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RESEARCH ARTICLE

Oesophageal chemoreceptors of blue crabs, *Callinectes sapidus*, sense chemical deterrents and can block ingestion of food

Juan F. Aggio*, Ryan Tieu, Amy Wei and Charles D. Derby

Neuroscience Institute and Department of Biology, Georgia State University, 850 Petit Science Center, 100 Piedmont Avenue, Atlanta, GA 30303, USA

*Author for correspondence (jaggio@gsu.edu)

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SUMMARY

Decapod crustaceans such as blue crabs possess a variety of chemoreceptors that control different stages of the feeding process. All these chemoreceptors are putative targets for feeding deterrents that cause animals to avoid or reject otherwise palatable food. As a first step towards characterizing the chemoreceptors that mediate the effect of deterrents, we used a behavioral approach to investigate their precise location. Data presented here demonstrate that chemoreceptors located on the antennules, pereiopods and mouthparts do not mediate the food-rejection effects of a variety of deterrents, both natural and artificial to crabs. Crabs always searched for deterrent-laced food and took it to their oral region. The deterrent effect was manifested as either rejection or extensive manipulation, but in both cases crabs bit the food. The biting behavior is relevant because the introduction of food into the oral cavity ensured that the deterrents gained access to the oesophageal taste receptors, and so we conclude that they are the ones mediating rejection. Additional support comes from the fact that a variety of deterrent compounds evoked oesophageal dilatation, which is mediated by oesophageal receptors and has been linked to food rejection. Further, there is a positive correlation between a compound's ability to elicit rejection and its ability to evoke oesophageal dilatation. The fact that deterrents do not act at a distance is in accordance with the limited solubility of most known feeding deterrents, and likely influences predator–prey interactions and their outcome: prey organisms will be attacked and bitten before deterrents become relevant.

Key words: behavior, chemoreceptor neurons, *Callinectes sapidus*, feeding deterrents.

INTRODUCTION

Animals often use chemical deterrents in defense against predators (e.g. Paul et al., 2007; Ferrer and Zimmer, 2009), which the predators detect and respond to through a diversity of chemoreceptor cells. In terrestrial animals, aversion can be evoked by both volatile odorants (Laska et al., 2005) and water-soluble tastants (Spector and Kopka, 2002). In some cases, the same compound may work as both an odorant and tastant. For example, insects can both smell and taste DEET (Ditzen et al., 2008; Syed and Leal, 2008; Lee et al., 2010), although it is unclear whether both senses play ecologically relevant roles in the responses to DEET under natural conditions, because their taste receptors may never contact it.

In the aquatic environment, chemical stimuli are typically water soluble and thus can access most sensory organs (Carr, 1988). Thus, one might expect that a deterrent would act through a variety of receptor cells on different organs. This would be advantageous to both predator and prey: the former would not search, track and attack an organism that it will eventually not consume; the latter would not be attacked. However, this is not always the case. For example, fish do not reject quinine-laced food until they have put it in their mouth (Lamb and Finger, 1995), perhaps because, being a relatively insoluble compound (Koyama and Kurihara, 1972; Ogawa et al., 1997), quinine does not diffuse into the surrounding water in high enough quantities. Conversely, aplysioviolin (APV) and phycoerythrobilin (PEB), which are active deterrents released with the ink of sea hares (*Aplysia* spp.) and are also relatively insoluble in water, significantly increase the time it takes fish to reach a food pellet by acting through their olfactory receptor neurons (Nusnbaum and Derby, 2010a). These compounds are also capable of stopping the search behavior of blue crabs when applied to their anterior region, which contains their olfactory organs, the antennules, as well as many other chemoreceptor neurons (Kamio et al., 2010a). In both studies, the deterrents were released from a pipette and formed a 'cloud', distinct from the food stimulus (Nusnbaum and Derby, 2010a; Kamio et al., 2010a). In contrast, when the deterrents are presented within food (e.g. added to pellets or freeze-dried shrimp), organisms such as fish [sharks (*Sphyrna tiburo*), wrasses (*Thalassoma bifasciatum* and *Oxyjulis californica*), mummichogs (*Fundulus heteroclitus*) and pinfish (*Lagodon rhomboides*) (Nusnbaum and Derby, 2010a)], crabs (Pennings, 1994; Kamio et al., 2010a) and spiny lobsters (Kicklighter et al., 2005; Aggio and Derby, 2008) reject it, but often after biting or eating portions of it. Catfish, which have an extensive array of extraoral taste cells (Atema, 1971; Caprio et al., 1993), do not have to take deterrent-laced food into their oral cavity to reject it; they do so after contacting it with their barbels (Nusnbaum et al., 2012).

Decapod crustaceans possess a variety of chemoreceptors (Derby and Steullet, 2001), which can be categorized according to their localization and/or function. The aesthetasc sensilla located in the lateral flagella of the antennules are the only sensilla that house exclusively chemoreceptor neurons (Grünert and Ache, 1988). These neurons project to the olfactory lobes, which have a glomerular

architecture (Schmidt and Ache, 1996b), a characteristic shared with the olfactory system of vertebrates (Pinching and Powell, 1971), insects (Tolbert and Hildebrand, 1981) and mollusks (Chase and Tolloczko, 1986). All other types of sensilla appear to house both mechanoreceptor and chemoreceptor neurons, and most are widely distributed. They are found in both antennular flagella and project to the lateral and medial antennular neuropils (Schmidt and Ache, 1996a), and in the mouthparts (Derby and Atema, 1982; Garm et al., 2005), pereiopods (Bauer and Hatt, 1980; Hatt and Bauer, 1980; Bauer et al., 1981; Derby and Atema, 1982) and oesophagus (Robertson and Laverack, 1979b; Altner et al., 1986). Although very little is known regarding the targets for the axons of non-antennular chemoreceptors, they are assumed to be different from those of the antennular ones, and the small amount of information available points to targets located in the ganglia of the ventral nerve cord (Ott et al., 2007).

Receptor neurons located on different appendages mediate different behaviors. In clawed and spiny lobsters, the receptor neurons in the antennules, both olfactory and non-olfactory, are sufficient to perform long-distance behaviors such as searching (Reeder and Ache, 1980; Derby and Atema, 1982; Steullet et al., 2001; Horner et al., 2004), and complex behaviors such as learning and discrimination (Steullet et al., 2002). Aesthetascs alone mediate the responses to intraspecific chemicals (Johnson and Atema, 2005; Horner et al., 2008; Shabani et al., 2008). In crabs, the aesthetascs also mediate responses to intraspecific signals such as sex pheromones (Gleeson, 1982; Gleeson, 1991). The antennules of crabs play a somewhat different role in chemo-orientation, although ablation of the antennules slows the crabs' upstream progress (Keller et al., 2003; Dickman et al., 2009). Another example of a different organization of the food search is found in the kelp crab *Pugettia producta*, in which low concentrations of chemical stimuli elicit a local search (e.g. raking the substrate) while higher concentrations are required to initiate locomotion, which in this context is considered a long-range searching behavior (Zimmer-Faust and Case, 1982).

Once the animals reach the vicinity of a food odor source, they switch their search strategy and use their pereiopods to rake and probe the substrate. This switch in search strategy is reminiscent of that used by ants of the genus *Cataglyphis* when looking for their nest after a long-range foraging trip (Wehner and Srinivasan, 1981; Wehner, 2003): in both cases, the animals cease to move in a particular direction and begin searching their surroundings, ants by moving in ever-widening circles and crabs and lobsters by probing the substrate. At this point, a different mechanism appears to underlie the search behavior. When a piece of food is contacted (usually with a pereiopod, see below), it is taken to the oral region where it in turn contacts the mouthparts (Hazlett, 1968; Derby and Atema, 1982). The mouthparts handle the food and position it so that the mandibles can bite it and it can then be swallowed (Garm, 2004; Garm and Høeg, 2006). Derby and Atema reported that on rare occasions lobsters make first contact with food with their mouthparts (Derby and Atema, 1982), but we have never observed this in crabs, where all initial contact was made with a pereiopod. Finally, the piece of food cut by the mandibles reaches the lumen of the digestive tract where it can come into contact with chemoreceptor neurons located in the oesophagus (Robertson and Laverack, 1979b; Altner et al., 1986). These receptor neurons are organized into two bilateral groups, the anterior and posterior oesophageal sensors (AOS and POS), and they have also been implicated in feeding control. Robertson and Laverack note that their stimulation has opposite effects on oesophageal peristalsis: the POS increases it while the AOS decreases it (Robertson and Laverack, 1979b). They speculate that the AOS could be implicated in feeding cessation, and note that these chemoreceptor neurons are only accessible if the cardiac sac is filled to capacity (Robertson and Laverack, 1979b). Additionally, in the shore crab *Carcinus maenas*, chemoreceptor neurons located inside the oral cavity have been reported to respond to ecdysteroids, which are feeding deterrents found in pycnogonids, by evoking oesophageal dilatation (OD), a wide opening of the anterior oesophagus (Tomaschko et al., 1995; Tomaschko, 1997).

In decapod crustaceans, the mouthparts are six paired appendages that share the task of manipulating, biting and aiding in the ingestion of food items. The mandibles lack setae and are used to bite or crush food items. Each one bears a slender palp with setae that help in pushing food into the mouth. The maxillae 1 and 2 and maxilliped 1 are small, have setae, and function in retaining food particles close to the mandibles (Caine, 1974), positioning small food particles and manipulating larger items (Garm, 2004). Lastly, maxillipeds 2 and 3 are very robust, manipulate larger food items (Salindeho and Johnston, 2003; Garm, 2004) and pull the food ventrally, away from the mandibles, so that it breaks close to them. The chelipeds often aid in this process, especially as the food items get bigger (Caine, 1974; Garm, 2004).

Feeding deterrence in spiny lobsters and crabs is not always expressed as an outright rejection of treated food (Aggio and Derby, 2008; Kamio et al., 2010a). Indeed, in many cases the addition of a deterrent compound to food causes the animals to significantly increase the time they spend manipulating it in what appears to be the result of a compromise between the effects of the deterrent and food-related compounds on the chemoreceptor neurons responsible for feeding. Thus, the time spent manipulating a piece of food is a good measure of its palatability.

With the aim of characterizing the mechanism of action of feeding deterrents in blue crabs, we investigated the localization of the chemoreceptor neurons responsible for the rejection of a variety of compounds. As a biologically relevant stimulus, we chose the defensive ink of the sea hare *Aplysia californica* (Nolen et al., 1995; Coelho et al., 1998; Ginsburg and Paul, 2001; Kicklighter et al., 2005). Sea hares possess a variety of compounds and mechanisms that enable them to escape predation (Derby et al., 2007; Derby and Aggio, 2011), and the principal deterrent components of the ink for both blue crabs and bluehead wrasses have been identified as aplysioviolin (APV) and phycoerythrobilin (PEB) (Kamio et al., 2010a). We also investigated the effects of several other compounds: quinine, caffeine and denatonium benzoate (hereafter denatonium), which are well-known feeding deterrents that humans perceive as bitter (Chandrashekar et al., 2006; Yarmolinsky et al., 2009; Carleton et al., 2010). In addition, we tested cinnamaldehyde, a compound that belongs in a group called reactive electrophiles, which cause tissue damage and elicit pain (Basbaum et al., 2009), are detected through TRPA1, and inhibit the sucrose-evoked proboscis extension response in *Drosophila* (Basbaum et al., 2009; Kang et al., 2010). Finally, we tested nicotinamide because it is one of the few compounds capable of eliciting an electrophysiological response from oesophageal receptor neurons in crayfish (Altner et al., 1986). Our results indicate that blue crabs use receptors located in their oesophagus to detect, and respond accordingly to, deterrent compounds.

MATERIALS AND METHODS

Animals

Adult male and female blue crabs, *Callinectes sapidus* Rathbun 1896, of carapace width 10–16 cm, were purchased at a local market

and kept individually in 40 l aquaria (50×25×30 cm) filled with artificial seawater (ASW, Instant Ocean, Aquarium Systems, Mentor, OH, USA) at approximately 25°C for at least a week prior to use, under a 12 h:12 h light:dark cycle (lights on at 07:00 h). A substrate of gravel was used. Crabs were fed shrimp every other day, and all experiments were performed on non-feeding days to ensure that crabs had an adequate level of hunger. They remained healthy in the laboratory, and pilot experiments showed that they were indistinguishable from those purchased from science suppliers (Gulf Specimen Marine Laboratories, Panacea, FL, USA).

Sea hares, *A. californica* Cooper 1863, 200–300 g, were purchased from Marinus Scientific (Long Beach, CA, USA) and upon arrival they were chilled, injected with 50 ml of 0.37 mol l^{-1} $MgCl_2$, and the ink glands removed and stored at –80°C until used. Ink was obtained by freeze drying the glands, crushing them with a mortar and pestle, and extracting them with 100% methanol. The methanol was removed in a rotary evaporator, and the resulting methanol-soluble material is called 'ink'. A fraction enriched in APV+PEB was obtained as follows: dry ink was resuspended in 40% methanol and adsorbed onto a Diaion HP20SS gel column (Mitsubishi Chemical USA, Inc., Chesapeake, VA, USA), and eluted with 100% methanol.

Chemicals

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and were at least 99% pure, unless otherwise stated

Behavioral assays

Searching behavior

These experiments were performed to investigate the effect of ink on the chemoreceptor neurons that elicit searching behavior in response to food. The food was a small piece (approximately one abdominal segment, dry mass 180–250 mg) of locally purchased frozen shrimp that had been freeze dried. This shrimp was laced with 500 µl of either ASW or full-strength (100%) ink and dropped into the crab's aquarium on the opposite side to where the crab was located, and the crab's behavior was observed for 1 min. For each animal, we recorded whether the animal initiated searching behavior and, if it did, the time that elapsed between presentation of the shrimp and the start of the search. The crabs tended to remain immobile when not stimulated, and the beginning of the search was defined as a sudden upward movement of the body followed immediately by initiation of locomotion. To avoid any interference of the experimenter, crabs were temporarily blinded with eyecaps made from custom-fitted, heat-shrink tubing. Because freeze-dried shrimp float, even after the addition of a liquid, the pieces offered here were weighted with a small piece of gravel to ensure that they would sink and remain immobile.

Feeding and grasping behaviors in freely moving crabs

These experiments were designed to evaluate the role of contact chemoreceptor neurons in the rejection of food laced with putative deterrents. We offered a crab a small piece of freeze-dried shrimp laced with 500 µl of ASW (control), the full-strength APV and PEB-enriched fraction of ink, a series of chemicals that deter feeding in other animals (quinine, caffeine, denatonium and cinnamaldehyde, all at 5 mmol l^{-1}) or nicotinamide at 5 mmol l^{-1}. We ensured that the first point of contact was one of the crab's pereiopods. In this case, it was not necessary to weigh down the shrimp because all crabs seized and secured it upon contact. The size of the shrimp was approximately one-half of an abdominal segment (dry mass 90–140 mg), which is small enough to be handled by crabs lacking maxillipeds 2 and 3 and to not require the aid of the chelipeds for its consumption (see Introduction). Each animal was observed for 120 s after receiving the food item, and we recorded whether the animal took the food to its mouth or not, if it eventually ate or rejected the food, and the time for the crab to do so, which is also a measure of palatability (Aggio and Derby, 2008; Kamio et al., 2010a). Each crab's response to a particular stimulus could fall under three broad categories: shrimp eaten in less than 120 s; shrimp continued to be handled until the time limit was reached; or shrimp rejected before the time limit was reached. In the first case, the time entered was the time to eat; in the second, 120 s was used; in the third case, a value of 121 s was used to reflect that this was a different behavior. In some cases, this led to non-normality of the data, which we then transformed to perform parametric tests (see below).

Ablation of mouthparts was performed by amputating the desired appendages on crabs that had been chilled by placing them on ice for 5–10 min. In pilot experiments, crabs had all their mouthparts except the mandibles ablated. However, because these animals did not eat normal shrimp, our experiments were conducted with animals that belonged to one of three ablation groups: (i) no inner mouthparts: ablation of the mandibular palps, maxillae 1 and 2 and maxilliped 1; (ii) no outer mouthparts: ablation of maxillipeds 2 and 3; and (iii) control animals that were placed on ice for 5 min and returned intact to their aquaria. The rationale for dividing them in this fashion was that the outer mouthparts perform highly variable patterns of movement that depend on the characteristics of relatively large pieces of food while the inner ones perform much more stereotypical movements in the handling of smaller items (Garm and Høeg, 2001; Salindeho and Johnston, 2003; Garm, 2004). Antennules were ablated in a similar fashion.

Food rejection *via* oesophageal stimulation in immobilized animals

These experiments were designed to evaluate the role of chemoreceptor neurons located within or at the opening of the digestive tract (i.e. oesophagus) in the rejection of food. Crabs were secured ventral side up by tying their pereiopods to a plastic grid. The mandibles, which cannot be ablated without severely damaging the animals, were kept open by inserting a small plastic rod between them. This procedure allowed us to introduce a small piece of freeze-dried shrimp laced with the chemical of interest directly into the crab's mouth. We recorded whether the animal swallowed the shrimp (acceptance) or failed to do so (rejection). This relatively weak measure of rejection was adopted because animals in this condition were not able to remove the piece of food from their mouth. Five of the six crabs lacked all mouthparts except the mandibles, and the remaining one lacked the inner ones. In this latter case, maxillipeds 2 and 3 were secured away from the mouth. No differences between this crab and the ones in the completely ablated group were noticed, and data from them were grouped.

We also used this immobilization technique to apply small volumes (50–100 µl) of chemicals to the mouth and record whether crabs displayed the OD behavior, which, as stated above, has been linked to stimulation with deterrent stimuli in crabs (Tomaschko et al., 1995). We compared the effectiveness of the different compounds in eliciting OD by calculating the concentration required to evoke it in 50% of the animals (ED_{50}) using a probit regression. In some experiments, the oesophagus was coated with silicone (silicone vacuum grease, Beckman Instruments, Palo Alto, CA, USA) using a Q-Tip™.

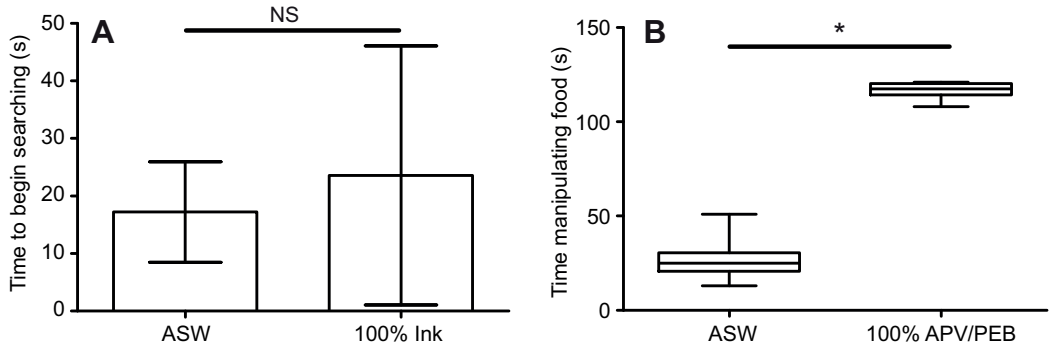

Fig. 1. Deterrents do not act through antennular chemoreceptors. (A) The mean time for intact crabs to begin searching for artificial seawater (ASW)- or ink-laced food does not differ (one-tailed paired *t*-test: t=1.462, P=0.0837, N=14). Values are means ± s.d. (B) Crabs without their antennules spend significantly more time manipulating shrimp laced in a fraction of ink enriched in aplysioviolin (APV) and phycoerythrobilin (PEB) than ASW-laced shrimp (one-tailed *t*-test on the square root of the time spent manipulating food: t=18.95, *P<0.0001, N=10). Box plots show median (solid black line), interquartile range (box length), and minimum and maximum values (error bars).

Mandibular muscle recordings

Crabs were chilled in ice for 5–10 min, then a small hole was drilled in the carapace, taking care not to damage the pericardial sac, and a small stainless steel electrode was glued in place. At least 24 h after this procedure, crabs were moved to an aquarium located in the experimental room, the electrode was connected to an amplifier (Grass P511, Quincy, MA, USA), and they were allowed to acclimate for at least 1 h. The reference electrode was placed in the water surrounding the animal. After the acclimation period ended, crabs were offered small pieces of freeze-dried shrimp laced with ASW or 5 mmol l^{-1} quinine, and the activity of the mandibular closer muscle was digitized (Digidata 1420 and pCLAMP 8.0, Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). For each type of shrimp (i.e. laced with ASW or quinine), we calculated the proportion of animals that bit at least once and the number of bites per episode, as evidenced by the number of bursts of activity in the closer muscle.

RESULTS

Antennular chemoreceptors

When a small piece of shrimp was introduced into their aquaria, crabs searched for it even when it was laced with full-strength ink. All 14 animals tested left their resting position and began to walk around their aquarium while probing the substrate with their pereiopods. In addition, ink added to shrimp did not significantly delay the time it took for this behavior to begin when compared with the ASW-laced control shrimp (Fig. 1A). Further, the lack of the antennules, which are considered the bearers of most if not all distance chemoreceptor neurons and are responsible for long-distance food search (Keller et al., 2003; Horner et al., 2004), did not abolish the deterrent effect of full-strength ink: ablated animals spent significantly longer manipulating shrimp laced with the APV+PEB-enriched fraction of ink than with ASW (Fig. 1B). We conclude that the antennular receptor neurons are not responsible for the feeding deterrent effect of ink.

Pereiopod chemoreceptors

Similarly, ink did not affect the grasping of food. All 14 tested crabs, upon first contact with ink-laced shrimp, immediately grasped it and passed it to their mouthparts, a behavior that is the same as that seen when the shrimp is laced with ASW. Further, in all the experiments described below, presenting shrimp laced with different compounds did not modify this behavior. We conclude that pereiopod receptor neurons do not play a role in the feeding deterrence of ink.

Maxilliped receptors

Because all crabs took deterrent-laced food to their oral region, we next investigated the role played by chemoreceptor neurons located in the mouthparts in mediating deterrence to ink and to several compounds known to deter other species. First, we offered crabs with different ablations freeze-dried shrimp laced with ASW or full-strength ink. Crabs with all pairs of mouthparts ablated except the mandibles did not finish eating even the smallest pieces of ASW-laced shrimp and so we were forced to perform partial ablations. We created three groups of animals: (i) intact (no ablations); (ii) inner mouthparts ablated (ablations of the mandibular palps, maxillae 1 and 2 and maxilliped 1); and (iii) outer mouthparts (maxillipeds 2 and 3) ablated. All sets of mouthparts are involved in food handling and contain chemoreceptor neurons (Garm, 2004; Garm, 2005; Garm and Høeg, 2006), with the outer ones being much bigger and playing a more important role in handling bigger pieces of food than the inner ones. Although it is not possible to ablate the entire mandibles, it has been shown in another species of portunid crab (Salindeho and Johnston, 2003) and a wide array of other decapods (Garm, 2004) that only their palps, which can be ablated, bear setae and, in *Homarus*, that the labrum does not respond to chemical stimulation (Robertson and Laverack, 1979a). Taken together, this suggests that no chemoreceptors were left in the outmost portion of the oesophagus (mandibular processes + labrum).

Our results show that the mouthparts are not needed to mediate the deterrent action of *Aplysia* ink (Fig. 2A). A two-way ANOVA with repeated measures (performed on the square root of handling times to ensure normality) showed that the stimulus had a significant effect (P<0.0001), while the ablation (P=0.078) and interaction terms (P=0.7473) did not. Animals in all three groups spent more time manipulating ink-laced than ASW-laced shrimp (P<0.05, ink *vs* ASW for each ablation group, Bonferroni). Although the ablation effect failed to reach significance, animals without inner mouthparts spent significantly more time handling ASW-laced food (P<0.05, no inner *vs* intact for ASW, Bonferroni). We do not have enough information to be able to tell whether the effect of ablating the inner mouthparts is due to the lack of afference (mechanosensory, chemosensory or a combination of both) or to the animals having

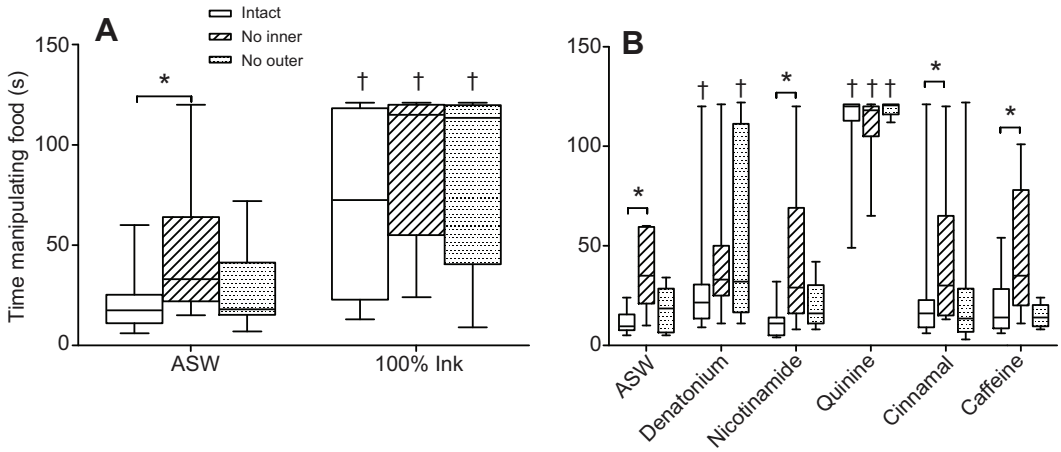

Fig. 2. Deterrents do not act through maxilliped chemoreceptors. (A) Ink increases the time that crabs manipulate food independently of the type of ablation: 'intact', no ablation (N=14); 'no inner', inner mouthparts ablated (N=11); or 'no outer', outer mouthparts ablated (N=13). See Materials and methods for description of ablations. Box plots show median (solid black line), interquartile range (box length), and minimum and maximum values (error bars). A square root transform was applied to the data before analysis. Two-factor ANOVA with repeated measures showed a significant stimulus effect ($P_{stimulus}$<0.0001), a non-significant ablation effect ($P_{ablation}$=0.078), and no stimulus × ablation interaction ($P_{interaction}$=0.7473). *Post hoc* comparisons for each factor were made using Bonferroni tests (P<0.05): * and † indicate significant differences from the intact group and the ASW group, respectively. (B) Quinine and denatonium benzoate are effective feeding deterrents, and the deterrence is not affected by the type of ablation (intact, N=16; no inner, N=11; no outer, N=8). Details as in A. $P_{stimulus}$<0.0001, $P_{ablation}$=0.0022, $P_{interaction}$=0.0017. All stimuli were presented at 5 mmol l^{-1}. All animals in A and B were monitored and, in all cases, took the shrimp to their oral region after making first contact with a pereiopod.

lost the ability to position the food correctly to gain entry to the mouth.

We then investigated whether these results were specific for ink or whether they were also seen for several compounds that deter feeding in other organisms: denatonium, quinine, caffeine and cinnamaldehyde, as well as nicotinamide (all 5 mmol l^{-1}), which is the only known compound that stimulates receptor neurons located in the crayfish oesophagus (Altner et al., 1986). The rationale behind this experiment is that in insects different deterrent compounds act through different chemoreceptors, located either on the tarsi or on the oesophagus (Kang et al., 2010). The results are presented in Fig. 2B. A two-way ANOVA with repeated measures of the square root of the manipulation times revealed a very strong stimulus effect (P<0.0001), ablation effect (P=0.0022), and stimulus × ablation interaction (P=0.0017). Denatonium and quinine significantly increased the handling times of intact animals (P<0.05 *vs* the ASW group, Bonferroni), and this effect was not abolished by ablation of the external mouthparts (P<0.05 *vs* the ASW group, Bonferroni). Ablation of the inner mouthparts resulted in a more complex pattern, in which quinine increased food-handling time (P<0.05 *vs* the ASW group, Bonferroni) but denatonium did not. This result is due to the fact that the ablation by itself increased the food-handling time. Indeed, for all stimuli that did not cause deterrence (i.e. nicotinamide, cinnamaldehyde and caffeine), animals without inner mouthparts took longer to manipulate food than did intact animals (P<0.05 *vs* the ASW group, Bonferroni).

As mentioned above, all animals in these groups also took the food to their oral region, generalizing the result that the pereiopods do not mediate rejection to other compounds.

Oesophageal receptors

Because we cannot gather reliable feeding data on freely moving crabs lacking mouthparts, and the mouthparts seem to play no role in mediating deterrence, we switched to a strategy in which crab movements were restricted and either small volumes of stimulus solutions or pieces of food were manually introduced directly into their oral cavity. We wanted to determine whether deterrents cause OD, which has been reported to be evoked by unpalatable compounds (Tomaschko et al., 1995; Tomaschko, 1997), and if those same stimuli cause animals to regurgitate (or refuse to eat) food placed in their mouth.

Ink was effective in eliciting OD in a dose-dependent fashion (Fig. 3A), and so were quinine, denatonium, cinnamaldehyde and caffeine (Fig. 3B). ASW and a 1% w/v shrimp extract both failed

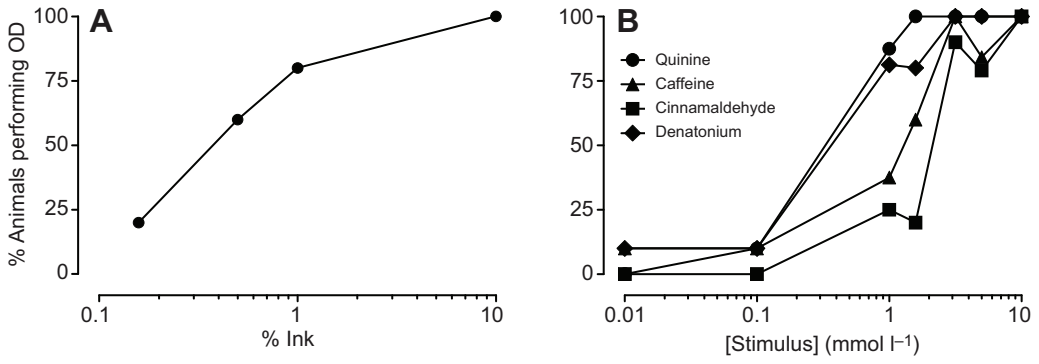

Fig. 3. Ink and other known deterrents evoke oesophageal dilatation (OD) in blue crabs. (A) The percentage of crabs performing OD increases with the concentration of ink (N=5). (B) The percentage of crabs performing OD increases with the concentration of stimulus (N=5–16). Quinine and denatonium, which are feeding deterrents in intact blue crabs, are more effective than caffeine and cinnamaldehyde, which are not.

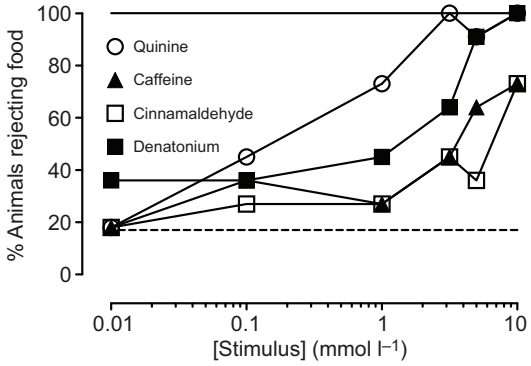

Fig. 4. Feeding deterrents act through oesophageal chemoreceptors. When the mouthpart receptors are bypassed by introducing food directly inside crabs' mouths, deterrents cause food rejection (N=6). All tested deterrents evoked rejection in a concentration-dependent manner. Ink (10% of full strength, solid horizontal line) caused 100% of the animals to reject the shrimp while only one of 6 rejected the ASW-laced stimulus (dashed horizontal line).

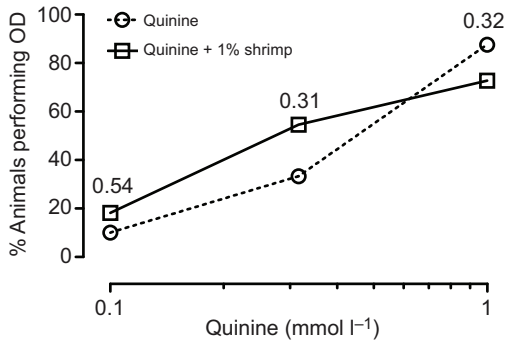

Fig. 5. An appetitive stimulus does not block the OD caused by a deterrent stimulus. The percentage of crabs that perform OD when stimulated with quinine alone does not change if a mixture of quinine and shrimp extract is used (N=9–16). For each concentration, the values are the one-tailed probabilities that the two groups differ significantly (Fisher's exact test).

to evoke this response (data not shown). Although all stimuli tested evoked OD, they fell into two categories with respect to their effectiveness, measured as the concentration that evoked OD in 50% of the animals (ED_{50}), and this is correlated with their ability to deter feeding in freely moving blue crabs. When we computed the probit regressions for all four of them, we found that the deterrent stimuli (denatonium and quinine) had lower ED_{50} values than stimuli that were not deterrent (caffeine and cinnamaldehyde) [denatonium: ED_{50}=0.246 mmol l^{-1}, 95% confidence interval (CI)= 0.10–0.48 mmol l^{-1}; quinine: ED_{50}=0.324 mmol l^{-1}, 95% CI=0.32–0.56 mmol l^{-1}; caffeine: ED_{50}=0.662 mmol l^{-1}, 95% CI=0.02–2.78 mmol l^{-1}; cinnamaldehyde: ED_{50}=1.920 mmol l^{-1}, 95% CI=1.22–2.68 mmol l^{-1}].

Tethered animals that had small pieces of food laced with different chemicals introduced into their oesophagus also rejected them in a dose-dependent fashion (Fig. 4). Once again, it is apparent that quinine and denatonium were more effective than cinnamaldehyde and caffeine, although in this case the effect was not as pronounced, possibly because some stimuli caused noticeable rejection even at the lowest concentration tested (probit regressions: denatonium: ED_{50}=0.188 mmol l^{-1}, 95% CI=0.02–0.70 mmol l^{-1}; quinine: ED_{50}= 0.033 mmol l^{-1}, 95% CI=0.00–0.17 mmol l^{-1}; caffeine: ED_{50}=0.429 mmol l^{-1}, 95% CI=0.07–1.32 mmol l^{-1}; cinnamaldehyde: ED_{50}=0.807 mmol l^{-1}, 95% CI=0.14–2.29 mmol l^{-1}). Ink (10% of full strength) was a very effective deterrent but ASW was not. Providing further proof that OD and rejection are linked, the ED_{50} values for the two behaviors are positively correlated (R^2=0.8944, one-tailed, P=0.0271), meaning that compounds that are more effective in evoking OD are also more effective in evoking rejection.

Cinnamaldehyde and caffeine, which were ineffective with intact animals, caused significant rejection under these conditions. One possible reason for this is the lack of appetitive input from the mouthpart chemoreceptor neurons.

Effect of an appetitive stimulus on OD

One of the main differences between the OD and feeding assays using tethered animals was the presence, in the former, of appetitive chemicals. To investigate the effect of these appetitive chemicals on the OD response, we tested whether it was modified by presenting deterrent shrimp mixtures. Fig. 5 shows that adding 1% w/v shrimp had no effect on the proportion of crabs performing OD when stimulated with quinine at concentrations that ranged from ineffective to fully effective (0.1, 0.316 and 1 mmol l^{-1}), indicating that there is no interaction, even though at this concentration shrimp extract evokes a robust response in intact crabs, consisting of rhythmic maxilliped movements.

Effect of blockage of oesophageal receptors

Blockage of the oesophageal receptors with silicone caused the proportion of animals performing OD to drop significantly. Fig. 6 depicts the results of the experiment in which nine crabs were stimulated with taurine (a well-known chemical stimulus for crustaceans) (Zimmer-Faust et al., 1984; Derby et al., 1991), denatonium and quinine, each at 5 mmol l^{-1}, and with ASW, before and after coating the oesophagus with silicone to hamper access to the chemoreceptors (Basil et al., 2000). The proportion of animals performing OD when stimulated with denatonium and quinine was reduced by treatment with silicone (one-tailed McNemar's test: quinine, P=0.0315; denatonium, P=0.0315). Neither ASW nor taurine evoked significant OD before or after treatment (binomial test: P>0.05 in all four cases when compared with the expected value of 0).

Access of chemicals to oesophageal receptors

Finally, we asked whether access of the deterrent compounds to the oesophageal receptors could be facilitated by the animals biting and attempting to swallow distasteful food. Because we could not directly observe access, we recorded from the mandibular closer muscles of eight temporarily blinded crabs that were free to move while they were manipulating shrimp laced with either ASW or 5 mmol l^{-1} quinine. Independent of the stimulus, all crabs bit the shrimp at least once (Fig. 7). Lacing the shrimp with quinine caused the number of bites per shrimp to fall from 10.63±6.278 (ASW) to 5.63±4.104 (quinine), a significant difference (paired two-tailed t-test, P=0.0144), explained by the fact that the crabs tended to reject the quinine-laced shrimp after a few bites.

DISCUSSION

Blue crabs, like all decapod crustaceans, use chemoreceptors of diverse types and locations to locate and consume food. Although

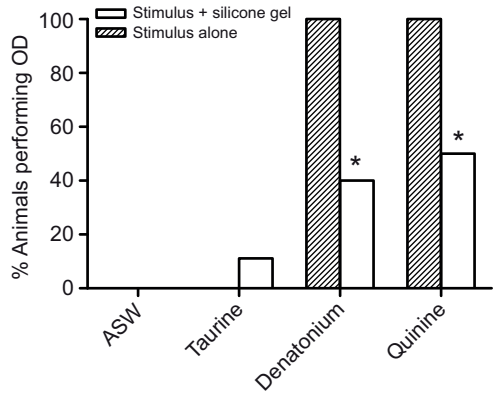

Fig. 6. Blocking the oesophageal receptors with silicone gel reduces the percentage of crabs performing OD when stimulated with feeding deterrents. Denatonium and quinine (5 mmol l^{-1}) evoked OD in all tested crabs, while ASW and taurine (5 mmol l^{-1}) did not evoke significant OD. After coating the animal's oesophagus with silicone gel, the proportion of animals performing OD was significantly reduced for both deterrents (*P<0.05, one-tailed McNemar's test), but it did not change for ASW or taurine. N=9.

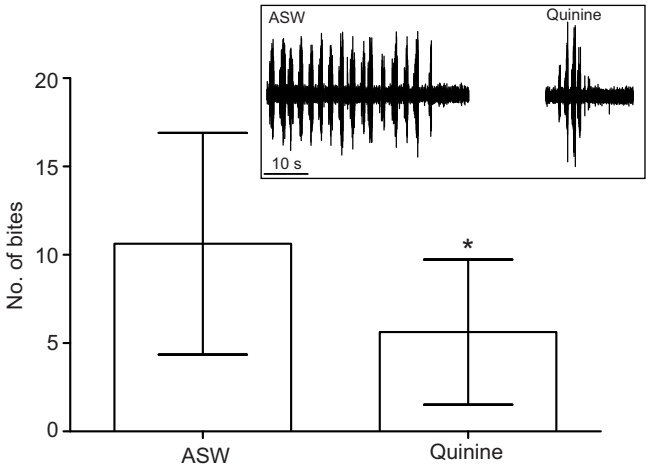

Fig. 7. Crabs bite quinine-laced shrimp. All 8 crabs bit the shrimp at least once, regardless of what it was laced with: ASW or 5 mmol l^{-1} quinine. The effect of the deterrent is evident in that the mean number of bites is greatly reduced (one-tailed paired t-test, *P=0.0144). This is because the crabs tend to reject the quinine-laced food instead of consuming it completely, as is the case with ASW. Values are means ± s.d. Inset: examples of two electrophysiological recordings from mandibular closer muscles: the crab bit a piece of ASW-laced shrimp 16 times (left) and then rejected a different piece of 5 mmol l^{-1} quinine-laced shrimp after biting it 4 times.

it has been known for some time that searching for food is driven by different subsets of these neurons depending on the distance between the animal and food, very little is known about any possible further specialization in their functions. All these receptor neurons are potential targets for chemical deterrent molecules, and the aim of this paper was to identify those that are used to decide whether a food item should be ingested or rejected. Our results show that if palatable food is laced with a deterrent, blue crabs use only chemoreceptor neurons inside the oesophagus to decide to eat or not. All appendages mediate the appetitive behaviors that are responsible for putting the food (and the deterrent) in close proximity with the oesophageal receptors. We currently do not know whether this is because the appendages lack deterrent-sensing chemoreceptor neurons altogether or because deterrent input from these appendages is processed differently. Our results imply that blue crabs will invest resources in tracking, attacking and attempting to consume an item that will eventually be discarded. It would be interesting to confirm whether the above is true for more biologically relevant deterrent molecules.

Receptors responsible for rejecting ink-laced food

Antennular chemoreceptor neurons, which include olfactory receptor neurons, do not mediate the effect of *Aplysia* ink or two of its major components. This is supported by the observation that intact blue crabs searched for ink-laced shrimp (Fig. 1A) and antennule-ablated ones did not eat APV/PEB-laced shrimp as readily as they ate ASW-laced shrimp (Fig. 1B), a result that closely resembles the behavior of intact crabs towards ink-laced shrimp (Fig. 2).

Chemoreceptor neurons located in the pereiopods are also not responsible for mediating the effect of feeding deterrents. All crabs passed deterrent-laced food to their mouthparts, where it was manipulated and bitten (Fig. 7).

Our results also support the idea that the deterrent-detecting receptor neurons are not located in the mouthparts. Even though we were unable to ablate all setae-bearing mouthparts simultaneously, ablation of some mouthparts did not abolish the deterrent effect of ink (Fig. 2A). If anything, the ablation of the inner mouthparts caused animals to spend significantly more time handling ASW-laced food than both intact and outer mouthpart-ablated crabs, and all three groups were affected by the presence of ink, and spent significantly more time handling ink-laced food than ASW-laced food (Fig. 2A). These results, and especially those with ASW-laced shrimp, suggest that at least the inner mouthparts mediate ingestion. This interpretation is confirmed by the results of feeding-restrained crabs (Fig. 4). Animals in this experiment were unable to use their mouthparts to handle the food, and 20% of them rejected the ASW-laced shrimp. These results are in keeping with what is known about the control of feeding behavior in decapod crustaceans. American lobsters with deafferented mouthparts pick up mussels but do not eat them, unlike control lobsters, which readily eat them (Derby and Atema, 1982). In addition, much of the literature, while not explicitly testing the hypothesis that the external chemoreceptor neurons are not responsible for the rejection behavior, presents the data in a way that shows that they are not; predators routinely attack and bite prey or artificial food before rejecting it. American lobsters reject a disk soaked in a mussel extract mixed with tannic acid, but only after repeatedly attempting to eat it (Derby et al., 1984). Spiny lobsters (Kicklighter et al., 2005; Aggio and Derby, 2008), freshwater prawn *Macrobrachium rosenbergii* (Steiner and Harpaz, 1987) and blue crabs (Kamio et al., 2010a) all attempt to eat deterrent-laced food. One possible exception is that APV interrupts the food-searching behavior in blue crabs (Kamio et al., 2010a). However, APV was delivered by squirting it to the mouth, so the possibility of it reaching the oesophageal receptor neurons cannot be excluded. Furthermore, crabs will search for ink-laced shrimp (Fig. 1A).

In insects, the situation is somewhat different. In the cockroach *Blatella germanica*, chemoreceptor neurons located in the labial palps are geared towards the reception of feeding deterrents, maxillary palps are geared towards feeding stimulants, and the paraglossae detect both (Wada-Katsumata et al., 2011), although

other authors have shown that insects use digestive tract-located chemoreceptor neurons to reject food (Thomas, 1966; Moulins, 1971; Kang et al., 2010).

Chemical senses-mediated, long-distance avoidance has been reported for decapod crustaceans. Blue crabs avoid traps that contain injured conspecifics (Ferner et al., 2005) and tend not to track a food odor if the scent of an injured conspecific is also present (Moir and Weissburg, 2009). Spiny lobsters avoid conspecific hemolymph (Briones-Fourzán et al., 2008; Shabani et al., 2008) and injured conspecifics (Behringer et al., 2006; Behringer et al., 2008). However, because these examples involve conspecific cues, these behaviors are probably mediated by aesthetasc chemoreceptors, a hypothesis that has only been tested, and found to be true, in one of the cases mentioned. Alarm cues released with conspecific hemolymph in spiny lobsters require the presence of the aesthetascs: their ablation reverses the behavioral response, and lobsters display appetitive responses to conspecific hemolymph (Shabani et al., 2008). There are also examples of long-distance avoidance in which predator odors, not conspecific odors, play a role. Juvenile spiny lobsters avoid dens that emanate octopus odor (Berger and Butler, 2001), and hermit crabs also avoid the odor of a predatory octopus (Brooks, 1991). To our knowledge, there is only one example of a feeding deterrent compound acting before it reaches the mouth of a decapod crustacean (Soti and Kem, 2001).

Rejection evoked by other compounds

The five other known deterrent or aversive compounds that we tested varied in their ability to affect food consumption by blue crabs. We tested three compounds perceived as bitter by humans (quinine, caffeine and denatonium), a reactive electrophile (cinnamaldehyde), and nicotinamide, which stimulates oesophageal receptor neurons in crayfish (Altner et al., 1986). Of these compounds, only quinine and denatonium were effective feeding deterrents for crabs (Fig. 2B) and, once again, this effect was not modified by ablation of the mouthparts. Interestingly, these compounds did evoke OD when applied to the oral region (Fig. 3B) and were effective deterrents when presented to immobilized animals (Fig. 4). Once again, the data point towards oesophageal receptor neurons being in charge of rejection behavior. The remaining compounds (caffeine, cinnamaldehyde and nicotinamide) did not evoke a significant rejection behavior in intact, freely moving crabs (Fig. 2B). The fact that all compounds tested evoke rejection in restrained animals may be due to the lack (or significant reduction) of appetitive input in that situation. Intact crabs take deterrent-laced food to their oral region and spend significant time handling it, which is evidence that the appetitive elements of shrimp are in fact having an effect. In natural conditions, of course, successful deterrence is only achieved if the unpalatable compounds are strong enough to fully counter the prey's palatable compounds.

Rejection and OD

The positive correlation between the ability of a compound to elicit OD and rejection is further proof that these two behaviors are linked, and argues for the existence of a common mechanism. Quinine and denatonium, the only compounds capable, at 5 mmol l^{-1}, of evoking significant rejection in freely moving crabs, are also more effective in evoking rejection and OD in immobilized crabs. Thus, OD provides a sensitive first approach to evaluate whether a given compound is deterrent or not. Because in a realistic context the deterrents are rarely, if ever, experienced without concurrent positive stimuli, we investigated whether the OD response would be affected by shrimp extract. The results of this experiment were negative, with a food stimulus unable to modify the response to quinine (Fig. 5). Robertson and Laverack showed that in *Homarus gammarus* stimulation of the two sets of sensors located in the anterior and posterior oesophagus cause opposite effects on peristalsis (Robertson and Laverack, 1979b). The posterior oesophageal sensors initiate it and the anterior ones terminate it, and the authors speculate that they might be involved in initiating and terminating feeding, respectively (Robertson and Laverack, 1979b). Our results show that there is no interaction between them. This is consistent with an 'all or nothing' or 'labeled line' interpretation of the bitter sense proposed for vertebrates (Chandrashekar et al., 2006; Yarmolinsky et al., 2009): food containing even minute amounts of 'bitter' (i.e. toxic) compounds must be rejected to avoid poisoning. Within this framework, it is to be expected that the presence of positive stimuli should not affect the OD reflex.

OD, food rejection and the oesophageal receptors

Further proof linking OD, rejection and the oesophageal receptor neurons comes from the fact that blocking the receptor neurons significantly reduces the proportion of crabs performing OD (Fig. 6). After having the oesophagus coated with silicone, approximately 50% of crabs ceased performing OD when stimulated with 5 mmol l^{-1} denatonium or quinine. The fact that all crabs bit quinine-laced shrimp at least once (Fig. 7) proves that even food that ultimately will not be consumed enters the oesophagus and is thus in a position to contact the oesophageal chemoreceptor neurons.

The known oesophageal chemoreceptors of decapod crustaceans are located in the anterior and posterior oesophageal sensors, paired structures located at the oesophageal–cardiac valve (Robertson and Laverack, 1979b). These sensors have opposite effects on oesophageal peristalsis: the AOS inhibits it while the POS stimulates it (Robertson and Laverack, 1979b). Although these authors were unable to record sensory activity from the axons of the sensory nerves that innervate the sensors, they obtained indirect evidence for this: stimulating the AOS with food extract or electrically stimulating its nerve caused peristalsis to stop, and the opposite effect was achieved when the stimuli (chemical or electrical) were applied to the POS (Robertson and Laverack, 1979b). To our knowledge, the only published report of successful recordings from these structures is the work of Altner and collaborators (Altner et al., 1986). They recorded from the AOS of the crayfish *Astacus astacus*, and reported that there are two types of chemoreceptor cells (Altner et al., 1986). These cells have unusual characteristics: one responds with a very long latency (~8 s) to stimulation with nicotinamide and related compounds; the other type responds with a somewhat shorter latency (~4–6 s), but only to crayfish gastric fluid. The authors tested many compounds and were unable to elicit responses from this cell type (Altner et al., 1986). These authors commented on the contradiction between their results and those obtained by Robertson and Laverack (Robertson and Laverack, 1979b); the two studies differ in the type of chemical stimuli required to evoke activity from these receptors. The issue remains unresolved.

Feeding rejection in other taxa

The existence of compounds that negatively affect animals acting through their olfactory systems has been demonstrated in other taxa. For example, *Aplysia* ink can prevent bluehead wrasses from reaching a food item, or increase the time it takes them to do so when released as a cloud between the animal and the food, and the olfactory epithelium is required for this effect (Nusnbaum and

Derby, 2010a). These compounds have multiple sites of action; they can also be tasted by the fish (Nusnbaum and Derby, 2010b). In addition, several fish species reject food laced with deterrents only after biting it or otherwise putting it in their mouth: goldfish (*Carassius auratus*) (Lamb and Finger, 1995), bluehead wrasses (*T. bifasciatum*), señorita wrasses (*O. californica*), mummichogs (*F. heteroclitus*), pinfish (*L. rhomboides*) and bonnethead sharks (*S. tiburo*) (Nusnbaum and Derby, 2010a; Nusnbaum and Derby, 2010b). This apparent paradox can be resolved if we consider that the two ways of administering the deterrent (as a cloud or lacing food) have very different consequences, an issue that will be discussed below.

In the terrestrial environment, insects avoid both DEET and CO_2 (Suh et al., 2007; Syed and Leal, 2008; Turner and Ray, 2009; Liu et al., 2010). DEET repels mosquitoes in the absence of an attractant (Syed and Leal, 2008) and inhibits feeding in *Drosophila* by acting through taste receptors (Lee et al., 2010). Lee and colleagues do not mention whether DEET has an olfaction-mediated effect, but if it does, their experimental design guarantees that it was equally distributed, and thus the effects they report are indeed gustation mediated (Lee et al., 2010). This is an example in which the magnitude of the olfaction *vs* taste effects can potentially be attributed, at least in part, to the differential distribution of a compound in two different media.

Not all damaging or aversive compounds are detected by all chemosensory subsystems, suggesting that there is specialization. In *Drosophila*, the bitter compound caffeine and reactive electrophiles such as cinnamaldehyde are detected with taste receptors located in different places (Kang et al., 2010). Whereas caffeine can exert its effect by contacting the tarsi, electrophiles have to contact receptor neurons located in the labral sense organ, whose sensilla open to the lumen of the oesophagus (Kang et al., 2010). This is very interesting, given that reactive electrophiles can damage tissues (Farmer and Davoine, 2007; Liebler, 2008), and one would expect that they would be detected (and thus avoided) earlier in the ingestion process.

There are other examples of rejection mediated by chemoreceptor neurons located within the digestive tract of insects (Thomas, 1966; Moulins, 1971), and Thomas (Thomas, 1966) mentions several earlier works in which insects were shown to bite food before rejecting it, which seems to indicate that internal receptors are required.

Why wait so long?

Given that many animals, and crabs in particular, possess chemoreceptor neurons distributed widely over their bodies, the question of why food is rejected only at the last possible moment arises. In terrestrial animals, the different chemical characteristics of odorants and tastants may explain why many feeding deterrents do not act through the olfactory system; being non-volatile, they are unable to reach the olfactory receptor neurons. This would seem to not be the case for aquatic animals, because both their olfactory and non-olfactory systems are tuned towards water-soluble compounds. Closer inspection, however, reveals that known feeding deterrents for aquatic animals are much less soluble in water than known feeding stimulants such as amino acids. Indeed, Koyama and Kurihara showed that bitter compounds such as quinine, caffeine and nicotine penetrate into the non-polar section of a lipid monolayer (Koyama and Kurihara, 1972). These authors, working long before the discovery of gustatory receptors, remarked that the taste thresholds for the different compounds were linked to their ability to penetrate membranes (Koyama and Kurihara, 1972). In our own experiments leading to this paper, we found that compounds such as capsaicin, menthol, allyl isothiocyanate and *N*-methylmaleimide, which are known irritants and deterrents, were almost insoluble in ASW and were not pursued further because they tended to aggregate in big clumps, making it impossible for us to deliver them consistently (data not shown). Interestingly, although PEB and APV are equally potent, *A. californica* transforms the former into the latter by changing a carboxyl group into a methyl ester (Kamio et al., 2010b), a reaction that may make the molecule even less soluble in water.

A priori, it would seem beneficial to both predator and prey for the deterrence to be effective from a distance. The predator does not expend energy and resources in finding prey it will not consume, and the prey avoids the proximity of the predator and the risks this entails. This, however, seems not to be norm, perhaps because of the characteristics of the deterrent compounds. It is interesting to speculate that the lack of solubility of deterrent compounds in aquatic environments allows the defended organism to retain them until needed, thus reducing the need to acquire or synthesize them. The oesophageal receptor neurons of decapods crustaceans may provide a model to further elucidate this issue.

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