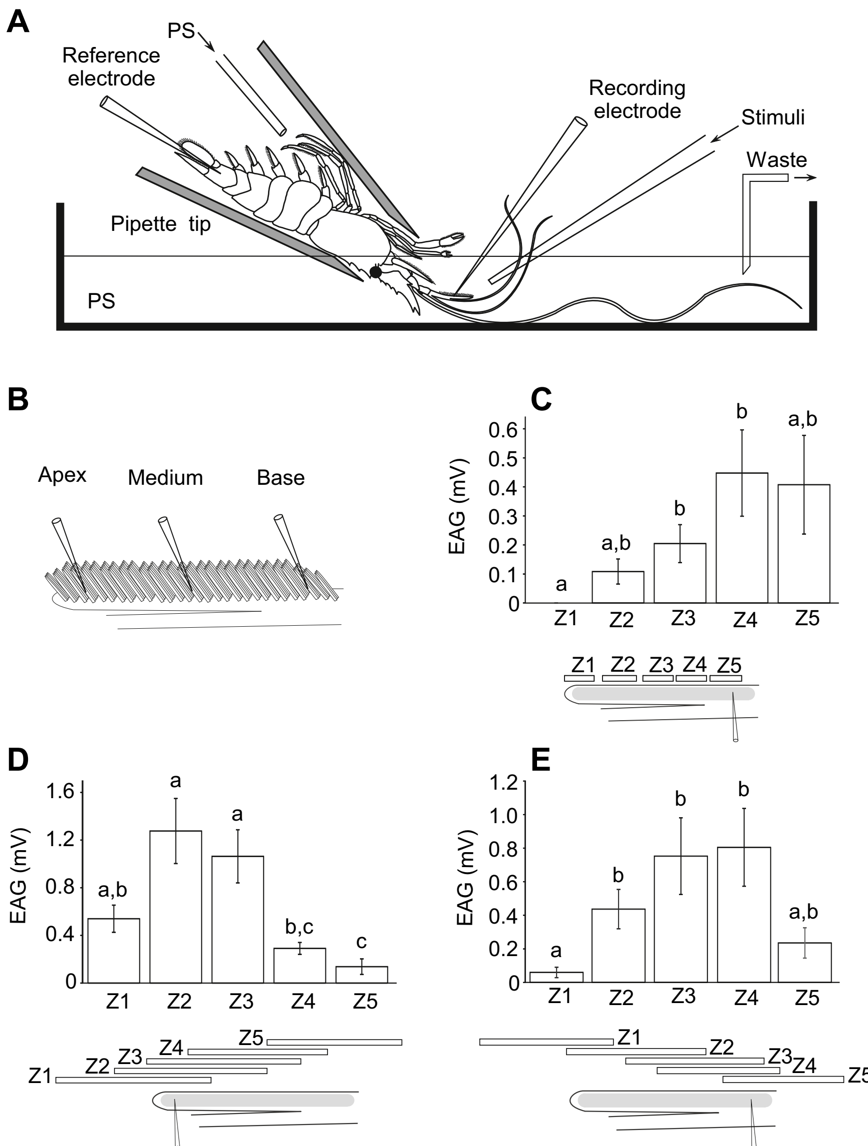

Fig. 1. Setup, electrode placements and electroantennography (EAG) responses from the marine shrimp *Palaemon elegans*. (A) Sketch of the setup used to record EAG responses. The shrimp, restrained in a cut pipette cone, is fixed head down in the bath solution, ventral side up, and is perfused with *Panulirus* saline (PS) solution near the branchial cavity. The recording electrode is inserted in the antennular lateral flagellum that bears the olfactory aesthetasc sensilla, and the reference electrode is inserted between the telson and the abdomen. The stimulus delivery system is placed approximately 1.5 mm from the lateral flagellum. Shrimp size (from telson tip to eyes) is 2.7±0.4 cm; antennae, antennules and materials are not to scale. (B) Sketch of *P. elegans* aesthetascs bearing antennular flagellum with the different electrode placements at the base, middle and apex regions. Not to scale. (C) EAG amplitudes recorded from the base region to narrow stimuli at different zones along the antennule (Z1, Z2, Z5, *n*=4; Z3, Z4, *n*=5). (D) EAG amplitudes recorded from the apex region to wide stimuli at different zones along the antennule (Z1, Z4, Z5, *n*=5; Z2, *n*=7; Z3, *n*=6). (E) EAG amplitudes recorded from the base region with wide stimuli at different zones along the antennule (Z1, Z2, Z3, *n*=7; Z4, *n*=6; Z5, *n*=4). In C–E, EAGs were recorded in response to Novo Prawn (NP) stimuli (0.2 g ml^{-1} of NP extract for 1 s in C, 0.5 s in D and E). Fast Green was used to visualize stimulated zones; it did not modify responses to NP stimuli. The gray zone on the antennule sketch represents the aesthetascs bearing area. Means±s.e.m. were compared with a one-way ANOVA with permutation test (C, P=0.04; D, P=0.002; E, P=0.01) and by multiple comparisons with two-sample permutation *t*-tests; Benjamini–Hochberg corrections were applied. Means with different letters are significantly different (P<0.05).

Electrophysiological recordings and data analysis

Electrodes were pulled from GB150F-8P glass capillaries (Science Products, Hofheim, Germany) using a P-97 puller (Sutter Instrument, Novato, CA, USA). They had a tip diameter of 1 to 2.5 µm and were filled with PS. The reference electrode was introduced through the soft articular membrane between the telson and the abdomen. The recording electrode was inserted with a NMM-25 micromanipulator (Narishige, London, UK) in the base, middle or apex region of the flagellum area bearing the aesthetascs, between two aesthetasc rows (Fig. 1B). Signals were amplified (×100) and filtered (0.1–1000 Hz) using an EX1 amplifier with a 4002 headstage (Dagan, Minneapolis, MN, USA), and digitized at 2 kHz by a 16-bit acquisition board (Digidata 1440A) under Clampex 10.3 (Molecular Devices, Sunnyvale, CA, USA). Data were analyzed using Clampfit (Molecular Devices). Signals were low-pass filtered offline at 20 Hz. Data are given as means±s.e.m.

Chemical stimuli preparation and delivery

An aqueous extract of shrimp food NP was used as odorant in most experiments. NP pellets were dissolved for 48 h at room temperature at 0.2 g ml^{-1} in PS. The extract was then centrifuged at 5900 ***g*** for 10, 15 and 20 min and the supernatant was collected after each centrifugation and filtered (0.45 µm), aliquoted and stored at −20°C until use. For dose–response experiments, NP extracts were diluted from 1:2 to 1:200 with PS. Aqueous extracts of green crab *Carcinus maenas*, blue mussel *Mytilus edulis* and dead *P. elegans* individuals were prepared from fresh material at approximately 0.1, 0.5 and 0.6 g ml^{-1} in PS, respectively, using the same protocol as for NP extract. Osmolarity (1050 mOsm l^{-1} with mannitol) and pH (7.85 with NaOH) were adjusted for all solutions.

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

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

RESULTS AND DISCUSSION

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

Determination of recording parameters

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

To estimate whether the recording electrode sampled the responses from a large or small portion of the antennule (i.e. global versus local response), different portions were stimulated with 0.2 g ml^{-1} of NP extract. When narrow stimuli were applied along the antennule while recording from the base region (Fig. 1C), the electrode recorded responses from zones stimulated far from the electrode location. Then, increasing lengths of the antennule were stimulated by moving a larger stimulation capillary along the antennule while recording from the apex (Fig. 1D) or the base (Fig. 1E) regions. For both electrode placements, the EAG amplitude increased with the size of the stimulated area. Thus, there is a good propagation of the chemosensory response within the antennule, so a large fraction of receptor neurons can be recorded. To maximize the amplitude of recorded responses (i.e. to record the largest number of receptor neurons), subsequent experiments were performed by placing the recording electrode in the middle region of the antennule (Fig. 1B).

In decapods, the antennule is not only equipped with unimodal (chemosensory) aesthetascs housing ORNs. It also carries bimodal (chemo- and mechanosensory) non-aesthetasc sensilla; however, in much lower density than aesthetascs (Cate and Derby, 2001; Hallberg et al., 1997; Obermeier and Schmitz, 2004; Steullet et al., 2002). Hence, both neurons innervating the aesthetascs and the non-aesthetasc sensilla can contribute to recorded chemical responses; selective ablation experiments could clarify this point. To verify whether control responses were mechanical, PS flows were applied at increasing pressures (2 to 10 psi). PS responses were pressure-dependent, with no significant response for the lowest pressure (2 psi) and significant response for pressure values of 4 psi and more (Fig. 2A,B), indicating that PS responses were likely mechanical. The response increase from 4 (−0.17±0.04 mV, n=24) to 10 psi (−0.32±0.09 mV, n=25) was not significant (one-way ANOVA with permutation test, P=0.8) because of a high variability of the amplitude of PS responses across recordings. We thus decided to adjust the pressure of all stimuli to 5 psi to facilitate stimulus access to aesthetascs through their dense packing and to distributed chemosensilla without eliciting strong mechanical responses that would have impeded the correct measurement of chemical responses.

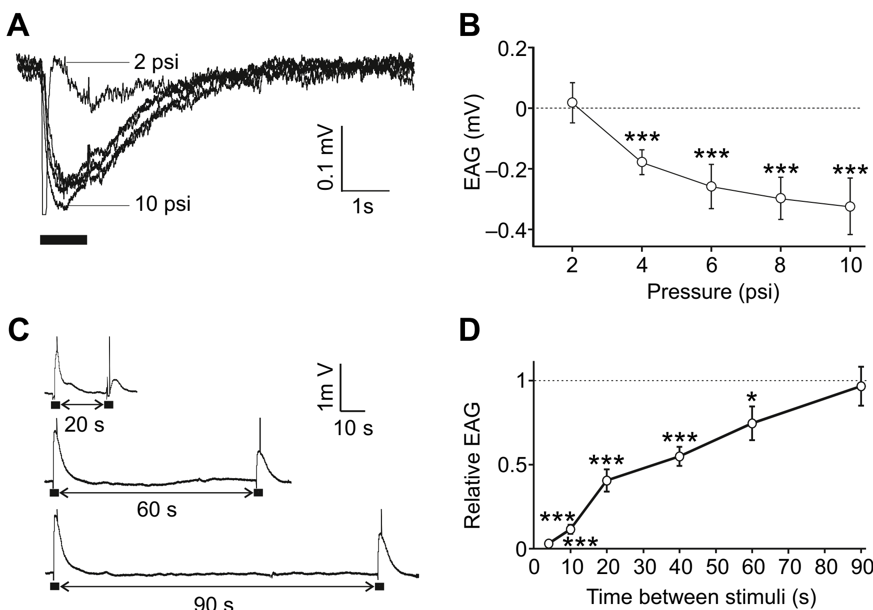

Fig. 2. EAG responses to mechanical and consecutive chemical stimuli. (A) Superimposed traces of EAG responses to *Panulirus* saline with increasing pressure, the upper trace corresponding to 2 psi, and the others to 4, 6, 8 and 10 psi. (B) EAG pressure–response curve to *Panulirus* saline (n=21 for 2 psi; n=24 for 4 psi; n=25 for 6, 8 and 10 psi). (C) Examples of EAG responses to two consecutive NP stimuli (0.1 g ml^{-1} of NP extract for 1 s at 5 psi) separated by 20, 60 and 90 s. (D) Amplitude of EAG responses to NP stimuli (same as in C) relative to the amplitude of EAG response to a previous identical stimulation, as a function of the time between 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 and C, bars indicate the stimulus delivery. Transient peaks are valve opening artefacts. Means±s.e.m. were compared with a one-way ANOVA with permutation test (B, P=0.004; D, $P<10^{-15}$), and with a one-sample permutation t-test to reference values (0 in B, 1 in D). *P<0.05; ***P<0.001.

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Responses of chemoreceptor neurons depend not only on the stimulus characteristics (quality, quantity), but also on previous chemosensory experience via the process of adaptation (Kaissling et al., 1987). When chemoreceptor neurons are adapted by a stimulus, responses to subsequent stimuli are reduced. The recovery from adaptation is time-dependent. To define a time interval between consecutive stimuli that could prevent measuring responses from adapted sensory neurons, we measured EAG responses to pairs of identical stimuli (0.1 g ml^{-1} of NP extract) with increasing inter-stimulus intervals (4 to 90 s) (Fig. 2C,D). As the inter-stimulus interval was increased, the average amplitude of the response to the second stimulus increased and eventually did not differ significantly from the amplitude of the first response when the interval was at least 90 s. For safety, in the following we kept an interval of at least 2 min between consecutive stimuli.

EAG responses according to defined recording parameters

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

EAG responses to food-related odor in *P. elegans* are reproducible, dose-dependent and exhibit sensory adaptation, confirming we indeed recorded chemosensory responses. We could not reach the saturation level in the dose–response curve because we reached the saturating concentration of the NP extract. All stimuli we tested (extracts of NP, crab, mussel and dead shrimp) elicited positive EAGs in *P. elegans* whereas insect EAGs are usually negative (Roelofs, 1984), with some exceptions related to the chemical structure of single odorants (e.g. Pavis and Renou, 1990). The EAG response is assumed to represent the summation of receptor potentials of many synchronously activated chemoreceptor neurons (Schneider, 1957, 1999; Nagai, 1983; Mayer et al., 1984). As slow electrical events (e.g. receptor potentials) travel better than action potentials because of low-pass filtering of the extracellular space (Bedard et al., 2004), the fact that odor-evoked signals travelled far within the antennule suggests that EAGs in shrimp are also summed receptor potentials. In the terrestrial hermit crab *Coenobita clypeatus*, compounds with different chemical properties elicited EAG responses of opposite polarity (Krång et al., 2012), which has been proposed to result from activation of different signal transduction pathways. In lobsters, odors may depolarize or hyperpolarize ORNs, and different pathways are directly linked to opposing outputs: excitatory and inhibitory receptor potentials coexist in the same ORN and the response type (excitation or inhibition) is not a property of the stimulant but it depends on the ORN (Doolin et al., 2001; McClintock and Ache, 1989; Michel et al., 1991). Positive EAG responses were obtained in robber crab, hermit crab and daphnia (Krång et al., 2012; Simbeya et al., 2012; Stensmyr et al., 2005). In the marine hermit crab *P. bernhardus*, EAG responses to amines were positive, and responses to acids were negative (Krång et al., 2012). Here we worked with complex mixtures. Comparison of EAG responses to single odorants will allow determining whether different chemical groups also elicit different response polarities in *P. elegans.* Mechanical stimuli elicited negative EAGs. Recordings from single chemoreceptor neurons and single mechanoreceptor neurons could help clarify why chemical and mechanical responses have opposite polarities.

The development of the EAG method in shrimp opens a large field of possibilities for studying antennular chemoreception in decapods and maybe other crustaceans. This method is relatively easy to use on species larger than 1 cm, and it enables work on intact animals. EAG is complementary to other existing electrophysiological methods. It gives a more general idea of the animal sensitivity than single chemoreceptor neuron recordings, and is thus relevant in an ecological approach. EAG allows the screening of compounds eliciting electrophysiological response, before testing potential odor-gated behavioral responses.

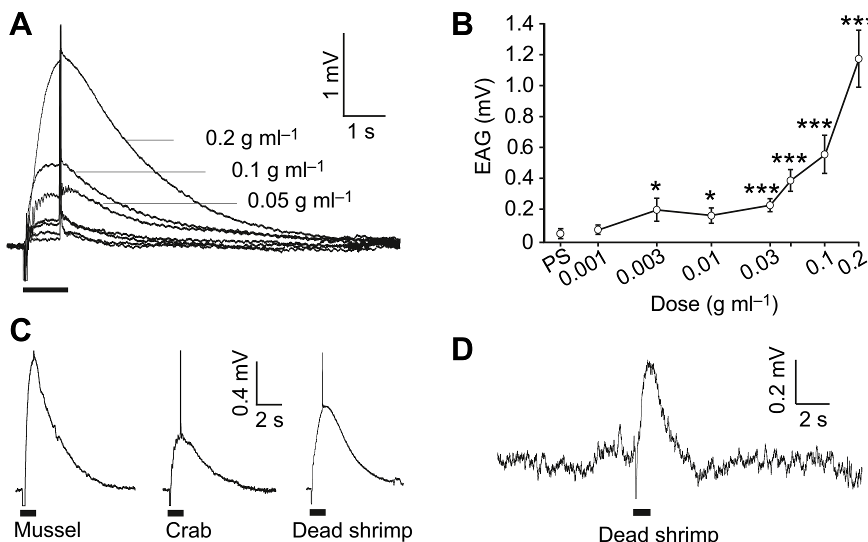

Fig. 3. EAG responses to odorant extracts in the marine shrimp *Palaemon elegans*. (A) Superimposed traces of EAG responses to dilutions of NP extract (0.001, 0.003, 0.01, 0.03, 0.05, 0.1 and 0.2 g ml^{-1}). (B) EAG dose–response curve to dilutions of NP extract (*n*=10 for 0.05 g ml^{-1}; *n*=11 for 0.001 and 0.003 g ml^{-1}; *n*=12 for control; *n*=14 for 0.2 g ml^{-1}; *n*=16 for 0.01, 0.03 and 0.1 g ml^{-1}). Means±s.e.m. were compared with a one-way ANOVA with permutation test ($P<10^{-15}$), and with a two-sample permutation *t*-test to control stimuli (PS). **P*<0.05; ****P*<0.001. (C) EAG responses to fresh mussel extract, fresh green crab extract and extract of dead *P. elegans* individuals. The order of stimuli (mussel, crab, dead shrimp) was randomized. (D) EAG response to dead shrimp extract from the deep hydrothermal shrimp *Mirocaris fortunata*. In A, C and D, bold horizontal bars indicate the stimulus delivery. Transient peaks are valve opening artefacts.

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Competing interests
The authors declare no competing or financial interests.

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

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