**REVIEW ARTICLE**

# Under threat… Chemical communication in decapods. A minireview on the role of olfaction during agonistic interactions.

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**Abstract**

Decapod crustaceans is an animal group whose functional characteristics make it an ideal model for many neurophysiological studies, from basic cellular function to integrative and the so-called superior brain functions. Olfaction and chemical detection are two faces of detection of water-dissolved compounds that are determinant for the survival of the individual as well as the species. Olfaction in particular, shares many anatomical commonalities with insects but also with vertebrates. The elaborated coding and the integration process of olfactory information seem both basic and highly complex to identify and differentiate unknown dissolved molecules that participate in mating, mating selection, and agonistic encounters. Not a single compound or a mixture of compounds associated with winning or losing agonistic encounters have been isolated. When two size-matched unknown winners or two size-matched unknown losers are paired a new winner emerges, which implies new coding/decoding and integrative processes have occurred. We do not know what these processes are, but pinpoint to the more important events in these relationships quite important in the maintenance of territoriality, access to food and mate, etc.

## 1. Introduction

In the wild, a male crayfish comes out of its burrow in the river sand. The animal patrols its territory and forages. Abundant food and a well oxygenated stream of water make its habitat ideal. The absence of predators around allows it to patrol its entire territory and to get the right amount of food. This animal can detect the nutrient substances dissolved in water that are useful to its metabolic necessities. Its attention is focused in the chemical information, but this animal is also capable to attend and process other sensory stimuli at the same time. It can detect shadows; moving or motionless shadows that can indicate the presence of danger, predators or enemies. Mechanical sensitivity is also present detecting water currents, underwater plants moving, and bubbles emerging from the bottom of the river. The water stream carries many molecules, some of them significant for the crayfish existence. These molecules impinge on the chemical receptors from the mouth parts, the walking legs and antennules. These chemical receptors are of particular importance because they relay until the brain and they carry information related to food sources but also from predators, possible mates and possible enemies, foreign crayfish just passing the territory or clear contenders trying to steal the territory and everything inside.

Suddenly a different odor impinges its antennules, it belongs to a foreign crayfish whose clear intentions are to take possession of the entire territory, even the females that soon will be sexually receptive. The intruder locates itself inside the visual field of the resident animal and immediately presents its most powerful weapons: both chelae, that open and close slowly in a threatening display. The resident crayfish displays the same weapons and it also elevates on its walking legs increasing its size. However, the intruder does not give up and advances, approaches the resident crayfish. Both animals are of the same size, more or less the same weight, healthy, all appendages complete, both "willing to keep the territory". Who will be the winner, who the loser? What are the determinants of winning or losing? It is possible to state that the best fitted will be the winner. But, under equality of capabilities, which are the determinants? When an animal recognizes its contender as the dominant? What are the signals?

Despite many studies on agonistic relations in crayfish and lobsters the previous questions seem open **(1)**. Recently we demonstrated that crayfish has recognition memory that is fully functional during agonistic encounters, although we do not know yet if this recognition is for individual or for status **(2)**. Although crayfish can detect shadows and objects to a certain distance its vision seems quite poor both on air or underwater conditions. Many ommatidia have to build a complex colored image which becomes more blurred as both animals come close together. Why they keep moving their chelae even at close distance? We do not know! However, we know that when they are very, very close they aim their nephropores towards the rostrum of the contender, specifically to the antennules, the olfaction detection organs. Big urine streams bath the antennules from both fighters. What's in the urine? Once again, we do not know.

Two forms of chemical information are arriving at this moment. A close-contact one that stimulates each part of the animal body and tells the crayfish what is around, food, detritus, everything that is dissolved in the water and is exciting the chemical detectors distributed all along the body. A second source of chemical information comes from the distance and is mixed with visual signals of great importance for the survival of the animal. Streams of chemicals accompany the visual display of a would-be contender. The main olfactory organs, the antennules, flick frantically increasing the contact between the aesthetascs from the inner branch of the organ with the streams of chemicals produced by the intruder. This is the olfactory information that will work simultaneously with the distributed system when both animals are in close contact in an agonistic encounter.

Chemical detection seems to be the most important sensitivity in aquatic decapods, particularly during, but not exclusively, agonistic encounters **(3)**. In this paper we will review the status of information about chemical detection in decapods with the necessary comparisons between and among species **(4)**and try to point out the biggest problems that remain in this field.

## 2. Chemoreception and Olfaction: two systems, two purposes?

Decapod crustaceans detect biologically significant stimuli under different conditions, as, for example, mate detection **(5;6)**, conspecific recognition **(7;8)**, food and shelter location **(9;10;11)**, and predators or dead conspecifics **(12;13)**. The most used crustaceans are crayfish, lobster and crab. Studies are mostly behavioral and therefore the integration mechanisms are unknown. We also ignore many of the important substances that trigger a specific behavioral response, beyond the obvious as food.

The first stage in chemical communication in decapod crustaceans is restricted to reception and classification of odor molecules, this involves peripheral receptors that carry the initial information to the olfactory lobes. At this point we want to emphasize that in decapod crustaceans, chemical communication and chemical detection are "used with distinct purposes". Chemical detection is valuable, detected and processed for survival; chemical communications has social purposes. We are interested in social problems (memory recognition, dominance-submission relationship), therefore we will dedicate the remaining of this review to chemical communication, that is olfaction in decapod crustaceans.

Crustaceans get chemical information through two main pathways **(14)** the distributed and the olfactory pathway **(4;3)** (Figure 1).

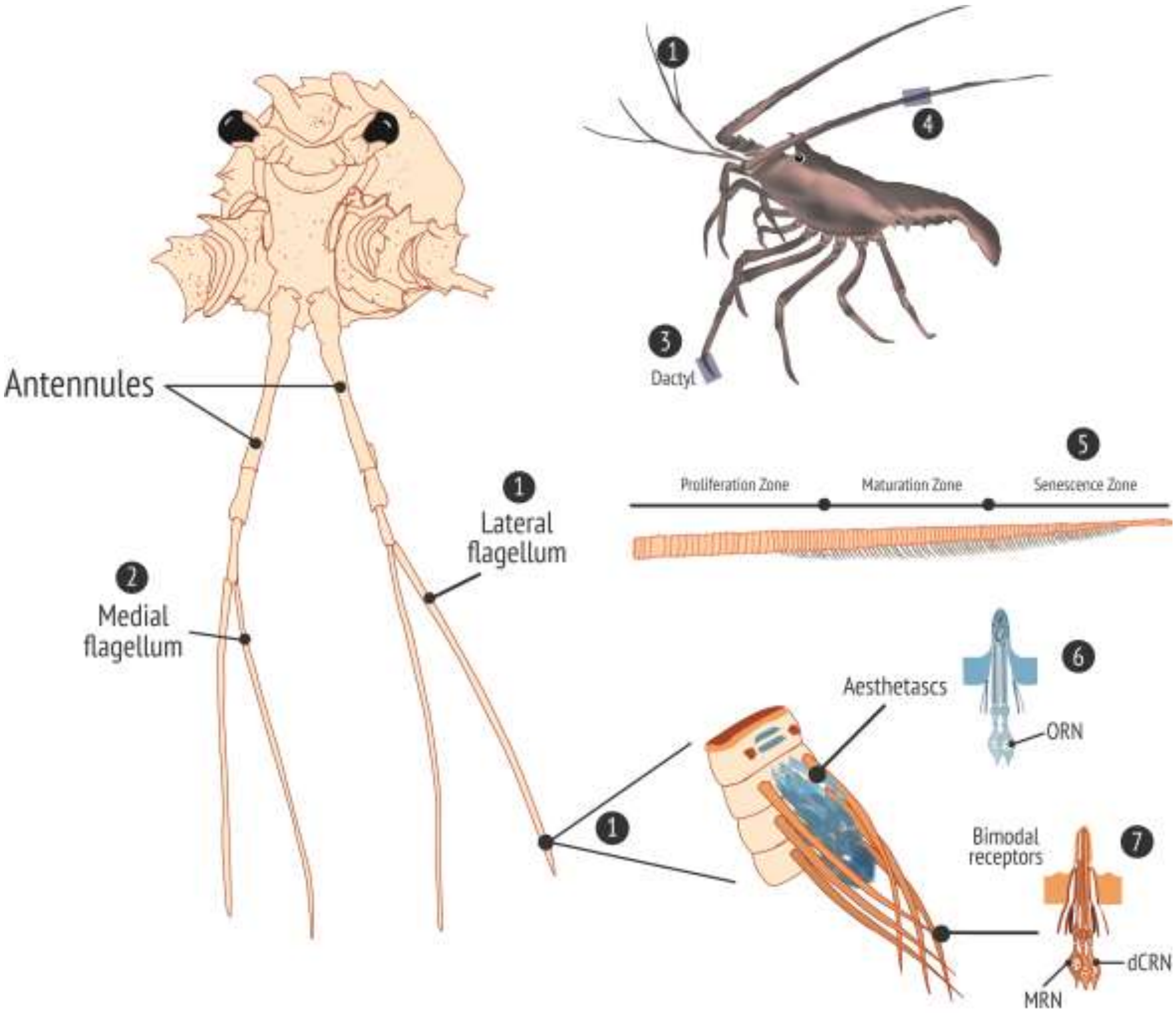

**Figure 1. Distribution of chemoreceptors in decapod crustaceans. 1) Lateral antennular flagellum. 2) Medial antennular flagellum. 3) Walking legs (dactyl). 4) Antenna. 5) Three development zones can be observed. The aesthetascs are located in the lateral flagella of the antennules. Each one are formed by cuticular rings that contains numerous aesthetascs. 6) Olfactory Receptor Neurons (ORN) are under the cuticular surface of aesthetascs. 7) Outside the aesthetascs there are bimodal receptors (mechanical -MRN, and deutocerebral chemical -dCRN).**
**Modified from Beltz and Sandeman, 2003 and Schmidt and Mellon, 2011.**

The distributed system is comprised by structures placed out of the main olfactory organ, the antennules, and consists of both chemical and mechanical receptors that are located in antennae, mouthparts, fans, dactyl claws, and maybe other parts of the animal, and that can be sensitive to a single modality, mechanical or chemical, or to both simultaneously (mechanical and chemical)(15;16;17). Information gathered by antennal bimodal receptors is sent to tritocerebrum through chemoreceptor neurons and mechanoreceptor neurons into somatotopically organized neuropils and in subesophageal ganglion, and thoracic and abdominal ganglia where they serve as local motor centers for the appendages **(18;4;3).** Behaviorally, crayfish show differential responses to chemicals placed in its aquarium (ammonium, glucose, glutamate, glycine, maltose, trehalose) and it is proposed that the animal uses chemoreceptors from pereiopods, walking legs and mouthparts **(17).**

Axons from the distributed system innervate the lateral antennal neuropiles (LAN) **(19)**.

The olfactory system of crustaceans is located in the lateral flagellum of the biramed antennules, short appendages that emerge from the rostrum. This lateral flagellum contains unimodal chemical sensitive units called aesthetascs (unimodal sensilla), that comprise only olfactory sensilla and are innervated by olfactory receptor neurons (ORN) that project into olfactory lobes organized into glomeruli in the deutocerebrum. The medial flagellum has also bimodal sensilla (chemical and mechanical sensitives) **(18;3)**.

Aesthetascs in antennulae are grouped in tuft-like structures in whose distal end are highly branched dendrites from the olfactory receptor cells (ORN) (Figure 1) **(20;21;22;23;24;25;3;18)**. Under the aesthetascs' cuticle are the ORN somata grouped in big sets of 100 to 400, depending on the species.

In crayfish and lobster there are three development areas along the antennule, the proliferative zone which produces constantly new aesthetascs; the maturation zone, with fully functional aesthetascs, and the senescence zone where the old damaged aesthetascs are discarded (Figure 1). It is not clear if there is a constant re-change of aesthetascs, only the damaged aesthetasc or the continuous grow of antennule **(26;27)**. The olfactory receptor neurons (ORN) leave the aesthehascs and their axons project towards specific neuropils (Figure 2) in both olfactory lobes of deutocerebrum **(28;29;30;31;32;33)**.

The total amount of ORN in crustaceans shows an evolutive increase in different species, such that they reach its maximum in decapods, which seems related to the greater importance of olfaction in this group of animals **(4)**.

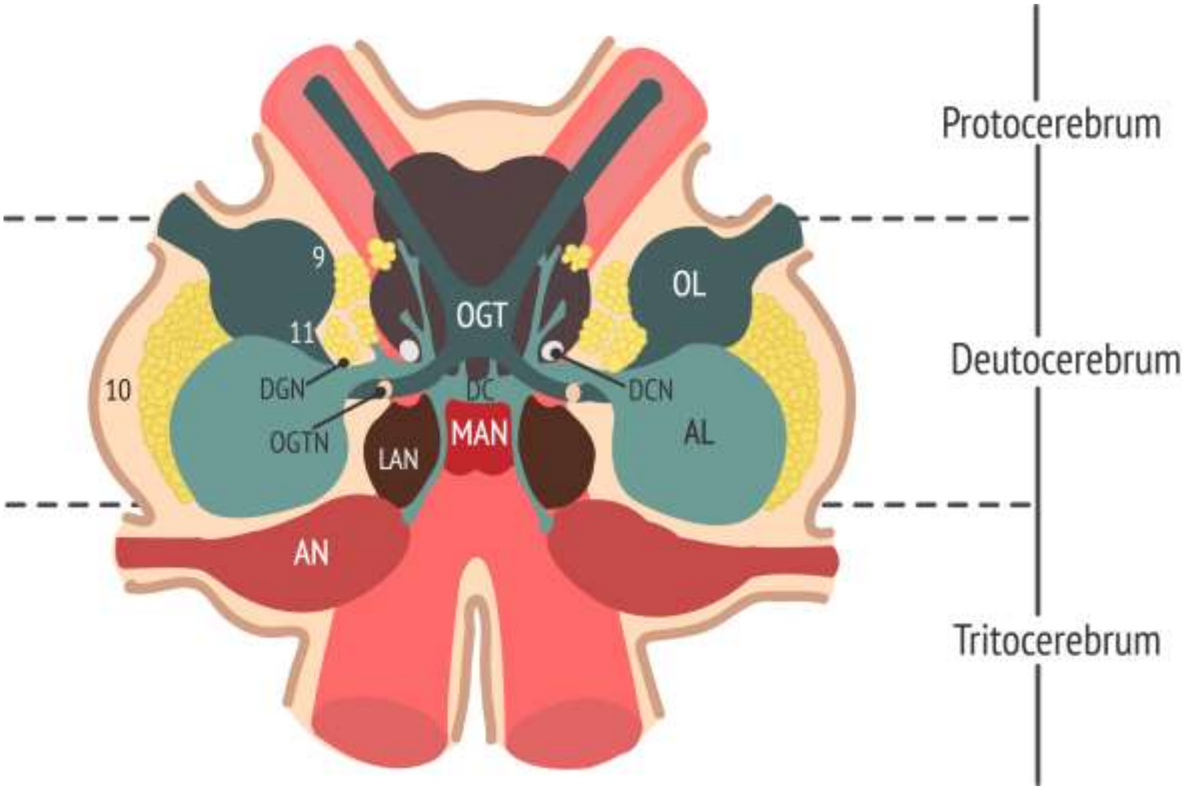

**Figure 2. Brain in decapods. It is made up of a set of neuropils and clusters, with the neural bodies in the second one. Three divisions can be observed: protocerebrum, deutocerebrum and tritocerebrum. Afferent extensions of ORNs reach the olfactory lobe (OL) in the deutocerebrum. Projection neurons send axons to the protocerebrum through the olfactory globular tract (OGT).**
**AL: accessory lobe, AN= Antennal neuropil, OGTN= Olfactory globular tract neuropil, LAN= Lateral antennular neuropil, MAN= Medial antennular neuropil, DC= Deutocerebral commissure. DCN= Deutocerebral commissure neuropil, DGN= Dorsal giant neuron. The clusters are represented by numbers.**
**Nomenclature after Beltz and Sandeman, 2003.**

It is convenient to point to a complementary function of these two chemoreceptor systems. On one hand we have a well-developed olfactory system that is capable to locate all the relevant information for socialization. On the other hand, a chemosensory contact system, with receptors all around the body and capable to find food among other things. Both systems working in parallel during the search for food, mate, conspecifics, etc. **(34;23;27)**

It has been proposed that the function of these two systems is cooperative although they can function in independent ways, as needed. When in cooperative form, the olfactory system would provide a chemical representation of the complex external world but without a specific behavioral response. Meanwhile, the distributed system would provide the somatotopic chemical compounds response, which allows the integration of closer chemical stimuli that can, or not, complement the olfactory ones but that have the specificity of the body parts **(3)**.

**2.1. Olfactory Lobes.** As in insects or some mammals, olfactory lobes are organized in glomeruli, highly synaptic areas that populate the cortex of the glomeruli. With an almost conic shape, the tip of each glomerulus points to the lobe's center **(4;35;36)**.

In each glomerulus (lobsters, crabs and crayfish) three longitudinal regions can be distinguished, a basal region, a layer and a sub-layer, this last is divided in a central nucleus and an external ring **(37,38)**. Both olfactory lobes are identical, and no regionalization or lateralization have been described **(27).**

When ORN axons reach the glomeruli, they invade the periphery. Here, they form a plexus at the level of the layer which becomes the region with the highest number of afferent synapses, some terminals penetrate deeply up to the sub-layer and even to the basal region **(39)**.

In lobster, argentic techniques have shown that ORN axons have a multiglomerular distribution **(40)**. Similar distribution has been described in crayfish **(41; 42)**. Steullet et al. **(43)** suggested that each aesthetasc has an identical combination of olfactory receptor cells where each one is different to the rest in the aesthetasc because each one has a different type of membrane receptors. In this proposal each glomerulus would receive the same kind of olfactory receptor cells. This would imply a direct relation between the number of glomeruli and the number of olfactory receptors in the antennule **(23)**, and that the increase in aesthetascs observed along the life of a crustacean would not imply a greater number of odorants detected since birth, but it does a higher sensitivity to the important compounds.

"The odor world is sampled by huge numbers of ORN, which converge type-wis upon a much smaller number of neurons within the glomeruli. There, odor information is distributed and transformed by cross-glomerular circuit interactions mediated, in part, by inhibitory interneurons, which impose fast oscillations and slow temporal structures on the principal neuron firing patterns…these transformations sparse the neural representations of odors, improve the signal-to-noise characteristics, define broad odor categories, achieve precise odor identification, extract invariant features and specializations…"**(44).**

As in insects, once inside the glomerulus, sensory afferents synapse with local interneurons (LNs) (that interconnect glomeruli) and projection neurons (PNs). In both cases, the neural somata are located in the cell groups called "clusters" (Figure 2). Somata from local interneurons are placed in clusters number 9 and 11, those from projection neurons are in cluster 10**(4; 45; 46; 36; 3).**

Local interneurons innervate the cortex of the glomerulus (layer and sublayer, 'rim interneurons') or the basement of the glomerulus ('core' interneurons). In both groups of neurons Sandeman, Beltz and Schmidt have demonstrated active neurogenesis along the animal's life. This neurogenesis is directed by neurogenic niches located into the clusters **(47; 48; 49; 36).**

Each local interneuron innervates many glomeruli, up to 85% in accordance with

Wachowiak et al. (50); while rim interneurons send lateral connections. Some of the interglomerular fibers are GABAergic cells, which suggests a lateral inhibition mechanism that modulate the afferent input from ORNs (50). In all studied decapods this radial arrangement of glomeruli is present with constant spaces and positions of glomeruli and connections which could facilitate the lateral inhibition functioning (39).

Projection neurons (PN) have their somata inside cluster 10. Their dendritic trees originate at the glomerular base, their very thin axons (200 nm diameter in the crayfish) carry sensory information to the terminal medulla/hemiellipsoidal body located in the protocerebrum (Figure 3). These axons join to other projection fibers to form the olfactory globular tract (OGT) which projects to protocerebrum. In its way to protocerebrum, the OGT finds another synaptic region in a neuropile, the Olfactory Glomerular Tract Neuropile, OGTN, here there are many interactions among local and projection neurons (51; 37; 52).

Roughly 100, 000 projection neurons can be found on each side of deutocerebrum, most of them send their axons to both sides of protocerebrum so that each hemiellipsoidal body receives around 200,000 axons (51; 37; 52). In this area, the hemiellipsoid body, arriving axons establish connections with protocerebral interneurons and form microglomerular structures (53).

At this moment we have an idea of the first stages of olfaction in decapod crustaceans. What happens later? The information gathered and classified by glomeruli is of social importance, it is not related with food or characteristics of the immediate environment, it can be related with dominant-submissive relationships, mating, identifying other conspecifics. Therefore, the next steps in the processing of information imply to integrate olfactory, visual, tactile, information in order to emit the appropriate response.

**2.2. Integration centers.** Integration seems to occur in three regions of the decapod brain, the terminal medulla, the hemiellipsoidal body, and the accessory lobe. These three neuropiles do not receive direct sensory inputs but from projection or second order interneurons which is associated with some variability in their building (35); these structures are associated with complex functions that imply decision making, conspecific recognition, etc. (27).

The Accessory Lobe (AL) is located in the deutocerebrum in a medial position as related to the olfactory lobe. In most decapods this structure shows a big size, that in lobsters and crayfish is bigger than the olfactory lobe (45,35). Thousands of tiny spherical glomeruli organize in columns and layers to form the AL, inputs coming from local interneurons and projection neurons. Local interneurons transmit information from the olfactory lobe while projection neurons transmit signals from the deutocerebral commissure. This last seems to be the most important input to the AL (54;28;29;55;51;38;56; 52). Olfactory signals reach the external cortex of the

AL while other sensory signals (mechanical and visual) reach the core. AL's columns are distributed in both AL zones and seem to filter the different modalities of sensory information (Figure 3). Signals from tritocerebrum arrive bilaterally and those from proto- or deutocerebrum make it unilaterally **(35;55)**.

The outputs from the AL are projection neurons that reach the hemiellipsoidal body/terminal medulla complex in protocerebrum. Multimodal signals arrive to the accessory lobe and modulate the projection neurons response to olfactory stimuli **(27)**.

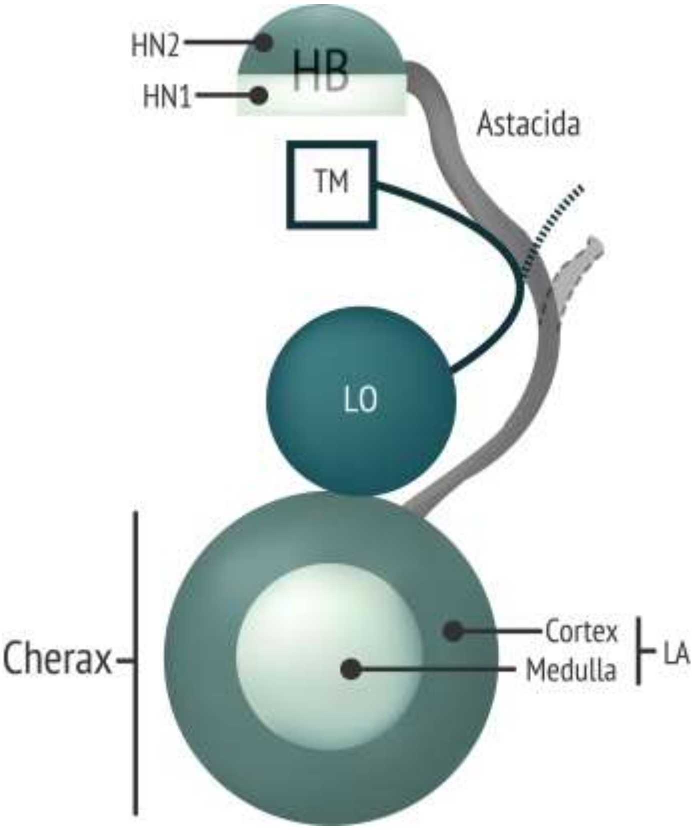

**Figure 3. Projections to integration centers. From the accessory and olfactory lobes in the deutocerebrum the projections go to the terminalis medulla/ hemielipsoidal body complex in the protocerebrum. In *Astacida* (crayfish) the projections have different targets. From the accessory lobe the projections arrive to the hemiellipsoidal body, while those coming from the olfactory lobe arrive to the terminalis medulla.**
**LA= Accessory love, HB= Hemiellipsoidal body, TM= Terminalis medulla, HN= Hemielipsoidal body neuropils.**
**Modified from Sandeman et al., 2014.**

Sandeman et al., suggest that AL receive multiple sensory inputs incoming from exploratory activities, then evaluate this information referred to previous experiences, and finally producing a corresponding output **(35)**.

The second integrative center is the hemiellipsoidal/terminal medulla complex located in protocerebrum. The terminal medulla receives axons from the accessory lobe and from the visual neuropiles located in the eyestalk **(32;57;35)**. Located very close to the terminal medulla, the hemiellipsoidal body (HB) is divided into two areas, the neuropil I and the neuropil II in the case of *P. clarkii* and *O. rusticus,* it receives projection neurons coming from the accessory lobe, but not from the olfactory lobe. Those from the olfactory lobe innervate mainly the terminal medulla. In lobsters the terminal medulla and the HB receive innervation from the olfactory lobe (Figure 3) **(32;57;58;59;56)**. Local interneurons (parasol cells) perform multimodal, higher-order sensory integration. Cell somata have been found at the base of the neuropils I and II, they have long dendrites that arborize extensively on the neuropils. Both neuropils receive asymmetrical input from contralateral and ipsilateral accessory lobes which is associated with functional separation of input information. Electrophysiological studies have shown different response patterns for chemical, visual or mechanical stimuli, and, in decapods, could be related with memory **(60;61;62;63;64;65).**

This complex arrangement of connections and synapses from the olfactory receptor cells to the upmost levels of brain integration imply the identification and recognition of an unknown variety of odors, images and tactile or mechanical stimuli that define the decapod behavior.

## 3. Olfactory receptors and decodification

In the odor domain it must start with the activation of molecular receptors and its transduction into action potentials or slow potentials along the olfactory pathways. In *Panulirus argus* there are ionotropic receptors (IRs) located in most of the ORN**(66)**; these receptors could have evolved from the ionotropic glutamate receptor. Their similarity with insect IRs could make them the first stage in olfactory transduction although there must be other olfactory proteins still undiscovered **(67;68;27)**. No olfactory binding proteins have been discovered although it is difficult to propose them in an aquatic environment full of currents. This environment is precisely which makes things more complicated. Odor molecules must be water-soluble and quite different from any others also dissolved in water **(27)**. As well as signaling diverse and complex messages, odor signals themselves are often very complex. Many classes of molecules fall within the theoretical limits of molecular size and type for olfactory signal function. These limits expand further when one considers that for aquatic species odors can travel by bulk flow in aqueous media. The information content of the signal is enhanced by the fact that real-world odors are rarely, if ever, single compounds, where related signals can contain many of the same components in different ratios. Single chemical compounds can elicit physiological and behavioral responses, but complete biological activity often requires stimulation with complex, multicomponent mixtures of chemical compounds (69).

Deciphering odor signals presents a common challenge to all animals. Odor signals serve to communicate in a diverse array of informationally demanding behavioral contexts. Odors of conspecific origin, known collectively as pheromones (70) denote the identity of individuals, social status, social group, and place. In addition to these "triggering" functions, pheromones also serve "priming" functions, in which the stimulus additionally or alternately initiates longer-term changes in the recipient animal rather than just eliciting immediate, overt responses (71;69).

All larger animals experience turbulent air or water flow, where local currents and eddies perturb stimulus clouds emanating from point sources, resulting in highly discontinuous odor plumes (72). Turbulent fluid motion on a scale of meters to millimeters determines the patchy intermittent structure of odor plumes in the environment (72). This is evident in the antennules from crustaceans, dye trapped, parcels penetrating among, along antennule.

There is strong consensus that odorants are coded in a combinatorial manner. This long-standing idea received strong support from evidence that individual mammalian olfactory receptor cells expressing a single, identified receptor protein can be activated by multiple different odorants, and that individual odorants activate multiple receptor cells expressing different receptor proteins (73). The olfactory system uses a combinatorial receptor coding scheme to encode odor identities; slight alterations in an odorant, or a change in its concentration, can change its "code", potentially explaining how such changes can alter perceived odor quality (73).

## 4. Olfaction in social behaviors

Chemical communication reaches critical levels under two circumstances, pheromone detection and agonistic interactions. In the first case it has been proposed that the distributed chemical system is in charge of detecting the chemical mixtures typical of female or male crustacean decapods (74;75; 3).

But our main interest is in the chemical communication during agonistic interactions. As previously stated, when two decapods face each other they orientate their nephropores aiming to the aesthetascs of the opponent antennules (Figure 4). Several studies have shown a principal role of urine in the establishment of a hierarchical order in different crustacean species. The general scheme escalates from long distance visual displays (meral spread, tip-toing on dactyls from ambulatory legs), to chemical communication that release some kind of compound directly to the olfactory organs, and finally the physical contact with its own levels of aggression.

**Figure 4: Agonistic encounter in crayfish. When two crayfish face each other, they orientate their nephropores aiming to the aesthetascs of the opponent antennules. Both crayfish present its chelae, that open in a threatening display. The urine is shown in green.**

What´s in urine? Is urine essential for recognition/establishment of hierarchical status? Many studies in lobsters and crayfish have shown that urine carries 'something' that identifies a given animal. Blocking olfaction or blocking contact with antennules induce long-lasting battles that normally are resolved in less than 15 minutes (75;76) recovering of olfactory function reestablishes duration of agonistic encounters. This shows that chemical signals released during offensive behavior are effective in reducing the aggression of an opponent.

Animals recognize each other but we do not know if this recognition is based on status or individual (2). Even more, the winner of an agonistic encounter is always the winner and the loser is also always a loser and all this seems to be dependent of the olfactory information. No differences have been identified in urine composition from winners or losers. Even more striking is the finding that when two dominant or two submissive animals face each other inevitably a new dominant and a new submissive animal emerges. Does this imply that the new dominant-dominant can produce more, or different mixtures added to its urine? Or that the new dominant-submissive learnt to produce the 'winner' mixture? Implications are multiple and we still do not have a clue on what's happening.

Chemical signals appear to play a major role during agonistic interactions between decapods. The study of the chemical nature of aggressive signals promises insight into these still unsolved questions of crustacean agonistic behavior.

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