A method for selective stimulation of leg chemoreceptors in whole crustaceans

Running title: Leg response in crustaceans

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Summary statement:

The presented technique opens possibilities for further studies on sensory-motor integration evoked by leg stimulation in whole aquatic animals under natural conditions to supplement, with a direct approach, current ablation/silencing techniques.

Abstract

The integration of sensory information with adequate motor outputs is critical for animal survival. Here, we present an innovative technique based on a non-invasive closed-circuit device consisting of a perfusion/stimulation chamber chronically applied on a single leg of the crayfish *Procambarus clarkii*. Using this technique, we focally stimulated the leg inside the chamber and studied the leg-dependent sensory-motor integration involving other sensory appendages, such as antennules and maxillipeds, which remain unstimulated outside the chamber.

Results show that the stimulation of a single leg with chemicals, such as disaccharides, is sufficient to trigger a complex search behaviour involving locomotion coupled with the reflex activation of antennules and maxillipeds.

This technique can be easily adapted to other decapods and/or other sensory appendages. Thus, it has opened possibilities for studying sensory-motor integration evoked by leg stimulation in whole aquatic animals under natural conditions to supplement, with a direct approach, current ablation/silencing techniques.

INTRODUCTION

The ability of animals to successfully cope with complex and dynamic habitats depends on their prompt production of adaptive responses that integrate sensory information with adequate motor outputs as they search for suitable resources and avoid potentially dangerous contexts (Proekt et al., 2008; Hoke et al., 2017; Chen and Hong, 2018). In this regard, motor control is closely linked to two sensory modalities, namely, chemo- and mechanoreception and chemo/hydrodynamic sensory-motor integration can be comprehensively studied using decapod crustaceans, including lobsters and crayfish, as excellent models (Mellon, 2012, Schmidt and Mellon, 2011). These organisms rely on waterborne chemical cues to produce appropriate behavioural responses, such as social communication, orientation, predator avoidance, sex recognition and localisation of suitable habitats and food resources in their environment (Hartman and Hartman, 1977; Schmidt and Mellon, 2011; Thiel and Breithaupt, 2011; Peddio et al., 2019). Stimulus detection is mediated by a large number of peripheral chemoreceptor neurons (CRNs) grouped within a vast array of cuticular sensory hairs called sensilla. These hairs are mainly located on cephalothoracic appendages, such as antennae, maxillipeds (mouthparts), antennules and pereiopods (major claws and walking legs) (Schmidt and Mellon, 2011).

The biramous antennules of decapods are considered the primary sensory organs for olfactory chemoreception. On their outer flagellum, they exclusively contain the aesthetasc sensilla, which are innervated by hundreds of CRNs. They mediate many complex odour-evoked behaviours, such as pheromone-guided courtship, social recognition, aggregation, agonistic interactions, alarm responses, (; Gleeson, 1982; Johnson and Atema, 2005; Horner et al., 2008; Shabani et al., 2008; Bauer, 2011; Solari et al., 2017), associative learning (Steullet et al., 2002) and food search (Steullet et al., 2001).

A well-known stereotypical behaviour exhibited by decapods is antennular flicking, which is a sniffing strategy that they apply so that they can explore their water environment to detect relevant chemical cues (Schmitt and Ache, 1979; Reidenbach et al., 2008). It consists of rapid alternating downward and upward movements of the aesthetasc-bearing flagellum. This organ helps reset the sensitivity of fast-adapting CRNs by continuously exposing them to novel aliquots of odour-containing fluid. An increase in the basal antennular flicking in crustaceans classically indicates the presence of a chemical (Daniel and Derby, 1991). Other appendages, such as maxillipeds, are involved in chemical detection because of their specific sensitivity to chemical stimuli (Garm et al., 2005). Furthermore, they indirectly play a role in this process because of their function as fan organs that can generate water currents and facilitate the transfer of chemical cues to nearby chemosensors in stagnant environments (Denissenko

et al., 2007, Breithaupt, 2011). In crayfish, CRNs distributed in the legs significantly contribute to sexual discrimination (Belanger and Moore, 2006). They also serve as potential food detectors because of their marked sensitivity to a number of disaccharides and amino acids (Corotto and O'Brien, 2002; Solari et al., 2015). However, leg sensitivity was indirectly assessed through a whole-animal bioassay based on the chemosensory block of legs or electrophysiological determination on an isolated appendage. As such, these approaches have not provided information about the specific contribution of the legs to the overall circuitry of sensory-motor integration involving other CRNs-containing organs, such as antennules and maxillipeds.

Here, we present an innovative technique to evaluate the behavioural responses evoked by chemical stimulation of single legs in a whole decapod crustacean. It is based on a non-invasive closed-circuit device consisting of a perfusion/stimulation chamber that can be chronically applied on a single leg of animals. In this way, the focal perfusion of chemoreceptors is limited to the leg inside the chamber. This approach may also help obtain more information on complex leg-dependent sensory-motor integration that involves or recruits other sensory appendages, such as antennules and maxillipeds, of a whole animal underwater, that is under natural environmental conditions. Even if this technique has been developed on red swamp crayfish *Procambarus clarkii*, it can be easily adapted to other marine or freshwater decapods and even other sensory appendages.

MATERIALS AND METHODS

Animal collection and rearing conditions

Wild intermoult adult red swamp crayfish *P. clarkii* of both sexes (35–40 mm in carapace length) were collected using a backpack electrofishing unit (5.2–2.8 A, 230–400 V and 1300 W) in Molentargius-Saline Regional Natural Park (Southern Sardinia, Italy) during the spring season in 2019. The crayfish were kept in Plexiglass® tanks (100 cm long, 50 cm wide and 20 cm deep) containing 60 L of aerated and bio-conditioned (Aquasafe, Tetra, Melle, Germany) tap water at 22–23 °C in a 16 h light/8 h dark photoperiodic regime and fed with lettuce, squid or a highly appetitive commercial pellet food (Shrimps Natural, SERA, Heinsberg, Germany) three times a week. Uneaten food was removed within 1 h after delivery. The crayfish were not fed for 48 h preceding the experiment to prevent the potential adaptation of their CRNs to food odours. Individuals were kept separated to avoid the reciprocal exposure of males and females and prevent attacks or cannibalism.

Design and application of the device for the perfusion/stimulation of single crayfish legs

A closed-circuit device consisting of a chamber that could be applied chronically on the leg and thus induce exclusive perfusion and stimulation was developed to chemically stimulate the chemoreceptors from only one crayfish leg at a time (Fig. 1).

The crayfish were removed from their tanks and immobilised dorsal side up on a rigid support by using Velcro® strips to expose the selected leg for the device application. The perfusion/stimulation chamber was composed of a segment of a flexible silicone tube for aquaristic use (AH 50-400 Air Pump Hose, Tetra, Melle, Germany; diameter 4/6 mm inside/outside and a suitable length of typically 2.5–3 cm) applied on the leg and nicely fitted to its two distal-most segments, the propodus (comprising a movable finger, i.e. the dactyl) and the carpus (Fig. 1A) with their chemosensillar populations. With the aid of a stereomicroscope (Stemi 2000-C, Zeiss, Oberkochen, Germany), two more thin flexible silicon tubes (code 289201300, Carlo Erba, Milan, Italy; diameter 0.5/1.3 mm inside/outside) running parallel to the merus were inserted into the chamber throughout its proximal end (Fig. 1B). The chamber and the two flexible tubes were secured onto the carpus by using 5 min epoxy resin (Devcon®). The distal end of the chamber was left open for the continuous hydration of the distal tip of the leg, but it was closed during the experiments with a removable male Luer Lock cap from a standard infusion set (Pentaven Scalp Vein Set, Pentaferte, Ferrara, Italy), in order to ensure a closed circuit for the exclusive perfusion/stimulation of the CRNs housed in the leg inside the chamber (hereafter referred to as the intubated leg). The two thin tubes were previously cut at a suitable length (typically 10-11 cm) so that they opened within the chamber at the distal and the proximal ends of the leg propodus. They guaranteed continuous perfusion in the entire portion of the intubated leg by acting as the inflow and outflow terminals through which water and chemical stimuli could be respectively delivered into and removed from the chamber. The two thin silicone tubes were further secured to the merus and the carapace surface by using cyanoacrylate glue (Loctite, Super Attak Power Flex) and were connected to suitable fittings, consisting of the terminal female Luer Lock PVC fittings from the abovementioned infusion set (Fig. 1C).

Through these fittings, during the experiments the inflow and the outflow terminals could be respectively connected, by way of male Luer Lock fittings (code 13160-100, WPI, Friedberg, Germany) and Tygon® tubing (code T3601-13, Saint-Gobain; diameter 0.8/2.4 mm inside/outside) to the perfusion system for stimulus delivery (tube length of about 30 cm) and to the wastewater collection system (tube length of about 100 cm). The latter system consisted of a 1 L plastic bottle

placed below the experimental station, where wastewaters and chemicals coming from the device could be collected.

After the device was applied (application procedures lasted about 25-30 min), the crayfish were placed into a dry container, and the glue was cured for 1 h before the crayfish were returned to their holding tanks. Chelipeds of treated animals were always kept banded with Parafilm® (Sigma-Aldrich, Milan, Italy) strips in order to prevent them from removing the device or cutting the thin tubes connected to it. Even if the actual level of disturbance caused by the device to the animals could not be exactly quantified, from a visual analysis the treated animals appeared to be able to walk, move, eat and exhibit other basic behaviours such as grooming, regardless of the device presence. None of the treated animals took off the device during the experimental time. All these aspects may therefore be considered as advantages of this device, the only drawback consisting of its loss when the crayfish moults.

After leg intubation, the crayfish were acclimated for at least 3 days before they were used in the experiments. Only the second/right pereiopod was intubated and utilised throughout this study. Dye tests were performed to verify the effectiveness of the closed-circuit perfusion/stimulation device (Movie 1). At the end of the experiments, the device fittings were unplugged from the perfusion system, and the crayfish were transferred to the holding tank.

Stimulation and supply protocol

The crayfish were individually exposed to the test compounds in Plexiglass® tanks (20 cm long × 15 cm wide × 10 cm deep) containing about 2 L of tap water (22–23 °C). At the beginning of each experiment, the perfusion/stimulation device was connected to a peristaltic pump (Minipuls Evolution, Gilson, Milan, Italy) operating at a flow rate of 5 mL/min and ensuring the hydrostatic pressure for the flow of fluids throughout the whole circuit, up to the wastewater collection system.

The crayfish were then allowed to acclimatise until they became motionless, which typically occurred within 15 min. Aliquots of the stimuli were administered by switching the perfusion/stimulation system from tap water to a different reservoir, and each crayfish was given 1 min to respond, which began from the time the stimulus entered the perfusion/stimulation chamber. A recovery interval of 3 min was set between two successive stimulations to minimise adaptation effects.

Trehalose, maltose and cellobiose (Sigma-Aldrich, Milan, Italy), which are disaccharides commonly known to elicit responses from the leg CRNs of *P. clarkii* (Corotto and O'Brien, 2002; Solari et al., 2015, 2018), were used as stimuli. They were dissolved in tap water at 10^{-1} mol/L, frozen and stored as

stock solutions. On the day of the experiments, the stock solutions were thawed and serially diluted in tap water to obtain three different concentrations: 10^{-5}, 10^{-3} and 10^{-1} mol/L, which were supplied at increasing concentrations. The experiments at each sugar concentration were performed on 14 crayfish. Before stimulation, the response of each crayfish to the same aliquot of tap water (blank, negative control) was monitored (Movie 2). At the end of each stimulation series, a food extract was tested as a positive control (Movie 3), and the crayfish that did not respond to the food were excluded from data analysis. The food extract was prepared as follows: the same commercial pellet food used for rearing crayfish was finely hashed and suspended (10 mg/mL) in tap water, vortexed for 3 min at 30 Hz (TecnoKartell TK3S, Kartell, Milan, Italy) and centrifuged for another 2 min at 10,000 rev/min (Minispin, Eppendorf, Hamburg, Germany). The supernatant was then filtered, frozen and stored until it was used for stimulation.

Trials were video recorded for later analysis by using a Samsung SMX-F34 (Samsung, Seoul, Korea) colour digital camera mounted above the test tank. Video recordings were analysed by an independent observer blinded to the experimental treatment.

Behavioural responses were determined by measuring a three-level ranking score partly in accordance with the methods of Kreider and Watts (1998) and Solari et al. (2015): 1) the duration of the movement of the walking legs (seconds × min^{-1}), 2) the rate of antennular flicking (flicks × min^{-1}) and 3) the duration of the movement of maxillipeds (seconds × min^{-1}).

Statistical analysis

Data are expressed as mean ± SEM. They did not conform to normal distribution (Kolmogorov-Smirnov test for goodness of fit) and non-parametric statistics were therefore used. For each of the three appendage types (leg, maxilliped or antennule), significant response differences between consecutive stimulus concentrations and between each stimulus concentration and the relative blank or food controls were calculated using the Wilcoxon matched pairs signed ranks test. The Spearman rank test was used for correlation analysis in the responses of the different appendage types. All statistical analyses were carried out by using the Prism program (GraphPad Software, San Diego, CA, USA). P values <0.05 were considered significant.

RESULTS AND DISCUSSION

In the present study, we implemented a new technique for evaluation of the behavioural responses evoked by chemical stimulation of single legs in a decapod crustacean, the freshwater red swamp crayfish *P. clarkii*, whilst keeping the whole animal and the stimulus in their natural environment, underwater. This technique is based on a non-invasive, chronic, closed-circuit device consisting of a perfusion/stimulation chamber successfully applied on a single leg of the crayfish. This device guarantees a reliable and focal perfusion coupled with chemical stimulation of the chemoreceptors from the intubated leg, whilst all other sensory appendages/organs of the animal remained unstimulated (Fig. 1). This experimental approach also provides evidence of the existence of a reflex activation of antennules and maxillipeds in the leg-dependent sensory-motor circuitry.

Currently, the sensitivity profiles of CRNs from different appendage types in whole-animal bioassays are assessed through indirect approaches, which mainly involve ablation or experimental silencing of selected appendages (for a review, see Schmidt and Mellon, 2011). Therefore, the proposed technique is innovative.

Response of a single leg to chemical stimulation

After being acclimated in the experimental tank, the leg-intubated crayfish became nearly motionless. In the absence of chemical stimulation, they displayed only a basal level of antennular flicking or grooming activity. However, the focal stimulation of the single leg with the highest concentration (10^{-1} M) of trehalose, cellobiose and maltose evoked a stereotyped response initially characterised by the oscillatory movements of the stimulated leg (Fig. 2). This response was immediately followed by the movements of the other unstimulated legs that were outside the perfusion/stimulation device and then culminated in a prolonged locomotory phase of the animal within the experimental arena. The duration of leg movements elicited by trehalose (44.1 ± 5.2 s/min), maltose (41.4 ± 4.0 s/min) and cellobiose (23.7 ± 5.9 s/min) at 10^{-1} M was significantly longer than that of the blank control (2.21 ± 1.31 s/min). In the case of trehalose and maltose, the level of leg activation was even comparable with that triggered by food (42.4 ± 3.9 s/min), which is a highly appetitive and stimulating compound for crayfish and thus selected as a known responsiveness control. Conversely, none of the tested sugars evoked any significant responses when they were focally supplied to the leg at the two lower concentrations (10^{-3} and 10^{-5} M). Previous electrophysiological experiments on dissected legs and behavioural trials on whole animals showed that the legs of this crayfish species are markedly sensitive to trehalose, cellobiose and maltose (Corotto and O'Brien, 2002; Corotto et al., 2007; Solari et al., 2015). In

behavioural trials, disaccharides were effective even at lower concentrations, consistent with the fact that they were supplied in order to stimulate the whole crayfish or all the legs and not just one of them as in the present study.

Here, we disregard the actual contribution of each leg to the overall detection of a given compound, or whether all the legs may contribute to the same extent. Our results show that chemical detection by a single leg of crayfish is sufficient to start searching behaviour although an increase in the number of the legs included in the detection likely enhances the overall sensitivity and thus reduces the threshold to evoke a behavioural response. Previous studies involving chemosensory blocking procedures reported that the major chelae and the first walking legs of male crayfish participate in sexual discrimination and female odour recognition (Belanger and Moore, 2006). To date, our technique is the first to be used for investigating the behavioural responses evoked by chemical stimulation of single legs of whole crayfish within their natural environment taking advantage of a direct stimulatory approach.

Under this experimental condition, crayfish exhibited a locomotor phase characterized by the lack of any precise orientation and quite different from the stereotyped sniffing and well-oriented search strategy that they use when they track a three-dimensional plume of odour, which spreads underwater under natural conditions (Moore and Grills, 1999, Steele et al., 1999; Palmas et al., 2019).

Reflex activation of antennules and maxillipeds coupled with leg stimulation

In addition to the activating movements of all pereiopods leading to crayfish locomotion, a reflex response of maxillipeds and antennules that were not intubated was produced by the focal stimulations of the single legs with the tested sugars. As shown in Fig. 3A, all the disaccharides at the highest tested concentration evoked a reflex activity of maxillipeds (40.8 ± 5.3, 34.7 ± 6.5 and 22.9 ± 6.3 s/min for trehalose, maltose and cellobiose, respectively). This activity was significantly longer than that observed after the blank stimulation of the leg (2.2 ± 1.3 s/min). The response of maxillipeds to trehalose and maltose was comparable with that observed in leg stimulation using food (40.6 ± 5.4 s/min). Interestingly, the Spearman rank test showed that the maxilliped reflex activity was positively correlated with the leg response to all the tested sugars (Spearman r_s = 0.81, 0.77 and 0.85 for trehalose, maltose and cellobiose, respectively; $p < 0.0001$ in all cases).

Even if the maxillipeds of other crustaceans, such as lobsters, were not specifically tested with these disaccharides, these sensory appendages are known to contain chemosensory neurons that are mainly tuned to a number of nitrogen-containing compounds with low molecular weight, such as amino acids,

and other feeding stimulants, including amines, nucleotides and peptides (Corotto et al., 1992; Garm et al., 2005). These compounds are usually present in the tissues of lobster preys and considered good indicators of high-quality food according to their carnivorous habits (Zimmer-Faust, 1993; Schmidt and Mellon, 2011). Maxillipeds are also indirectly involved in chemical detection because of their role as fan organs that may generate water currents; consequently, they facilitate the transfer of chemical cues to nearby chemosensors under stagnant conditions (Denissenko et al., 2007, Breithaupt, 2011). These organs are strategically located below the frontal sensory organs, including antennules, which are considered the main chemoreceptive organ of decapod crustaceans (Schmidt and Mellon, 2011). Therefore, the reflexive movement of maxillipeds triggered by the leg detection of stimulants may enhance the overall chemosensory performance of the animal in terms of increased chance of encountering chemical stimuli.

Focal leg stimulation with our device also induced the reflex activation of antennules (Fig. 3B). Their flicking activity stimulated by the highest concentrations of trehalose (8.8 ± 1.5 flicks/min), maltose (8.4 ± 1.5 flicks/min) and cellobiose (6.9 ± 1.3 flicks/min) was significantly higher than that triggered by the blank control (2.0 ± 1.3 flicks/min). Unlike the response of maxillipeds, the reflex response of antennules was lower than that evoked by the focal stimulation of the leg with food (13.1 ± 1.8 flicks/min) as if the threshold of the antennules towards these stimulants was higher than that of maxillipeds. In all the tested sugars, reflex antennular flicking was positively correlated with movement of the leg (Spearman r_s = 0.68, 0.58 and 0.52 for trehalose, maltose and cellobiose, respectively; $p < 0.0001$ for trehalose and maltose, $p = 0.0004$ for cellobiose).

The flicking rate in crustaceans is considered an indicator of chemical detection (Schmitt and Ache, 1979; Daniel and Derby, 1991). Therefore, the leg-dependent reflex activation of antennular flicking, similarly to maxilliped activation, may be crucial to enhance the chances of antennules to enter relevant trails of chemical cues. Consequently, they provide animals with a better sampling of their environment and directionality in search strategies. Crustaceans use parallel chemosensory pathways characterised by unimodal chemo- and bimodal chemo-/mechano-sensory sensillar populations on mouthparts, antennae, antennules and legs, which are also characterised by functional redundancy (Steullet et al., 2001, 2002; Harzsch and Krieger, 2018). Ultimately, multiple detection by similar and different sensory appendages and their cross-recruitment may help create three-dimensional maps of the chemical environment for the prompt detection of a chemtrail and its source.

With the development of our technique for focal leg stimulation in crayfish, sensory-motor integration in decapods and other crustaceans may be further explored. Even if our technique was developed on *P. clarkii*, it could be easily adapted for use in other decapods larger than a few centimetres and other sensory appendages. As such, whole, intact animals may be comprehensively investigated. In principle, our technique could also be used to investigate the sensitivity of other sensory systems such as thermo-, mechano-, pH-, osmo-receptors, etc, provided that the chemical/physical stimulus of interest can be supplied under perfusion.

This technique should be used to complement other existing methods based on ablation or silencing of sensory appendages, not replace them. Thus, direct evidence of specific sensitivity in single appendages can be obtained, and sensory-motor integration amongst them can be evaluated. In further studies, this technique may be improved by applying two parallel devices onto two different legs or appendages to examine combined agonistic (stimuli with the same signs, i.e. both attractive and repulsive) or antagonistic (stimuli with opposite signs, i.e. one attractive and one repulsive) sensory inputs.

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Author Contributions

Conceptualization: P.S., A.S., R.C.; Methodology: P.S., A.S., R.C.; Validation: P.S., G.S., F.P., A.S., R.C.; Formal analysis: P.S., G.S., F.P.; Investigation: P.S.; Resources: P.S., R.C., Data curation: P.S., G.S., F.P.; Writing - original draft: P.S.; Writing - review & editing: P.S., G.S., F.P., A.S., R.C.; Visualization: P.S., R.C.; Supervision: P.S., R.C.; Project administration: P.S., R.C.; Funding acquisition: P.S., A.S.

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Data availability

The original data are available upon request from the corresponding author.

Competing interests

The authors declare no competing or financial interests.

Figure captions

Figure 1. Design and application of the device for the perfusion/stimulation of single crayfish legs used in this study. (**A**) Photograph of a second pereiopod showing the different segments. (**B**) Photograph of the closed-circuit device consisting of a perfusion/stimulation chamber for the intubation of the leg and two flexible silicon tubes for the delivery (inflow) and removal (outflow) of water/chemicals with the fittings to be connected to the perfusion and waste collection systems. (**C**) View of the whole crayfish with the device (arrow) applied on the leg, with the tubes secured on the carapace and the fittings connected to the perfusion system during the supply of a green liquid. Ch = cheliped, a = antenna and A-lf and A-mf = lateral and medial flagella of antennules, respectively.

Figure 2. Duration of leg movements determined over a 1 min interval during stimulation with increasing concentrations of trehalose (Tre), maltose (Mal) and cellobiose (Cell) compared with the blank (B-Ctrl) and food (F-CTRL) controls and supplied with the device for the perfusion/stimulation of a single leg. Values are means ± SEM (vertical bars) from 14 crayfish. For each tested sugar, * indicates responses significantly different from those at the next lower concentrations, whilst B and F denote significant differences compared with B-Ctrl and F-CTRL, respectively ($p < 0.05$, Wilcoxon matched pair signed rank test).

Figure 3. Duration of maxilliped movements (**A**) and frequency of antennular flicking (**B**) determined over a 1 min interval during stimulation with increasing concentrations of trehalose (Tre), maltose (Mal) and cellobiose (Cell) compared with those of the blank (B-Ctrl) and food (F-CTRL) controls and supplied with the device for the perfusion/stimulation of a single leg. Values are means ± SEM (vertical bars) from 14 crayfish. For each tested sugar, * indicates responses significantly different from those at the next lower concentrations, whilst B and F denote significant differences compared with B-Ctrl and F-CTRL, respectively ($p < 0.05$, Wilcoxon matched pair signed rank test).

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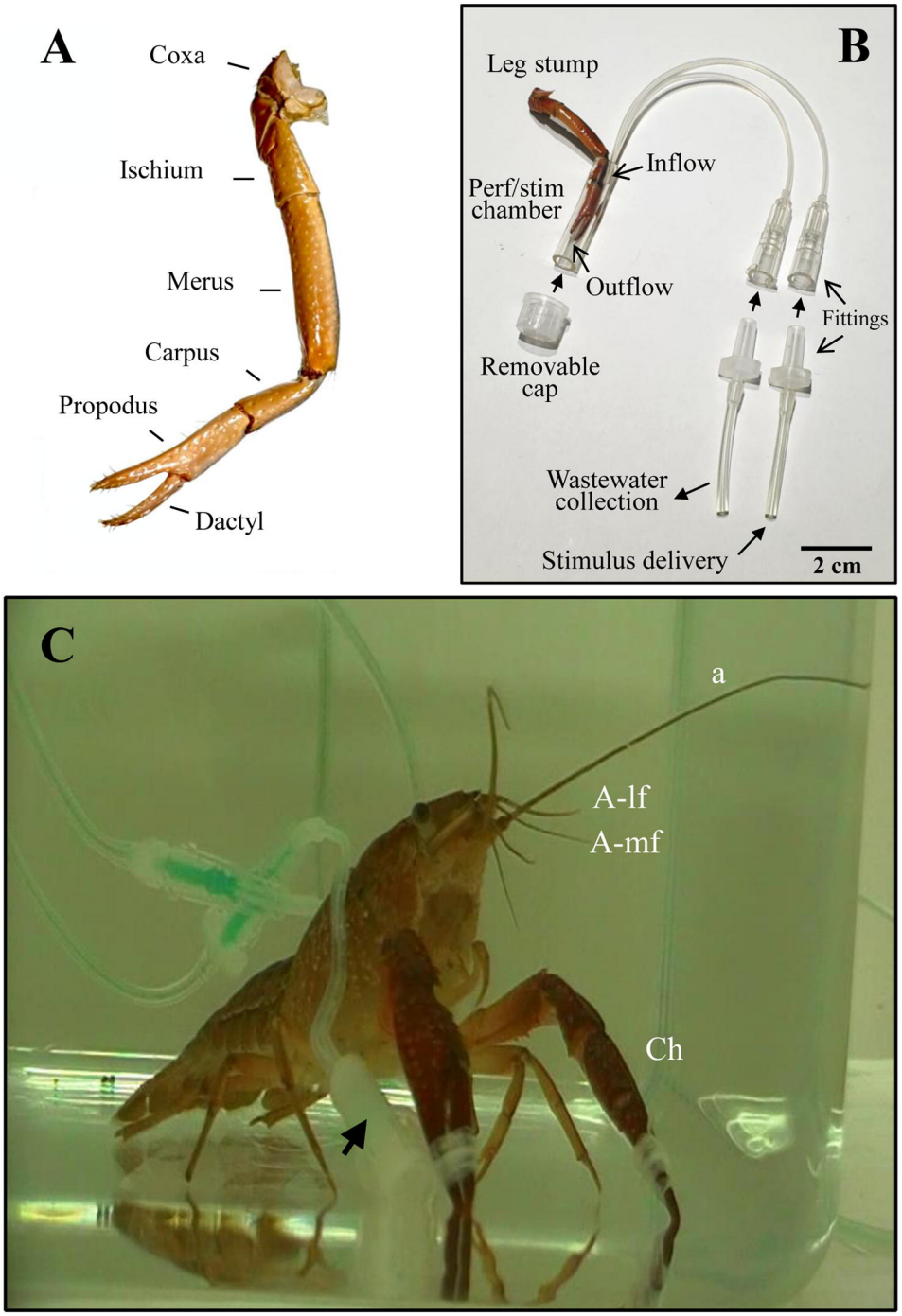
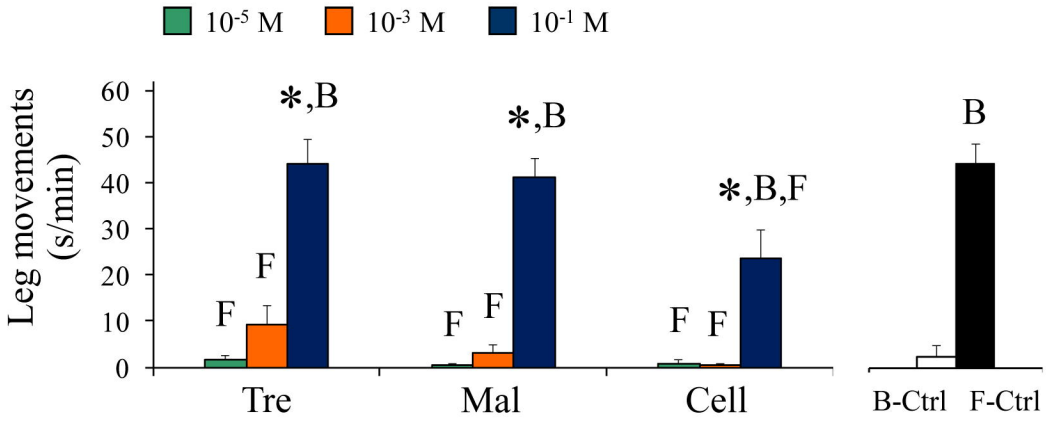

A
Coxa
Ischium
Merus
Carpus
Propodus
Dactyl
B
Leg stump
Inflow
Perf/stim chamber
Outflow
Fittings
Removable cap
Wastewater collection
Stimulus delivery
2 cm
C
a
A-lf
A-mf
Ch

Figure 1


10-5 M
10-3 M
10-1 M
Leg movements (s/min)
60
50
40
30
20
10
0
*,B
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*,B,F
B
F
F
F
F
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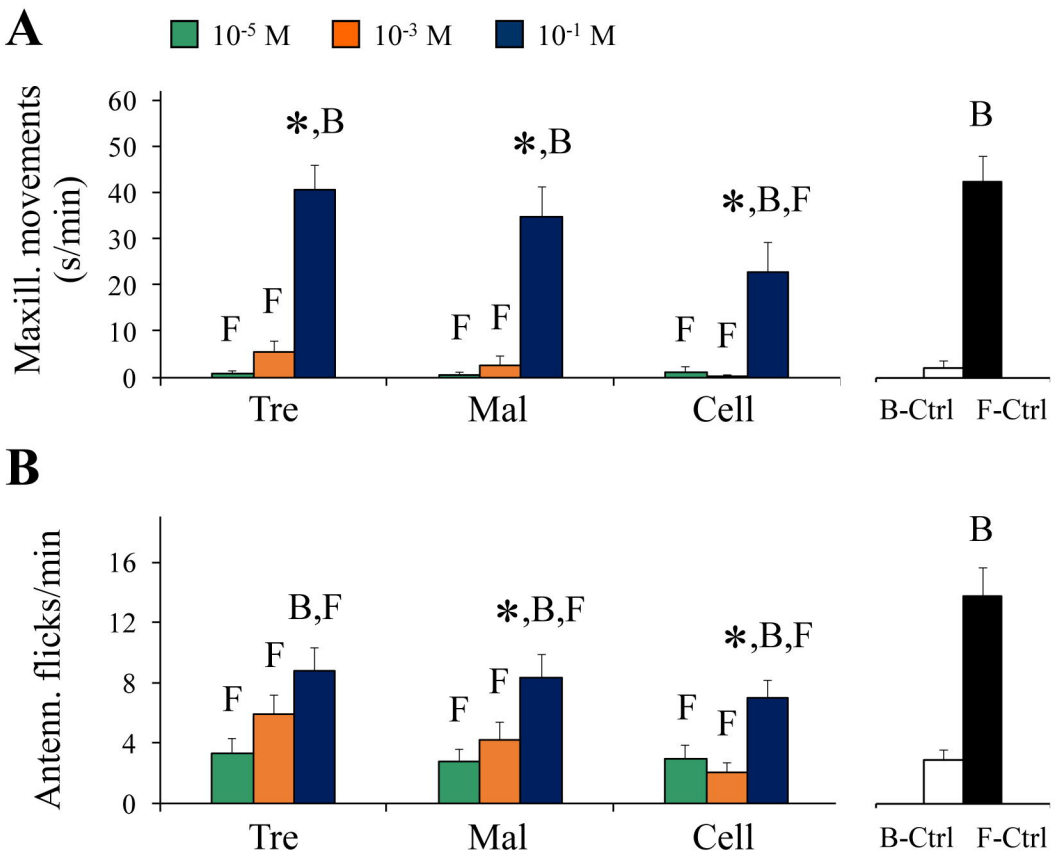
Figure 2


A
10-5 M
10-3 M
10-1 M
Maxill. movements (s/min)
60
50
40
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10
0
*,B
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F
F
F
F
F
F
B
Tre
Mal
Cell
B-Ctrl
F-Ctrl
B
Antenn. flicks/min
16
12
8
4
0
B,F
*,B,F
*,B,F
F
F
F
F
F
F
B
Tre
Mal
Cell
B-Ctrl
F-Ctrl

Figure 3