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**Electrophysiological and morphological analyses
in two species of insects,
Protophormia terraenovae and *Ceratitis capitata***

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List of Publications

This thesis is based on the work contained in the following papers:

Giorgia Sollai, Paolo Solari, **Valentina Corda**, Carla Masala, Roberto Crnjar (2012). The spike generator in the labellar taste receptors of the blowfly is differently affected by 4-aminopyridine and 5-hydroxytryptamine. *Journal of Insect Physiology* 58: 1686-1693.

Valentina Corda, Giorgia Sollai, Carla Masala, Paolo Solari, Roberto Crnjar. Morphological characterization of the antennal lobes in the Mediterranean fruit fly *Ceratitis capitata*. *Manuscript*.

Abbreviations

ORN	Olfactory receptor neuron
PBP	Pheromone-binding protein
OBP	Olfactory-binding protein
OR	Olfactory receptor
ODE	Odor-degrading enzyme
GPCR	G protein-coupled receptor
IP ₃	Inositol trisphosphate
AL	Antennal lobe
GRN	Gustatory receptor neuron
SOG	Subesophageal ganglion
MDT	Maggot debridement therapy
SIT	Sterile insect technique
K ⁺	Potassium
K _v	Voltage-gated K ⁺ channels
KA	A-type K ⁺ current
DKR	Delayed rectifier K ⁺ channels
5-HT	5-hydroxytryptamine, Serotonin
4-AP	4-aminopyridine
RP	Receptor potential
NB	Neurobiotin
DAPI	4',6-diamidino-2-phenylindole
PFA	Paraformaldehyde
PBS	Sodium phosphate buffer
PBST	Sodium phosphate buffer with 0.2% Triton X-100
BSA	Bovine serum albumine
AN	Antennal nerve
AC	Antennal commissure
MGC	Macroglomerular complex
DPX	Mountant for histology

Objectives

Chemoreception represents one of the most important sensory modality to guarantee animals survival, and it plays a fundamental role also in an insect life. In fact, the detection of food sources and proper sites of ovoposition, the identification of conspecifics for mating or aggregation, the recognition of prey or predators are all behaviors resulting from the activation of different processes after the exposure to the wide range of soluble and volatile chemical molecules in the environment. In this respect, insects present clearly separate senses of taste and olfaction comparable to those of vertebrates, and they represent an excellent experimental model to investigate the complexity of gustative and olfactory systems also thanks to the relative simple organization of their neuronal circuits, as well as the feasibility of the breeding.

On the basis of these considerations, aim of this work was to give a contribution on the general understanding of chemoreceptive mechanisms. In particular, two main aspects of chemoreception in insects were investigated, divided in two separate sections.

Section 1. Elucidation of the role of K^+ channels in the encoder repetitive firing in sensory receptors, by means of some K^+ channels inhibitors (4-aminopyridine and 5-hydroxytryptamine). This study was conducted in the labellar chemosensilla of the blowfly, *Protophormia terraenovae*.

Section 2. Study of the morphological properties of the olfactory system in the Mediterranean fruit fly *Ceratitis capitata*, by means of two different staining techniques, confocal microscopy and image analysis. The combination of these tools allowed the construction of three-dimensional maps of the first olfactory centre in the central nervous system of the medfly, the Antennal Lobe.

Introduction

1. Insect Chemoreception

The ability to sense various chemical substances in the environment is a fundamental requirement for animals survival. In this respect, chemoreception represents one of the most important sensory modality. Chemoreception is defined as the process by which organisms respond to chemical stimuli in their environments, and it basically relies on the chemical senses taste (gustation) and smell (olfaction), the most common and ancient sensory systems within the animal kingdom (de Bruyne and Warr, 2005; Strausfeld and Hildebrand, 1999). These senses convey information about the external world to the nervous system, inducing appropriate behavioral responses accordingly.

In general, the distinction of taste and smell is based on the anatomical location of the sense organs, as well as the chemical state of the stimulant molecules (gaseous for odorants and solid or liquid for tastants). However, this distinction is not valid for aquatic organisms, that live in an environment in which all chemicals are dissolved in the same medium, water. To solve this contradiction, gustation is better defined as contact-chemoreception since chemicals, usually at higher concentrations, are perceived when there is a direct contact between the organisms and a substrate. In contraposition, olfaction is defined as distance-chemoreception, allowing to sense chemicals present in the environment at relatively low concentration (de Bruyne and Warr, 2005). In the past years, a great deal of research has been conducted on vertebrates and invertebrates to elucidate chemoreceptive mechanisms at all the morphological, biochemical, functional and behavioral levels. The results obtained by these combined efforts confirmed the thought that, despite the fact that there are clear anatomical and molecular distinctions between vertebrates and invertebrates, their sensory systems evolved a common organization (Ache and Young, 2005; Hildebrand and Shepherd, 1997; Hildebrand, 1995; Strausfeld and Hildebrand, 1999). Among invertebrates, Arthropods are the most ubiquitous animal group, representing about 80% of all known living animal species (**Fig. 1**), likely involved in ecosystems conservation due to their ability to invade almost all niches (Prather et al., 2013). Within the Arthropods phylum, the most important and representative group is constituted by insects. Insects have captured the attention of researchers interested in chemoreception, since they show clearly separate senses of taste and olfaction comparable to those of vertebrates (Benton et al., 2006; Yarmolinsky et al., 2009). Moreover, they represent an excellent experimental model to investigate the complexity of gustative and olfactory systems, thanks to the relative simple organization of their neuronal circuits, as well as the feasibility of the breeding. *Drosophila melanogaster* is only an example of insect species massively used in research: due to the availability of genetic tools for the creation of several types of mutants and the possibility to employ sophisticated electrophysiological and

biochemical techniques, this insect has become one of the central model organisms nowadays (Vosshall, 2000).

Chemoreception plays a fundamental role in an insect life: the detection of food sources and proper sites of ovoposition, the identification of conspecifics for mating or aggregation, the recognition of prey or predators are all behaviors resulting from the activation of different processes after the exposure to the wide range of soluble and volatile chemical molecules in the environment. In particular, the first event involved in chemoreception is the interaction of those molecules with specific sensory neurons housed inside specialized cuticular structures, called sensilla, that are well defined chemosensory units. A sensillum constitutes the preferential pathway by which the information from the chemical world outside is carried into the internal environment.

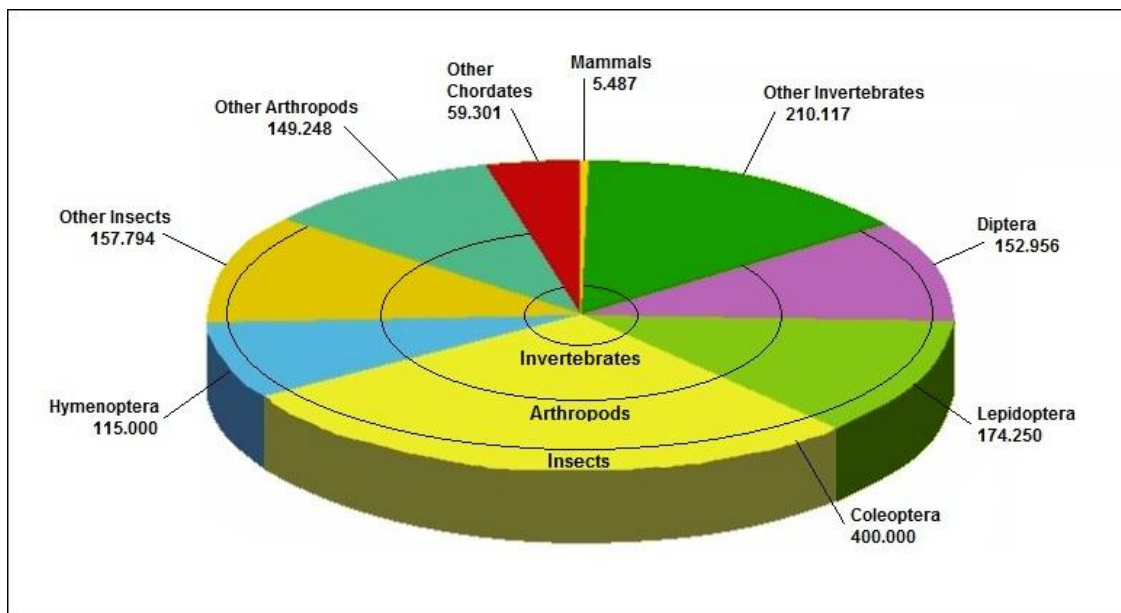


Figure 1. A pie-chart representation of animal groups according to their number of species. Arthropods constitute about 80% of all animal species.

2. General organization of insects sensilla

A sensillum is generally characterized by three main components, regardless of the typology. In fact, each of these small sensory organs consists of a certain number of receptor cells together with accessory cells, all surrounded by a layer of cuticle (Hallberg and Hansson, 1999; Keil, 1997a, 1997b).

In general, the cuticle represents the outer layer that covers the entire body of an insect, an interface to the environment that provides protection from the external insults. In the sensilla, cuticle is modified and takes the form of an extroflexion that protects the dendrites of the sensory neurons housed inside. Common to all the different types of sensilla is the presence of pores, that allow the passage of the stimulant chemicals from outside to the internal environment. The number of pores is strictly correlated to the sensory function: in particular, the cuticle covering olfactory sensilla contains numerous narrow pores, allowing access for the airborne molecules that stimulate the sensory cells inside (Basibuyuk and Quicke, 1999; Laue and Steinbrecht, 1997; Palma et al., 2013; Shanbhag et al., 1999; Steinbrecht, 1997). By the same principle, cuticle of taste sensilla presents a single larger pore at its tip (Hallberg and Hansson, 1999; Keil, 1997a, 1997b; Prakash et al., 1995).

The receptor cells inside sensilla are bipolar neurons, whose number is generally around 3-4, but it may vary greatly among species and, in some cases, also between sexes (Galizia, 2008). The soma of these cells is located at the base of the sensillum. The dendrites at the apical pole of the cell extend along the cuticular cavity, ending in a ciliated projection: dendrites are structured to increase the contact surface with the stimulants. The axons at the basal pole project into the central nervous systems directly, without making synaptic connections. All around these sensory neurons, usually three accessory cells with a secretory function are present, named on the basis of their role in morphogenesis. The outer cell is known as tormogen, and it forms the socket around the base of the sensillum. The trichogen cell is the middle one, and it produces the cuticular material that forms the hair. The innermost thecogen cell wraps the soma of the sensory neurons and forms the dendritic sheath that surrounds the dendrites (Hallberg and Hansson, 1999; Keil, 1997b). Once the sensillum is completely formed, these cells secrete the sensillum lymph, that protects the nerve endings from desiccation, and keep the constant ionic environment necessary for receptor cells to function properly (Isono and Morita, 2010; Kaissling, 1996; Pollack and Balakrishnan, 1997; Vosshall and Stocker, 2007).

Depending on the type of sensillum its shape can be different, usually hairs, pegs or other forms. Also the topological distribution on the body surface is variable, with the main density of olfactory sensilla in antennae and maxillary palps, and gustatory sensilla largely spread in mouthparts, tarsi and ovopositor (**Fig. 2**) (Dahanukar et al., 2005; de Bruyne and Warr, 2005).

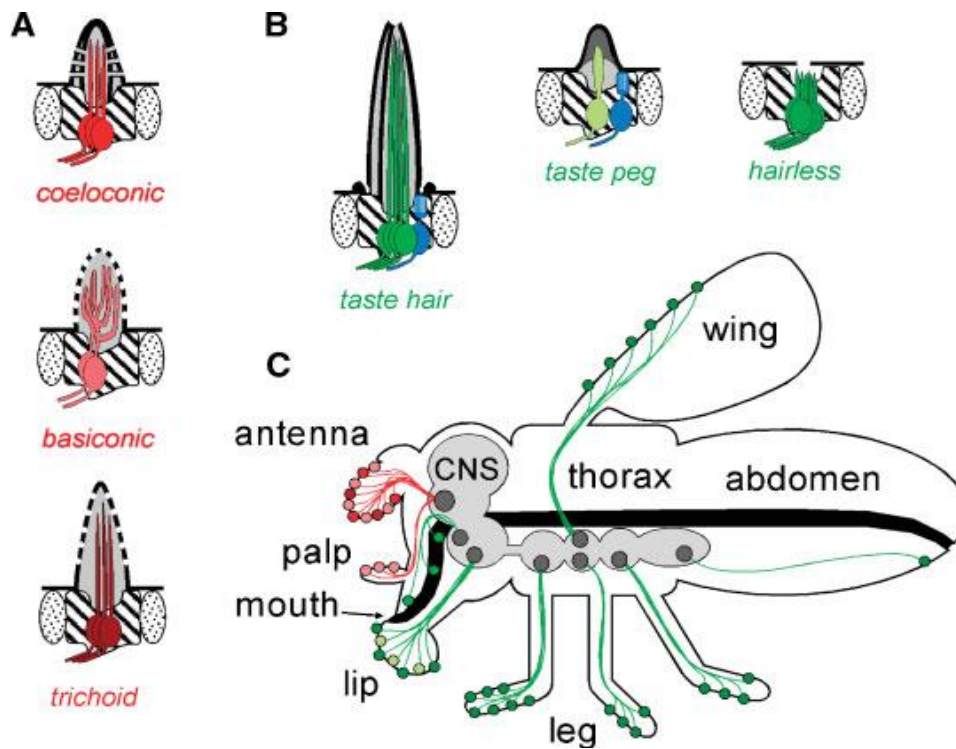


Figure 2. Example of different typology of sensilla and their distribution on the body surface of *Drosophila* (image from de Bruyne and Warr, 2005).

A. Different morphologies of olfactory sensilla, with sensory neurons in red. The cuticular layer presents several pores that allow the molecules to reach the dendrites inside the internal environment.

B. Gustatory sensilla are bimodal structures, with both a taste and tactile function. Sensory receptors are shown in green, mechano-receptors in blue. Cuticle is modified at the tip, forming a single pore for the entrance of molecules.

C. Anatomical distribution of sensilla on the body surface of the insect. Olfactory sensilla, in red, are located on antennae and maxillary palps, whereas taste sensilla, in green, are distributed on external and internal (hairless sensilla) mouthparts, tarsi, wings and ovopositor.

3. Olfactory system of adult insects

The peripheral olfactory organs in insects are located exclusively on the head of the animal, and consist primarily of a pair of appendages called antennae, but also of maxillary palps in some species (mainly dipterans) (de Bruyne et al., 1999; Galizia, 2008). The latter organ is characterized by a simple structure compared to that of the former, since it only houses one class of sensilla (generally basiconic) and a lower number of olfactory receptor neurons (ORNs) (Syed

and Leal, 2007; Vosshall and Stocker, 2007). On the contrary, antennae possess different types of sensilla, as already mentioned, and a subdivision in three main elements. The proximal segment attached to the head capsule is called the scape, the middle one is known as the pedicel and the distal part is the flagellum. While scape and pedicel are covered mainly by mechanosensory sensilla, the flagellum represents the real olfactory sensitive portion of the antenna. In fact, this is the section where olfactory sensilla are principally located, with a direct correlation between this number and the length of the segment (Wcislo, 1995). The flagellum is the most variable part of the antenna, not only in terms of distribution of sensilla, but also for shape and size (**Fig. 3**). This variability occurs among species, as well as between sexes, with different functional and ecological implications. In some species of bees, for example, the terminal part of the flagellum is modified only in males, and this modification appears related to courtship behavior (Wcislo, 1995). In the same way, males of moths generally possess big and magnificent feathery antennae, very different from the slender ones of females. In these insects, the pronounced sexual dimorphism is not correlated to courtship, as in the previous case, but is associated with the requirement of more efficient olfactory sensitivity. Such sensitivity is assured by an increase of the surface in contact with the external environment, therefore the odorants may interact with a greater number of sensilla (Symonds et al., 2012; Vogt and Riddiford, 1981).

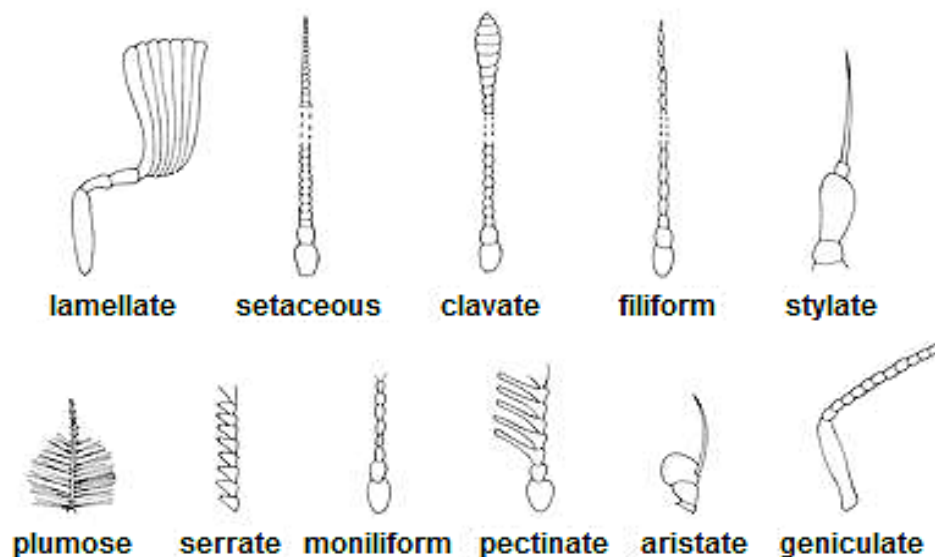


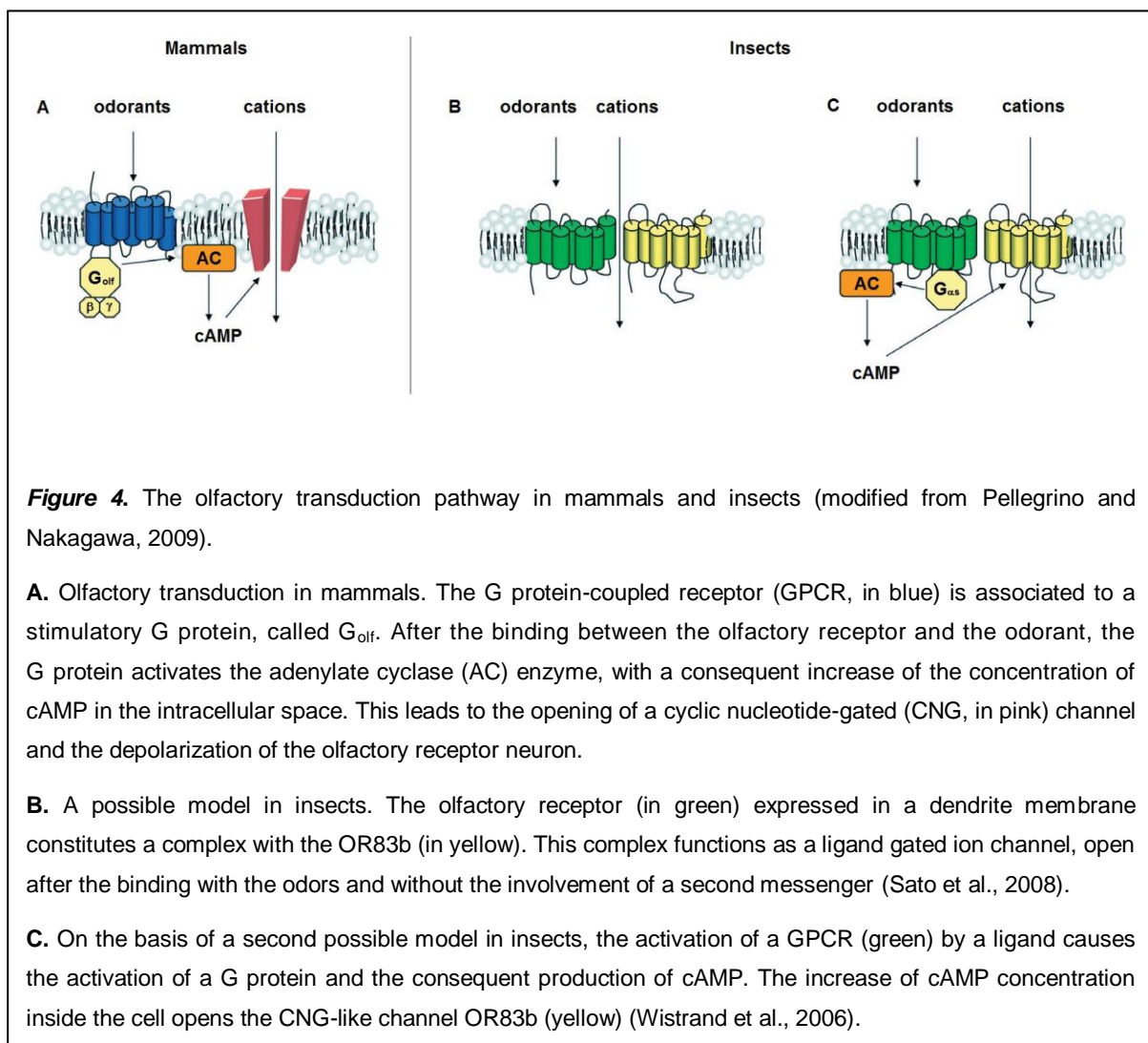
Figure 3. Different typology of antennae (picture from *H. Weber (1966). Grundriss der Insektenkunde; Gustav Fischer Verlag*).

Once the volatile molecules reach an olfactory sensillum, the contact between the stimulants and the dendrite of a sensory cell is a fundamental step for its activation. The passage of stimulants through the sensillar cuticle is possible thanks to the numerous pores distributed on the wall surface. This number is usually large, to enhance the possibility that the stimulants in low concentration in the air make contact with the sensillum. The main problem for researchers was to understand how the hydrophobic volatile olfactory stimulants may reach the dendrite membrane through an aqueous medium, such as the sensillum lymph. A first answer to this question was given in 1981, when Vogt and Riddiford showed in the wild silk moth *Antheraea polyphemus*, the presence of a specific class of soluble proteins in the sensillum lymph, that they called "pheromone-binding proteins" (PBPs). Afterwards, many studies have been conducted on a large number of insects species (Bohbot and Vogt, 2005; Györgyi et al., 1988; Krieger et al., 1996; Leite et al., 2009; Shanbhag et al., 2001; Vogt et al., 1999, 1991, 1989; Xu et al., 2010, 2009), thanks to which many types of PBPs and more general olfactory binding-proteins (OBPs) were discovered. The common thought that comes out from this great amount of work is that the main role of PBPs and OBPs is to solubilize odorants, in order to assure their transport through the sensillum lymph. A further evidence of the importance of this class of molecules was given by the case of the *Drosophila* LUSH PBP, a protein involved in the transport of the pheromone molecule cis-vaccenyl acetate to its specific olfactory receptor. The lack of this particular PBP was shown to seriously affect the possibility to recognize the pheromone (Xu et al., 2005). Furthermore, in a recent study the LUSH PBP has been determined not only to play a passive role in carrying the odorants, but also to participate actively in the activation of the odorant receptor, thanks to the conformational changes induced after the binding with its ligand (Laughlin et al., 2008). The olfactory pathway involves several proteins than the odorant-binding proteins, such as the olfactory receptors (ORs) located in the dendrite membranes, specific enzymes for the degradation of the odor molecules called odor-degrading enzymes (ODEs), and all the proteins involved in the transduction cascade (Hill et al., 2002; Ishida and Leal, 2005, 2002; Krieger and Breer, 1999; Vogt and Riddiford, 1981).

The olfactory receptors belong to a family of G protein-coupled receptors (GPCRs), characterized by the presence of seven-transmembrane domains in the structure, and are one of the most important elements of the olfactory system both in insects and vertebrates, since they are the first responsible for the recognition and discrimination of the odor stimulants (Mombaerts, 1999). The first ORs in insect were discovered in 1999, thanks to the work conducted on *Drosophila* by three different research groups (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). After those studies, many others were conducted, with the intent to completely understand the anatomical and functional properties underlying the olfactory transduction in insects. One interesting evidence found was that, although the similarity between GPCRs of mammals and insects, the latter developed a different protein topology, with the N-terminal located in the intracellular side and the C-terminal protruding in the extracellular surface (Benton et al., 2006).

Another important difference has been found regarding the expression of the receptors in the dendrite membrane: while in mammals every ORN expresses only one type of OR, in insects an additional receptor, called OR83b, is always expressed together with the regular one, both in antennae and maxillary palps. On the basis of these studies, it is clear that OR83b is a fundamental element for the correct function of the other ORs, although it does not serve as olfactory receptor by itself (Jones et al., 2005; Vosshall et al., 2000).

The mechanisms of signal transduction in mammals are well established, while in insects they are still not totally clear. In the past years, the general thought was that the biochemical processes involved in the olfactory pathway were based on the activation of a G protein and the subsequent reaction cascade involving the production of IP₃ as second messenger (Krieger and Breer, 1999). Recent studies, however, propose new insights, according to which the olfactory transduction pathway relies on a ligand-activated non-selective ion channel conduction (**Fig. 4**) (Sato et al., 2008; Wistrand et al., 2006).



After the binding between odorants and ORs, the receptor cells inside sensilla are activated, and the olfactory signal is sent to the higher brain centers where it is properly processed. In particular, the first center of olfactory information processing, into which ORNs project their axons, is the antennal lobe (AL) (Anton et al., 2003; Barrozo et al., 2009; Galizia et al., 1999; Hansson et al., 1995; Kristoffersen et al., 2008; Mustaparta, 1996; Rospars, 1988; Stocker et al., 1990), analogous in structure and function to the olfactory bulb of vertebrates (**Fig. 5**) (Strausfeld and Hildebrand, 1999). Inside the AL, the projections of the olfactory neurons synapse with the dendrites of two types of target neurons, into specific spherical subunits called glomeruli, in a variable number among species (Rospars, 1988). These central neurons are the local interneurons, whose axons remain within the AL and connect few or many glomeruli, and the projection neurons, which send their axons in the higher brain centers, the mushroom bodies and the lateral protocerebrum (Distler and Boeckh, 1997; Ng et al., 2002; Stocker, 1994). The mushroom bodies are multimodal structures, receiving information both from the olfactory and the visual system (Farris, 2005; Strausfeld et al., 1998). This part of the insect brain is responsible for the learning and memory of the olfactory stimuli (Akmal et al., 2006; McGuire et al., 2001; Wolf et al., 1998) The lateral protocerebrum is a part of the insect brain still not well understood. On a general thought, its main role seems to be related to innate olfactory behaviors (Gupta and Stopfer, 2012; Kido and Ito, 2002).

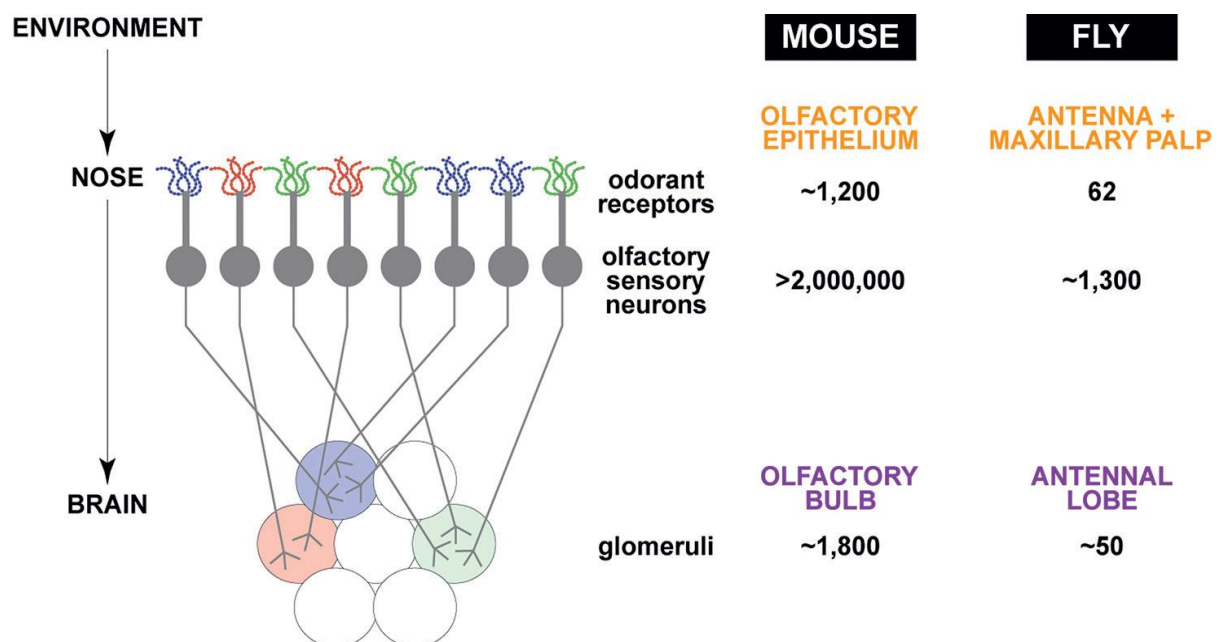


Figure 5. Schematic representation of the olfactory system. Vertebrates and insects possess a common organization. (Image from Benton, 2006).

4. Gustatory system of adult insects

Unlike olfaction, the gustatory system of mammals and insects evolved quite independently (Vosshall and Stocker, 2007; Yarmolinsky et al., 2009). While in mammals there is only one gustatory organ localized in the head, in insects taste sensilla are widely distributed over the entire body surface. The target area of these sites in the central nervous system may vary depending on the site itself: since insects are segmented animals, mostly with a ganglion in each segment, axons of the taste sensory neurons project directly in the ganglion in which they occur (Stocker, 1994). The different location of taste sensilla reflects their diverse function: for example, while sensilla on the ovopositor appear to be involved in the selection of proper ovoposition sites, those on the tarsi and the mouthparts are strictly related to the feeding behavior (Pollack and Balakrishnan, 1997; Stocker, 1994; van Loon, 1996).

In the past decades, the use of morphological and electrophysiological techniques allowed an extensive study of the gustatory system properties in many insects, in particular flies, and among these *Drosophila melanogaster* is certainly the best characterized. In *Drosophila*, and in flies in general, the taste sensory organ equivalent to the human tongue is called labellum, and it is located in the distal part of a muscular tube that forms the pharynx. All together, this parts constitute the proboscis that, thanks to its extension properties, in the absence of a proper stimulation is usually stowed at the base of the head. The labellum presents two pair formations, known as labial palps, on the surface of which three morphological types of taste sensilla are distributed: the small type (s) and long type (l) contain four sensory neurons, the intermediate type (i) only two (Stocker, 1994). A variety of other taste sensilla is widely distributed on several parts of the body, with sexual dimorphism due to the presence of sensilla involved in the sexual behavior on males (Bray and Amrein, 2003; Park et al., 2006).

From a functional point of view, the electrophysiological tip recording technique (Hodgson et al., 1955) has been one of the most useful experimental procedures employed to study the gustatory receptor neurons (GNRs) in insects. The evidence resulting from these studies is that taste cells respond mainly to food-related chemical stimuli, and each of them produces a stronger excitatory response after stimulation with a particular substance or category of substances. On the basis of these compounds, GNRs have been classified, although this specificity is not so strict and may be variable depending on the species (Evans and Mellon, 1962a, 1962b; Shiraishi and Kuwabara, 1970; van Loon, 1996). In a general way, in long and short bristles containing four neurons, one is sensitive to sugar (S cell), one to water (W cell), one to low concentrations of salt (L1 cell) and one is sensitive to high concentrations of salt and bitter compounds (L2 cell) (Amrein and Thorne, 2005; Vosshall and Stocker, 2007). Intermediate bristles contain only two taste neurones: one responds to stimuli considered attractants, such as sugars and salts at low concentrations, the other one is activated by stimulation with aversive tastants like bitter compounds and high concentration of salts (Yarmolinsky et al., 2009). As already mentioned, GRNs send their axons in different parts of the central nervous system. In particular, taste

neurons localized in the head appendages project in the subesophageal ganglion (SOG), the gustatory center comparable to the AL where the gustatory information is first processed, whereas fibers from taste sensilla located in other body areas reach the nearest thoracic ganglion. Unlike the ALs, the structure of the SOG is not so defined for two reasons: first, the SOG is not organized in morpho-functional units such as the glomeruli, and second, there are not clear margins that allow to distinguish a separate organ (Stocker, 1994).

The ability to respond to a variety of compounds, albeit mostly belonging to the same category, is due to the receptor composition of the dendrite membrane. After the discovery of the olfactory receptor genes, the same or other methodologies were used for the identification of genes responsible for the gustatory receptors expression (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001). From these studies taste receptors turned out to possess a quite similar structure to that of ORs, since they consist of proteins with seven transmembrane domains. So far, many receptors have been identified in the S and L2 cells of *Drosophila* and, recently, also one molecular marker for W neurons (Inoshita and Tanimura, 2006). The receptors involved in the sense of salt at low concentrations still need to be investigated.

5. Insects used in this study and their importance



***Protophormia
terraenovae***

(photo by James Lindsey)



***Ceratitis
capitata***

(photo by Enio Branco)

5.1. *Protophormia terraenovae*

Protophormia terraenovae is an insect species belonging to the Diptera order, Calliphoridae family (**Tab. 1**). Also known as blowfly or blackbottle fly (Taylor et al., 2007), this insect usually lives in the northern hemisphere, where temperatures are cool, given its high cold tolerance (Byrd and Castner, 2012).

Morphology. Adult insects of *P. terraenovae* (**Fig. 1**) are characterized by a dark blue to black metallic body color, with black legs and head. Among calliphorid species, its body size is one of the largest, with a total length between 6 and 12 mm and no differences in males and females. Sexes are distinguished mainly on the base of the distance between the eyes. (Byrd and Castner, 2012; Rognes, 1991; Wall and Shearer, 1997).



Figure 1. Adult stage in *Protophormia terraenovae* (photo by Nikita E. Vikhrev).

Life cycle. Females lay clusters of 100-200 eggs preferably on carrions, but also on living mammals as secondary invaders of myases (Taylor et al., 2007). After the hatching, larvae start feeding and develop until they reach their maximum size, turning into the pupal stage. During this phase, the outer cuticular case hardens and assumes progressively a darker brown color. Once the insect is mature, the case opens and the adult fly emerges. *P. terraenovae* in the adult stage may live for 30 days.

Importance for humans. *P. terraenovae* is considered an insect of great interest: not only it is a veterinary pest, dangerous mainly for sheep but also for cattle and reindeer, but (Taylor et al., 2007; Wall and Shearer, 1997), it is also used in forensic investigations as indicator of the time of death, given the importance of larvae in the evaluation of the post-mortem interval (Grassberger and Reiter, 2002; Myskowiak and Doums, 2002). Moreover, *P. terraenovae* is a blowfly species commonly used in the maggot debridement therapy (MDT). The MDT is a medical therapy applied in particular cases of chronic wounds and necrotic flesh, when the healing is not successful with the typical pharmacological and surgical treatments (**Fig. 2**) (Mumcuoglu et al., 1999). It is based on the use of blowfly larvae on infected wounds: larvae release digestive enzymes and feed on necrotic tissues, without damaging the healthy ones around. In addition, the secretion of antimicrobial peptides facilitate the disinfection of the treated wounds. After complete development, maggots leave the tissues and pupate (Sherman, 2002).

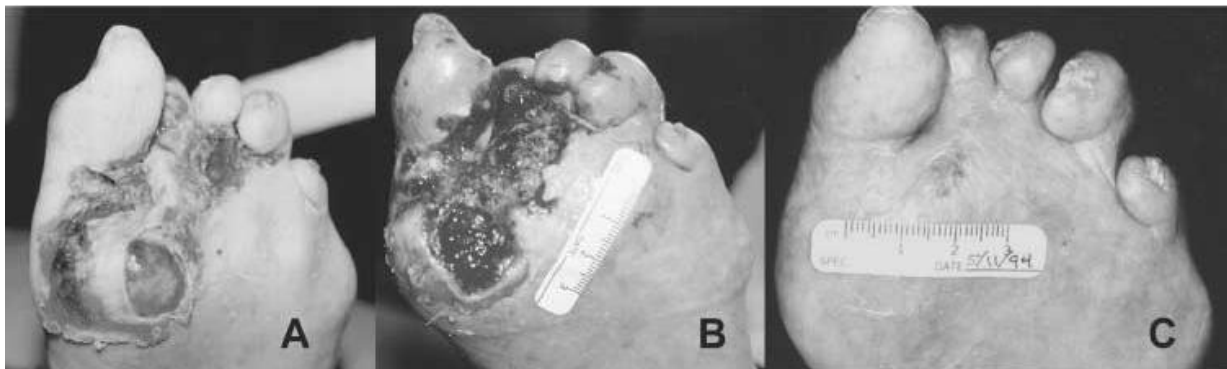


Figure 2. Application of the maggot debridement therapy (MDT) in a foot ulcer: initial condition (A), development of the therapy (B) and situation after 1 year treatment (C) (Sherman, 2002).

5.2. *Ceratitis capitata*

The Mediterranean fruit fly *Ceratitis capitata* is a phytophagous insect of the Tephritidae family, Diptera order (**Tab. 1**). According to the literature, this species originated in sub-Saharan and South Africa (De Meyer et al., 2008), but it is currently distributed worldwide, mainly colonizing tropical areas and mild temperate regions (Headrick and Goeden, 1996; Thomas et al., 2010; Vera et al., 2002).

Morphology. Adult medflies (**Fig. 3**) present a body length between 3,5 and 5 mm. The body color is mostly yellow, except for the dorsal part of the thorax in which a characteristic pattern of black spots is present (**Fig. 4A**). Also wings possess distinctive markings black, brown and yellow colored (**Fig. 4B**). Males and females show sexual dimorphism: besides the ovopositor, males can be distinguished from females by a peculiar pair of black modified bristles on the head (**Fig. 4C-D**).



Figure 3. Male and female adults of *Ceratitis capitata* (Diamantidis et al., 2011)

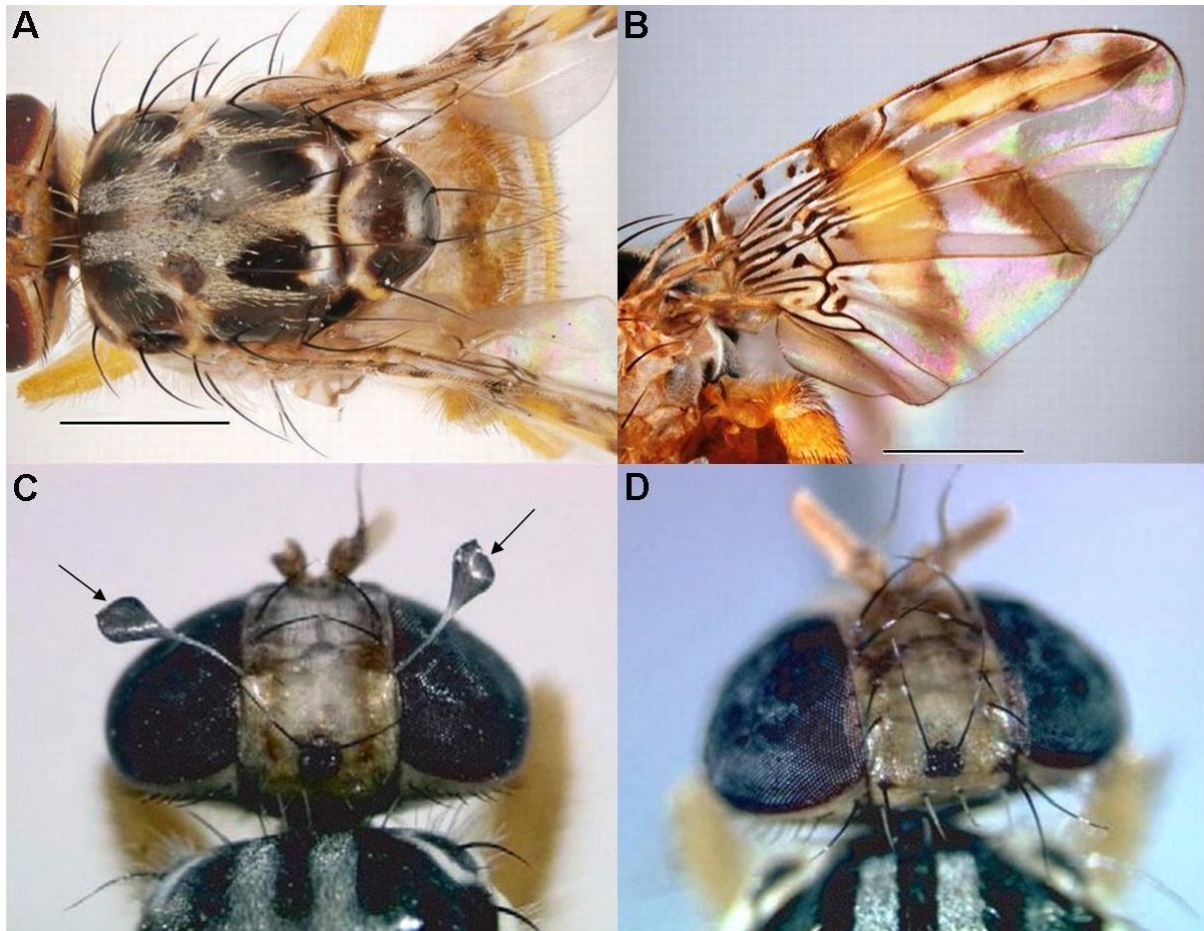


Figure 4. Morphology of *Ceratitidis capitata*. Distinctive markings on the dorsal part of the thorax (A) and wings (B) in adult medflies. Scale bar 1 mm. Photos by Ken Walker, Museum Victoria, Melbourne, Australia. Example of male (C) and female (D): males can be distinguished from female also by a pair of modified bristles on the head (black arrows in *fig. C*). Photos by OEPP/EPPO, 2011.

Life cycle (Fig. 3D). The time required for life cycle depends on different factors. One of them is temperature: in temperate climates, in fact, development of eggs, larval and pupal stages needs around 30 days, and it usually stops when temperature reaches 10° C. Also the type and condition of infested fruits can affect the time: in citrus, such as lemons, larval life seems to be longer than in sweeter fruits as peaches. Females of adult medflies lay their eggs under the skin of fruits, particularly when the fruit is ripen and the skin is already broken in some areas. The same spot can be used by different females, that may deposit a cluster of 75 or more eggs. The eggs hatch within three days, and the larvae start to eat and develop inside the fruit. For pupation, larvae leave the fruit and pupate possibly in the soil. The adult medflies possess a limited ability to disperse, but winds can transport them in long distances. Under favorable conditions of food and temperatures, adults can live for months (Thomas et al., 2010).

Importance for humans. *C. capitata* is considered one of the most important agricultural pests in the world (De Meyer et al., 2008; Malacrida et al., 2007), since the high number of infested species (more than 350, Liquido and Cunningham, 1990), the possibility of adaptation to diverse environments and the high rate of reproduction (Gasperi et al., 2002). The citrus fruits are reported as the major vehicles for the medfly colonization in Mediterranean and American subtropical areas (McPheron and Steck, 1996). In general, globalization and the fruit trade are considered the key factors in the dispersal of species outside their natural living regions (Wilson et al., 2009), and this is true also in the case of *C. capitata*. The need to limit the economic loss has led to increase the efforts in finding efficient control systems, but nowadays this control is still problematic. Some strategies are based on olfactory chemoreception, such as the use of chemicals and traps, but the efficiency of these methods is directly related to the level of attractiveness of the odorant molecules and sometimes they are not sufficient. The modern biological control programs gave just partial success in the limitation of *Ceratitidis* invasion, probably because, although the medfly has a wide range of competitors, a specific one has not been found yet. Another control system is based on the release of reared sterile insects in the environment to interrupt reproduction, and it is called sterile insect technique (SIT). In the case of medflies, this method is considered rather efficient but not sufficient alone for eradication, since sterile males are not competitive in mating as wild males (Headrick and Goeden, 1996).

Table 1. Scientific classification of *Protophormia terraenovae* and *Ceratitidis capitata*

Animalia	<u>Kingdom</u>	Animalia
Arthropoda	<u>Phylum</u>	Arthropoda
Insecta	<u>Class</u>	Insecta
Diptera	<u>Order</u>	Diptera
Calliphoridae	<u>Family</u>	Tephritidae
<i>Protophormia</i>	<u>Genus</u>	<i>Ceratitidis</i>
<i>Protophormia terraenovae</i>	<u>Species</u>	<i>Ceratitidis capitata</i>

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Section 1

The spike generator in the labellar taste receptors of the blowfly is differently affected by 4-aminopyridine and 5-hydroxytryptamine

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Abstract

In taste chemoreception of invertebrates the interaction of taste stimuli with specific membrane receptors and/or ion channels located in the apical membrane of taste receptor cells results in the generation of a receptor potential which, in turn, activates the 'encoder' region to produce action potentials which propagate to the CNS.

This study investigates, in the labellar chemosensilla of the blowfly, *Protophormia terraenovae*, the voltage-gated K^+ currents involved in the action potential repolarization and repetitive firing of the neurons by way of the Kv channel inhibitors, 4-aminopyridine and 5-hydroxytryptamine.

The receptor potential and the spike activity were simultaneously recorded from the 'salt', 'sugar' and 'deterrent' cells, by means of the extracellular side-wall technique, in response to 150 mM NaCl, 100 mM sucrose and 1 mM quinine HCl, before, 0-10 min after apical administration of 4-AP (0.01–10 mM) or 5-HT (0.1–100 mM).

The results show that the receptor potential in all three cells is neither affected by 4-AP nor by 5-HT. Instead, spike activity is significantly decreased, by way of blocking different Kv channel types: an inactivating A-type K^+ current (KA) modulating repetitive firing of the cells and responsible for the after hyperpolarization, and a sustained K^+ current that resembles the delayed rectifier (DKR) and contributes to action potential repolarization.

1. Introduction

The essential elements of taste sensory systems in all animals are neuronal receptor cells which respond to gustatory stimuli by way of changes of their membrane potential, known as receptor potential, and transmit their information contents to the CNS encoded as spike firing frequency. It is generally accepted that a direct correlation exists between receptor potential amplitude and spike firing frequency in both vertebrates and invertebrates (Erler and Thurm, 1981; Herness, 2000).

The region of the neuron where spikes are generated is called 'encoder' and is localized at the level of the dendrite, either in its proximal portion (Erler and Thurm, 1981) or in the distal one (Murakami and Kijima, 2000).

Neuronal encoders are endowed with two different voltage-gated channel families: Na⁺ channels, involved in the action potential depolarization, and K⁺ channels (K_v) which play an important role in various aspects of the bioelectrical activity of excitable cells such as: setting the resting membrane potential, shaping the action potential waveform and modulating the frequency of neuronal firing (Halliwell, 1990; Hille, 2001; Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999; Mathie et al., 1998; Mercer et al., 1995; Salkoff et al., 1992). Two different voltage-gated K⁺ currents have been observed in a number of types of insect cells: a fast, inactivating A-type K⁺ current (K_A) modulating repetitive firing of the cells and responsible for the after hyperpolarization, and a sustained K⁺ current that resembles the delayed rectifier (DKR) and contributes to action potential repolarization (Dacks et al., 2008; Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999; Mercer et al., 1995; Saito and Wu, 1991).

Recent studies have covered several functional aspects of neuronal K⁺ channels with various approaches such as electrophysiology, molecular biology, neuromodulation and classical pharmacology (Chandy and Gutman, 1995; Cook and Quast, 1990; Critz et al., 1991; Dacks et al., 2008; Dolly and Parcej, 1996; Harvey, 1993; Jan and Jan, 1992; Kloppenburg and Hildebrand, 1995; Kolb, 1990; Mercer et al., 1995; Pongs, 1992; Rehm and Tempels, 1991; Wolff et al., 1998).

Among the pharmacological probes, serotonin (5-HT), a known neurotransmitter, neurohormone and neuromodulator in the nervous system in vertebrates and invertebrates, has been found to modulate the activity of several K⁺ conductances, including voltage-independent membrane channels, voltage-dependent channels and Ca²⁺-activated channels (Birmingham and Tauck, 2003; Critz et al., 1991; Dacks et al., 2008; Gatellier et al., 2004; Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999; Mercer et al., 1995).

Another probe, 4-aminopyridine (4-AP), has been reported to selectively block K⁺ channels in axons of the cockroach (Pelhate and Pichon, 1974), squid (Meves and Pichon, 1975; Yeh et al., 1976), the fanworm *Myxicola* and the lobster neuromuscular junctions (Schauf et al., 1976), frog nodes of Ranvier (Wagner and Ulbricht, 1975), neurons of *Drosophila melanogaster* (Mercer et al., 1995), tail sensory neurons of *Aplysia* (Critz et al., 1991), as well as in mammalian taste buds

(Liu et al., 2005). In antennal lobe neurons of *Manduca sexta*, 4-AP has been found to be an effective blocker of the fast A-type K^+ current, leaving the slower-activating DKR current largely intact (Mercer et al., 1995).

Several studies on various chemo- and mechanoreceptors of insects reported that the encoder, where nerve impulses initiate, is located somewhere near the sensillum base (Dethier, 1976; Morita, 1992), in the proximal area of the dendrite (Erler and Thurm, 1981) and/or in the distal dendrite (Murakami and Kijima, 2000). They also concluded that spikes not only propagate centripetally, but also centrifugally into the apical region of the dendrite (Erler and Thurm, 1981). A similar location for the encoder has been shown in the spider mechanoreceptor sensilla, containing bipolar neurons, where the action potentials normally start at the distal dendrites and propagate regeneratively to the soma (Gingl and French, 2003).

With the purpose of studying the role of Kv channels in the encoder repetitive firing in sensory receptors, the labellar taste system of the blowfly, *Protophormia terraenovae*, represents a favorable and simple biological model.

The taste chemosensory system of blowflies is composed of cuticular hairlike structures, called sensilla, each containing five bipolar neurons. Three are specific chemoreceptors and have been named 'salt', 'sugar' and 'deterrent' cells depending on their best stimuli, one is a mechanoreceptor and the remaining one, called a "water" cell, is a chemo/osmoreceptor (Dethier, 1976; Evans and Mellon, 1962; Liscia and Solari, 2000; Rees, 1970; Solari et al., 2010; Wieczorek and Koppl, 1978).

The spikes recorded extracellularly from each of the four chemoreceptors differ in amplitude and waveform, can be separated from one another, and their relative amplitude ratios are a conserved feature for each species and sensillum type, regardless of the recording condition (Dethier, 1976; Liscia and Solari, 2000; Liscia et al., 1998; Smith et al., 1990).

Based on these considerations, the present work investigates the voltage-gated K^+ currents involved in the action potential repolarization and repetitive firing of the neurons. To this end, the responses of the "salt", "sugar" and "deterrent" cells in the blowfly *P. terraenovae* were recorded following stimulation with NaCl, sucrose and quinine HCl, respectively, before and after treatment with 5-HT, a well known neuromodulator, neurotransmitter and neurohormone that modulates the electrophysiological properties of sensory neurons by suppressing the K^+ current (Critz et al., 1991; Dacks et al., 2008; Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999; Mercer et al., 1995) and 4-AP, a known blocker of the voltage-gated K^+ channels (Connor and Stevens, 1971; Critz et al., 1991; Hille, 2001; Hodgkin and Huxley, 1952; Llinas, 1988; Mercer et al., 1995; Smith et al., 2000).

2. Materials and methods

2.1. Insects

Two- to five-day old adults of the blowfly, *P. terraenovae* (Robineau-Desvoidy), were used in all experiments. Flies were reared under standard conditions ($22^{\circ}\text{C} \pm 1$ and 70–80% of relative humidity) in the Dept. of Biomedical Sciences at the University of Cagliari, Italy. Insects were tested after being starved, but water satiated, for 24 h.

2.2. Recording technique

The receptor potential (RP) and the spike activity from labellar “Largest” (Wilczek, 1967) uniporous chemosensilla were simultaneously recorded by means of the extracellular ‘side-wall’ technique (Morita and Yamashita, 1959). All recording operations were carried out by means of micromanipulators under the field of a stereomicroscope. A thin Ag/AgCl wire serving as ground electrode was inserted into an isolated head through the ‘foramen magnum’. The recording glass capillary electrode (diameter at the tip $10\ \mu\text{m}$), filled with *P. terraenovae* saline (standard saline: NaCl 17.1 mM, KCl 5.6 mM, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 3.2 mM, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 2.1 mM, glucose 79.4 mM; pH 6–6.3), was brought in contact with a small crack in the sensillar side-wall cuticle, while the sensillum tip was independently stimulated with the various stimulants delivered by a micropipette.

Signals were recorded with a high input impedance ($10^{15}\ \text{X}$) electrometer (WPI Duo 773), band-pass filtered (DC to 100 Hz for RP and 0.1–3 kHz for action potentials), digitized by means of an Axon Digidata 1440A A/D converter (sampling frequency: 10 kHz), and stored on a PC for further analysis.

Both RPs and spike discharges were analyzed in the 1st s, starting 30 ms after the onset of the stimulation, in order to avoid the baseline shift induced by the contact artifact. RP amplitude values were measured by means of the Axoscope 10.0 software, while spikes in the discharges were sorted with the SAPID Tools software (Smith et al., 1990) and identified as the “sugar”, “salt” and “deterrent” units, by comparing them with those responding to pure salt, sugar or bitter solutions described in previous papers (Dethier, 1976; Liscia and Solari, 2000; Liscia et al., 1998).

Spike width was measured as difference between “time of end” and “time of peak” of the spikes by means of the Clampfit 10.0 software, before and after 4-AP or 5-HT administration.

2.3. Test compounds and experimental protocols

Both the RPs and the spike activity were recorded following stimulation with 150 mM NaCl, 100 mM sucrose and 1 mM quinine HCl aqueous solutions, before (control) and after the administration of

4-aminopyridine (0.01, 0.1, 1 and 10 mM) or 5-hydroxytryptamine (0.1, 1, 10 and 100 mM). The recordings were performed immediately ($t = 0$), 1, 3, 5 and 10 min after drug administration.

4-aminopyridine (4-AP) is a selective blocker of potassium conductance through voltage-gated K^+ channels (Hille, 2001; Kim and Mistretta, 1993; Liu et al., 2005; Mathie et al., 1998; Wolff et al., 1998), while 5-hydroxytryptamine (5-HT or serotonin) is an inhibitor of three K^+ currents (see review by Birmingham and Tauck, 2003): a voltage-independent K^+ current (Klein and Kandel, 1980), a voltage-dependent K^+ current similar to the delayed rectifier (Baxter and Byrne, 1990, 1989) and a calcium activated K^+ current (Walsh and Byrne, 1989).

4-AP and 5-HT were dissolved in *P. terraenovae* saline and administered to the preparations by diffusion for 1 min via the sensillum apical pore, via the dendritic liquor to reach the dendritic membrane of the receptor cells (Sollai et al., 2008).

4-AP and 5-HT concentrations were selected according to information reported in the literature (Dacks et al., 2008; Gatellier et al., 2004; Kim and Mistretta, 1993; Lucero et al., 1992; Meves and Pichon, 1977) and on the basis of preliminary experiments. All compounds were purchased from SIGMA–Aldrich.

2.4. Statistics

Differences in RP amplitude, spike frequency and spike width were evaluated by means of the ‘two-sided’ Student “t” test with a 95% confidence level ($P \leq 0.05$). Spike frequency vs. RP amplitude regression lines, were obtained from pooled data for all concentrations of each stimulus.

Lines were then cross-compared with the test of parallelism and coincidence, in order to evaluate differences among the different stimuli in the absence of pharmacological treatment by taking into consideration two parameters with a 95% confidence level ($P \leq 0.05$): differences between slopes (P_s , lines have different slopes) and elevations (P_i , lines are parallel but differ in Y-intercept). P_i was calculated only when P_s was not statistically significant.

3. Results

3.1. Relationship between receptor potential amplitude and spike frequency

The spike firing frequency is positively correlated with the RP amplitude. The linear regression plots between spike numbers and RP values were significant in response to NaCl ($R^2 = 0.0717$; $P < 0.01$), quinine HCl ($R^2 = 0.1576$; $P < 0.001$) and sucrose ($R^2 = 0.4436$; $P < 0.001$) (**Fig. 1**). The results of linear regression show that the difference between NaCl and sucrose slopes is not significant ($P_s = 0.2715$) and that between elevations is highly significant ($P_i < 0.0001$), while both NaCl/quinine HCl and sucrose/quinine HCl comparisons show that the slopes differ statistically ($P_s = 0.008613$ and $P_s = 0.000372$, respectively).

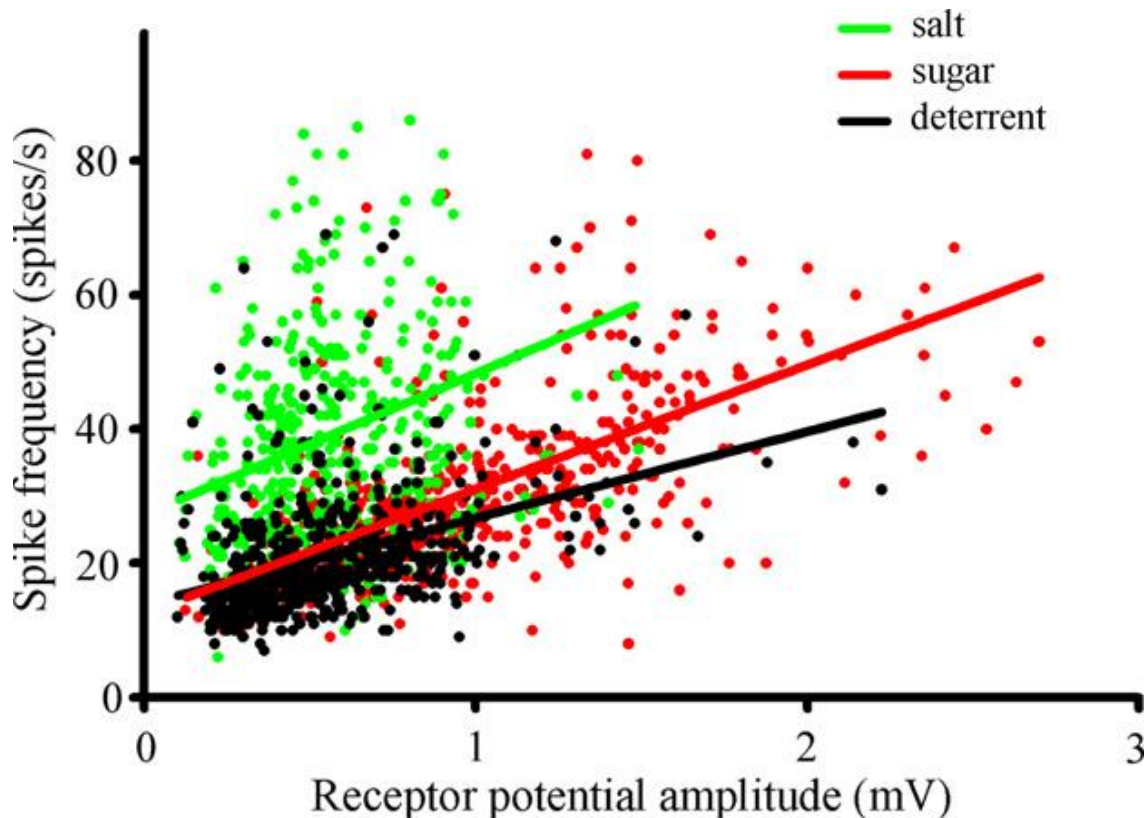


Figure 1. Receptor potential amplitude vs. spike firing frequency plot of the “salt”, “sugar” and deterrent” cells in the taste labellar chemosensilla of the blowfly *P. terraenovae*, in response to the NaCl, sucrose and quinine HCl solutions. Data referring to all concentrations tested are pooled.

3.2. Effects of 4-aminopyridine and 5-HT on ‘salt’ cell

4-aminopyridine 10 mM (4-AP) administered directly into the sensillum, via the apical pore, significantly reduced the spike frequency in response to 150 mM NaCl from 42.59 ± 3.25 to 30.09 ± 3.38 spikes/s, already 3 min after administration. 4-AP 1 mM caused the spike firing frequency to decrease from 50.11 ± 2.68 to 39.12 ± 3.71 spikes/s 5 min after administration, and 4-AP 0.1 mM significantly reduced the spike frequency from 51.28 ± 2.71 to 38.76 ± 2.44 spikes/s only 10 min after administration (**Fig. 2A** and **B**). **Fig. 2A** and **C** shows that spike frequency in response to 150 mM NaCl decreased significantly from 56.37 ± 3.77 to 44.90 ± 2.92 , from 53.96 ± 2.85 to 22.81 ± 2.62 and from 50.32 ± 2.59 to 37.99 ± 2.79 spikes/s after 3, 5 and 10 min of 100, 10 or 1 mM 5-HT, respectively. No significant decreases were obtained after 0.01 mM 4-AP or 0.1 mM 5-HT administration. Conversely, RP amplitudes in response to 150 mM NaCl tested were not affected by either compound at all concentrations (**Fig. 2A–C**). The spike width was increased, after 5 min 5-HT, from 1.223 ± 0.005 to 1.458 ± 0.018 ms ($N = 30$; $p \leq 0.0001$), while no statistical variations were found after 4-AP administration ($N = 30$; $p = 0.480$) (**Fig. 5A**).

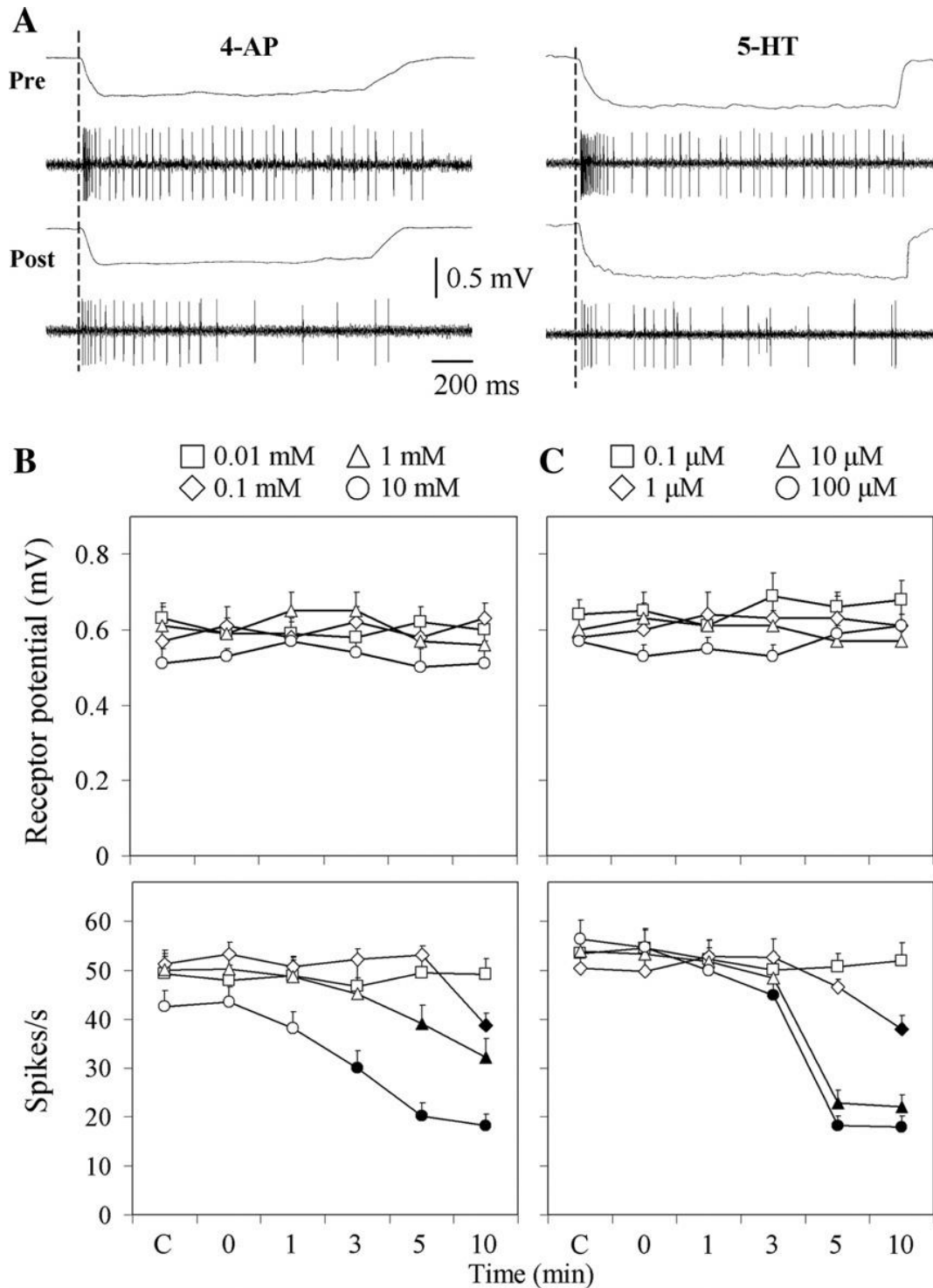


Figure 2. (A) Sample traces showing the receptor potential (mV) and the spike firing frequency following stimulation (vertical dashed line) with 150 mM NaCl, before (pre) and 5 min after (post) luminal administration of 10 mM 4-AP or 100 mM of 5-HT. (B and C) Mean values \pm S.E.M. of receptor potential and spike firing frequency following stimulation with 150 mM NaCl: before (C = control), 0, 1, 3, 5 and 10 min after drug apical administration of (B) 0.01, 0.1, 1 or 10 mM 4-AP and (C) 0.1, 1, 10 or 100 mM 5-HT. Filled symbols indicate significant differences from the control ($p \leq 0.05$). Experiments performed on 30–33 sensilla (1 per fly) for both pharmacological probes.

3.3. Effects of 4-aminopyridine and 5-HT on 'sugar' cell

Fig. 3A and **B** shows that, when 4-AP was directly administered into the sensillum, spike frequency in response to 100 mM sucrose decreased significantly from 36.04 ± 1.60 to 23.00 ± 1.07 , from 35.06 ± 2.08 to 20.32 ± 1.94 and from 36.47 ± 1.83 to 28.47 ± 1.43 spikes/s, already after 3 min of incubation with 10 mM 4-AP, after 5 min with 1 mM 4-AP and only after 10 min with 0.1 mM 4-AP, respectively. Similarly, spike frequency decreased significantly from 35.09 ± 2.03 to 27.02 ± 0.92 , from 33.83 ± 1.45 to 15.67 ± 1.23 and from 32.11 ± 1.20 to 19.70 ± 0.91 spikes/s, already after 3 min of incubation with 100 mM 5-HT, after 5 min with 10 mM 5-HT and only after 10 min with 1 mM 5-HT, respectively (**Fig. 3A** and **C**). No significant decreases were obtained after 0.01 mM 4-AP or 0.1 mM 5-HT administration. Instead, RP amplitudes, in response to 100 mM sucrose, were affected neither by 4-AP nor 5-HT at all concentrations tested. Spike width was increased from 1.273 ± 0.005 to 1.500 ± 0.008 ms ($N = 30$; $p \leq 0.0001$) after 5 min 5-HT, but did not change significantly after 4-AP administration ($N = 30$; $p = 0.211$; **Fig. 5B**).

3.4. Effects of 4-aminopyridine and 5-HT on 'deterrent' cell

Fig. 4A and **B** shows that spike frequency in response to 1 mM quinine HCl decreased significantly from 21.62 ± 0.87 to 14.70 ± 0.69 spikes/s, already after 3 min with 10 mM 4-AP, from 21.64 ± 1.17 to 10.88 ± 0.81 spikes/s, after 5 min with 1 mM 4-AP and, finally, from 22.09 ± 1.29 to 14.45 ± 0.73 spikes/s, only after 10 min of 0.1 mM 4-AP. Similar effects were obtained after 5-HT administration; in fact, spike frequency decreased significantly from 23.30 ± 1.22 to 16.93 ± 0.41 , from 24.93 ± 1.27 to 15.43 ± 0.71 and from 23.67 ± 0.97 to 13.10 ± 0.87 spikes/s, after 3, 5 and 10 min of 100, 10 and 1 mM 5-HT, respectively (**Fig. 4A** and **C**). No significant decreases were obtained after 0.01 mM 4-AP or 0.1 mM 5-HT administration. Instead, RP amplitudes, in response to 1 mM quinine HCl, were not affected after applications of all concentrations with 4-AP or 5-HT. Spike width was increased from 1.304 ± 0.006 to 1.580 ± 0.022 ms ($N = 32$; $p \leq 0.0001$) after 5 min 5-HT, but did not change before and after 4-AP administration ($N = 32$; $p = 0.865$) (**Fig. 5C**).

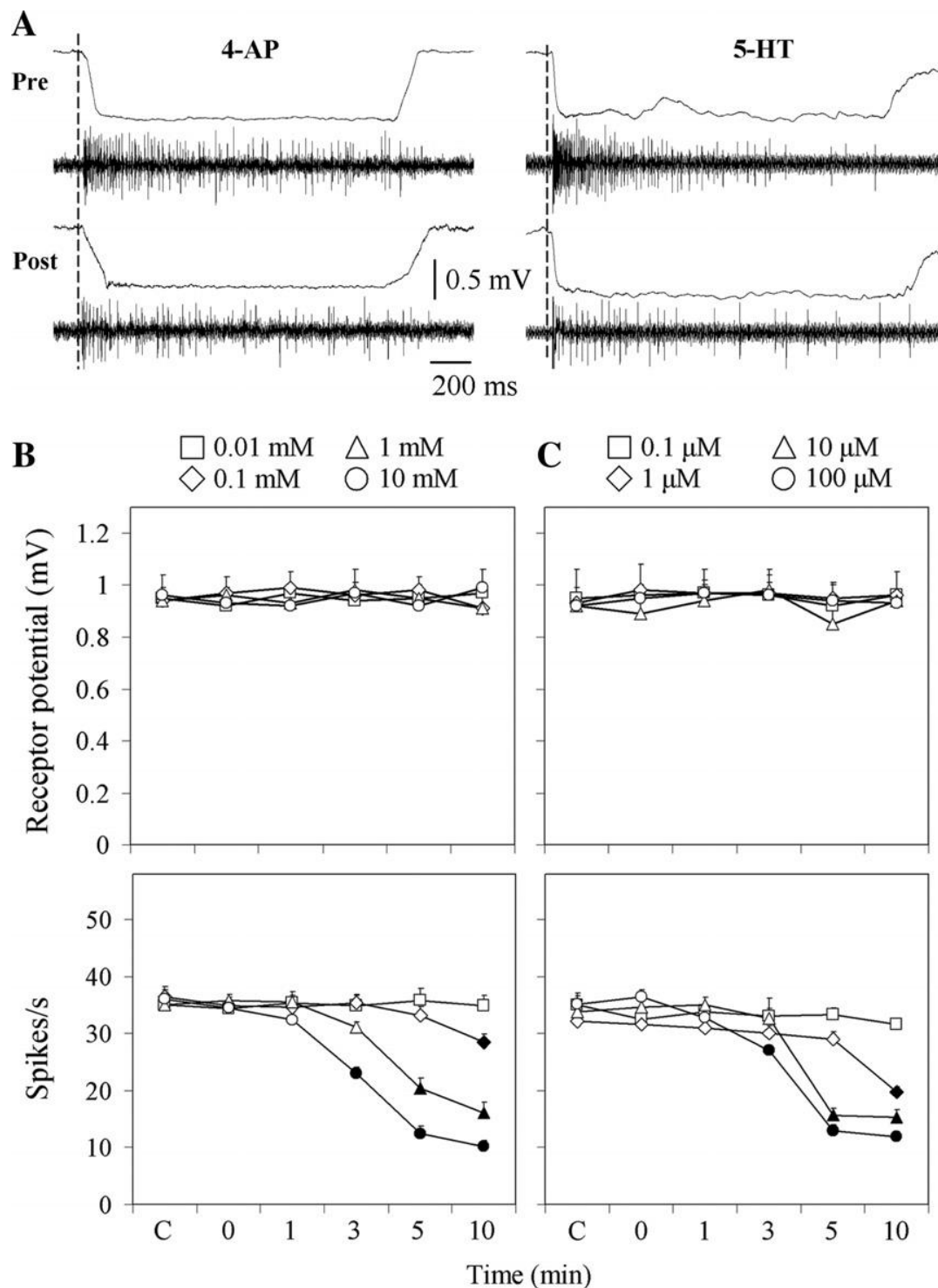


Figure 3. (A) Sample traces showing the receptor potential (mV) and the spike firing frequency following stimulation (vertical dashed line) with 100 mM sucrose, before (pre) and 5 min after (post) basolateral supply of 10 mM 4-AP or 0.1 mM of 5-HT. (B and C) Mean values \pm S.E.M. of receptor potential and spike firing frequency following stimulation with 100 mM sucrose: before (C = control), 0, 1, 3, 5 and 10 min after drug apical administration of (B) 0.01, 0.1, 1 or 10 mM 4-AP and (C) 0.1, 1, 10 or 100 mM 5-HT. Filled symbols indicate significant differences from the control ($p \leq 0.05$). Experiments performed on 30–35 sensilla (1 per fly) for both 4-AP or 5-HT.

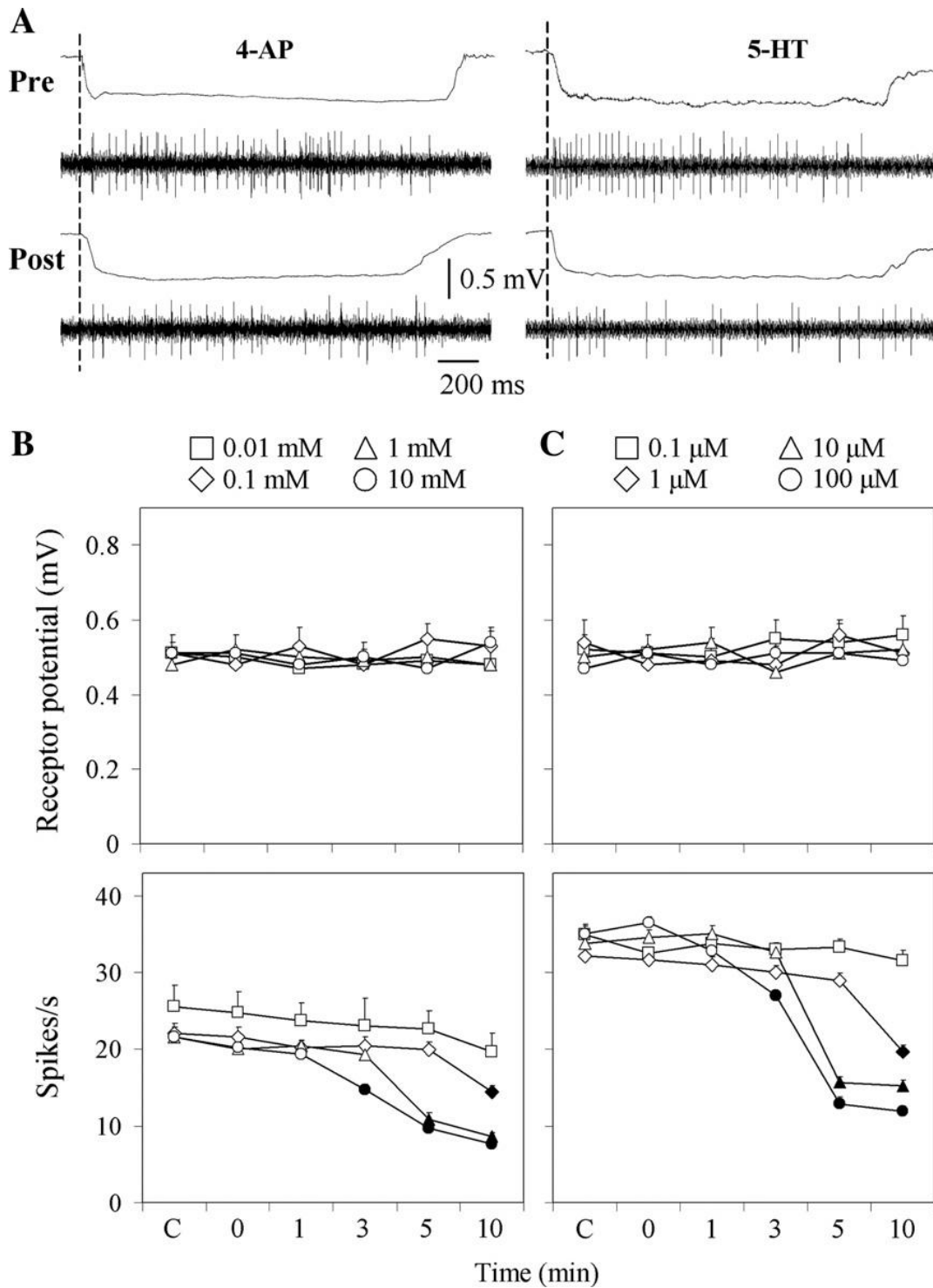


Figure 4. (A) Sample traces showing the receptor potential (mV) and the spike firing frequency following stimulation (vertical dashed line) with 1 mM quinine HCl, before (pre) and 5 min after (post) basolateral supply of 10 mM 4-AP or 0.1 mM of 5-HT. (B and C) Mean values \pm S.E.M. of receptor potential and spike firing frequency following stimulation with 1 mM quinine HCl: before (C = control), 0, 1, 3, 5 and 10 min after drug apical administration of (B) 0.01, 0.1, 1 or 10 mM 4-AP and (C) 0.1, 1, 10 or 100 mM 5-HT. Filled symbols indicate significant differences from the control ($p \leq 0.05$). Experiments performed on 30–35 sensilla (1 per fly) for both 4-AP or 5-HT.

4. Discussion and conclusions

In the taste chemoreceptors associated with labellar sensilla of blowflies the receptor potential, evoked by the interaction between taste stimuli with specific membrane receptors and/or ion channels located in the apical membrane of the dendrite, is converted into a discharge of action potentials that propagate to the CNS (Dethier, 1976; Eler and Thurm, 1981; Ishimoto and Tanimura, 2004; Morita, 1992; Murakami and Kijima, 2000). Spikes do not only propagate centripetally, but also centrifugally into the apical region of the dendrite (Eler and Thurm, 1981). Potassium (K^+) channels play an important role in various aspects of the bioelectrical activity of excitable cells; in particular, inactivating KA channels modulate the repetitive firing of the neuron, while DRK channels contribute to action potential repolarization and shaping the action potential waveform (Halliwell, 1990; Hille, 2001; Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999; Mathie et al., 1998; Mercer et al., 1995; Salkoff et al., 1992).

Our results show that the spike activity of the 'salt', 'sugar' and 'deterrent' cells, evoked by stimulations with several concentrations of NaCl, sucrose and quinine HCl, respectively, is selectively and significantly decreased by applications of both pharmacological probes, in a dose-dependent manner. In fact, the higher the drug concentration the shorter is the time required for observing a significant spike frequency decrease ranging, for both drugs, from a lack of response at the lowest concentration to a 3 min time at the highest. The effects of both drugs are completely reversed 5–20 min after administration of physiological saline (data not shown).

Based on these results, we hypothesize that 4-AP may act on KA channels, responsible of repetitive firing in many types of neurons (Adams et al., 1980; Connor and Stevens, 1971), by blocking them in an open steady state, thus prolonging the outward K^+ current during the after hyperpolarization phase and delaying repolarization, as suggested by Thompson (1982). According to Thompson "it is not yet clear how 4-AP prevents inactivation, but it may act by shifting the voltage dependence of inactivation gating toward more depolarized voltages so that channels with bound aminopyridines do not inactivate at voltages where inactivation normally goes to completion". On the other hand, we can exclude a blocking effect on DRK channels in a closed state, thus preventing repolarization (Hille, 2001; Mathie et al., 1998; Meves and Pichon, 1977; Smith et al., 2000; Wolff et al., 1998), since we did not observe an increase in spike width, which should follow from a reduction of the DKR current responsible of the action potential repolarization (Dacks et al., 2008; Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999; Mercer et al., 1995; Saito and Wu, 1991).

Instead, an increase in spike width was obtained after 5-HT administration, thus suggesting an effect on the DRK channels, in agreement with that reported in other systems (Dacks et al., 2008; Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999; Mercer et al., 1995).

In fact, a reduction of the K^+ current responsible for membrane repolarization slows down the return to the membrane resting potential, resulting in a longer duration of the action potential.

The data in the literature show that 5-HT produces different effects which depend on several factors: (a) the cellular system considered, (b) the concentration used and, in this regard, it should be kept in mind that the applied concentration does not necessarily reflect the actual concentration experienced by the neuron, and (c) different effects could be mediated by different receptors (Gatellier et al., 2004; Kloppenburg and Hildebrand, 1995; Teshiba et al., 2001). In *Bombyx mori*, 5-HT applied at 0.1 mM to the brain increased the sensitivity to female pheromone, whereas 1 mM serotonin had the opposite effect (Gatellier et al., 2004). In antennal lobe neurons in the brain of *M. sexta*, 5-HT applied at low concentration (0.01 μ M) reduced the excitatory responses evoked by electrical stimulation of the antennal nerve, while at high concentration (0.1 mM) it enhanced the responses and at 1 μ M had little apparent effect on the cell (Kloppenburger and Hildebrand, 1995). In the crayfish, the neuromodulatory effect of serotonin on the lateral giant neurons depends on its dosage, rate and duration: inhibitory effects are obtained when high concentrations are rapidly reached while excitatory effects occur when low or high concentrations are reached gradually (Gatellier et al., 2004; Teshiba et al., 2001). Serotonin has an excitatory effect on the biventer cervicis muscle of the chick at suitable concentrations and an inhibitory dose-dependent effect at high concentrations (Gatellier et al., 2004), but an irreversible toxic effect was observed with repeated exposures to serotonin (Teerapong and Harvey, 1977). In general, contradictory effects of different serotonin concentrations have been reported in vertebrates and invertebrates (Gatellier et al., 2004).

The encoder is presumably located at the level of the dendrite (Dethier, 1976; Morita, 1992), in the proximal (Erlor and Thurm, 1981) and/or in the distal portion of the dendrite (Murakami and Kijima, 2000), so that it can be easily reached by apical administration of the drug.

Our results show that the RP amplitude, in response to all tested compounds, is not affected by apical applications of both 4-AP and 5-HT, in agreement with the fact that Kv channels are not known to be involved in RP generation, but are variously involved in the termination of action potentials, repolarization and re-establishment of resting potential (Halliwell, 1990; Hille, 2001; Kloppenburg and Hildebrand, 1995; Kloppenburg et al., 1999; Mathie et al., 1998; Mercer et al., 1995; Rehm and Tempels, 1991; Salkoff et al., 1992). In fact, by the 'side-wall' technique one can simultaneously record the RP and the ensuing spike activity from the stimulated chemoreceptor cell (Kijima et al., 1988; Morita and Yamashita, 1959). Labellar chemosensory neurons are known to be best responding to stimulus categories such as the ones we tested (NaCl, sucrose and bitter compounds) (Dethier, 1976). It is reasonable to assume that if a specific stimulus triggers transduction mechanisms and receptor currents in one cell only, the sensillar potential recorded be equivalent to the RP of that cell, with the other sensillar cells providing a negligible or no contribution to ionic currents (Morita, 1992).

In conclusion, the present study reveals that both 4-AP and 5-HT alter the spike responses of peripheral chemosensory neurons to gustatory stimuli and suggest the presence of two different voltage-dependent K^+ currents: one fast, transient A-type K^+ current that modulates the frequency

of neuronal repetitive firing (Adams et al., 1980; Connor and Stevens, 1971; Salkoff et al., 1992) and a slower-activating K^+ current that resembles the delayed rectifier current and that plays a role in the membrane repolarization process (Mercer et al., 1995; Saito and Wu, 1991).

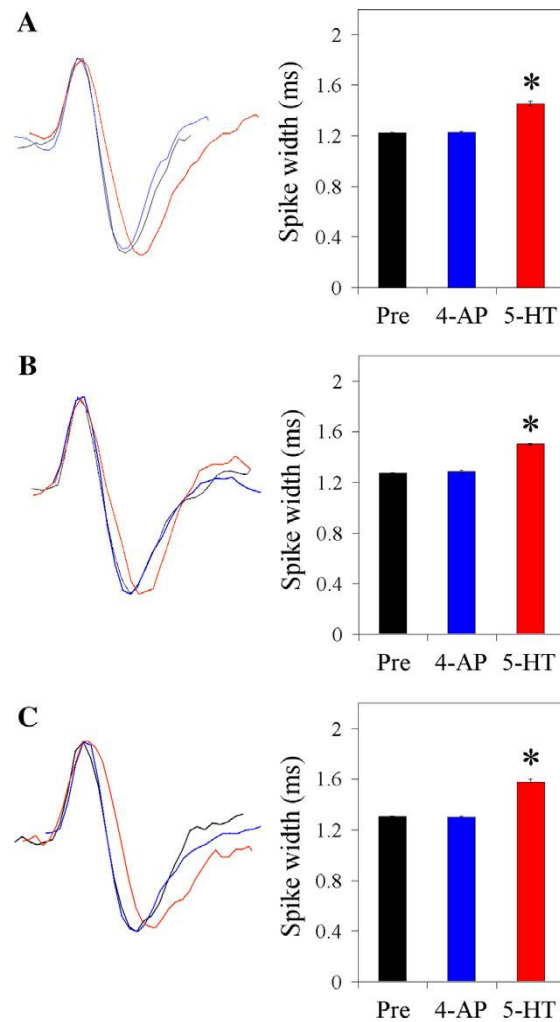


Figure 5. (A) Mean values \pm S.E.M. and sample traces showing the spike width evoked by stimulation with 150 mM NaCl (A), 100 mM sucrose (B) and 1 mM quinine HCl (C), before (black), after 4-AP (blue) or after 5-HT (red) administration. Pre = before drug administration (control). Asterisk denotes values significantly different from the control ($p \leq 0.05$).

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Section 2

Morphological characterization of the antennal lobes in the Mediterranean fruit fly *Ceratitis capitata*

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Abstract

The medfly (*Ceratitis capitata* Wied.) is one of the most important pest for horticulture, targeting a great variety of fruit and vegetables species worldwide. Due to its commercial relevance, many studies focused on the development and improvement of control strategies based on olfactory chemoreception. A complete knowledge of the anatomical and functional properties of the olfactory system is still lacking. Aim of this work is to give a morphological characterization based on the three-dimensional reconstruction of the antennal lobes (ALs) in adult medfly brains.

In order to reach this goal, we performed unilateral antennal backfills of olfactory receptor neurons (ORNs) in adult males and females by means of the neuronal tracer neurobiotin, revealed by streptavidin-Cy3 or avidin-Alexa Fluor 488 conjugated. In association with the anterograde staining, immunohistochemistry was applied in some brains. Confocal stacks acquired from whole-mount specimens were analyzed with the AMIRA software, using the Segmentation tool.

Unilateral neurobiotin and immunohistochemical stainings successfully revealed the AL structure of the adult medfly in all the specimens tested. As in other insects, the ALs of *C. capitata* are organised in glomeruli, more tightly packed in the anterior part than the posterior one. Axons of ORNs innervate a bilateral pair of homologous glomeruli and form a commissure between the two ALs, which is a typical feature of Diptera. We systematically counted a number of 53 glomeruli in each AL studied, with few exceptions.

Our results provide a basis for future investigations on the interactions with host plants of this important agricultural pest.

1. Introduction

The Mediterranean fruit fly *Ceratitis capitata* (Wied.) is a widespread pest for horticulture, targeting a great variety of fruit and vegetables species (Liquido et al., 1990). It is considered one of the most important agricultural pests in the world not only for its broad polyphagy, but also because of its biological potential, the possibility of adaptation to diverse environment and the difficulty of control (De Meyer et al., 2008; Diamantidis et al., 2011; Gasperi et al., 2002; Malacrida et al., 2007). Due to its commercial relevance, many studies focused on the development and improvement of different control strategies. Some of them are based on the use of biological competitors (Headrick and Goeden, 1996) or sterile adults, as in the Sterile Insect Technique (SIT, Ratios et al., 2001; Shelly and Kennelly, 2002; Shelly and McInnis, 2003; Shelly, 2005), others on chemicals, such as the mass-trapping (Cossè et al., 1995; Heath et al., 1991; Jang et al., 1994, 1989; Katsoyannos et al., 1997; Papadopoulos et al., 2001). Both the SIT and the mass-trapping involve the activation of the olfactory system, since they need efficient species-specific attractants to work properly. Therefore, a deep knowledge of the morphological and functional properties of the peripheral and central olfactory structures is a fundamental requirement to fully understand the mechanisms involved in odor information processing.

In this respect, in the past years several studies have been conducted on olfactory sensitivity of *C. capitata* towards different compounds of interest for this species. For example, electrophysiological recordings from antennae of medfly adults have shown a higher sensitivity of females than males for some Citrus peel oils and volatile compounds (Hernandez et al., 1996; Levinson et al., 1990; Light et al., 1992, 1988), although behavioral tests revealed a stronger preference of males for the volatiles of orange flavedo than females (Katsoyannos et al., 1997). In females, the odor preference may be affected by mating behavior: it has been observed that they are mostly attracted by the male-produced pheromone than the ripe guava odor during early sexual maturity, but this preference is inverted after mating (Jang, 1995). On the contrary, the exposure to some natural and synthetic compounds may influence the mating behavior in medfly males (Shelly et al., 2007, 2008, 2004, 1996).

From a morphological point of view, information about the olfactory system of *C. capitata* in the literature is dramatically incomplete. Only few works have described the peripheral structures in adult medflies (**Fig. 1A-C**), with particular attention to the typology and distribution of olfactory chemosensilla on the antennal surfaces of males and females. Briefly, four sensillum types (classified as basiconic, clavate, trichoid, and grooved) (**Fig. 1B**) cover the antennal flagellum (Bigiani et al., 1989; Crnjar et al., 1988; Mayo et al., 1987; Sollai et al., 2010), with no differences in number and distribution (**Fig. 1C**) between sexes, except for clavate sensilla, found in a statistically higher number in the lateral surface of females (Sollai et al., 2010). A gap of knowledge on the olfactory structures in the central nervous system of this species is still present. In insects, the first brain centre in which the olfactory information is primarily processed is the antennal lobe (AL), analogous in structure and function to the olfactory bulb of vertebrates

(Strausfeld and Hildebrand, 1999). The olfactory information is sent to the ALs after the interaction between the volatile stimuli and the olfactory receptor neurons (ORNs) on the antennae. ORN axons project into specific spherical structures inside the AL, called glomeruli, where they synapse with the dendrites of target neurons (local interneurons and projection neurons), the role of which is to convey the information processed inside the ALs to the higher brain centers, the mushroom bodies and lateral horn (Homberg et al., 1989, 1988; Ito et al., 1998; Stocker, 1994).

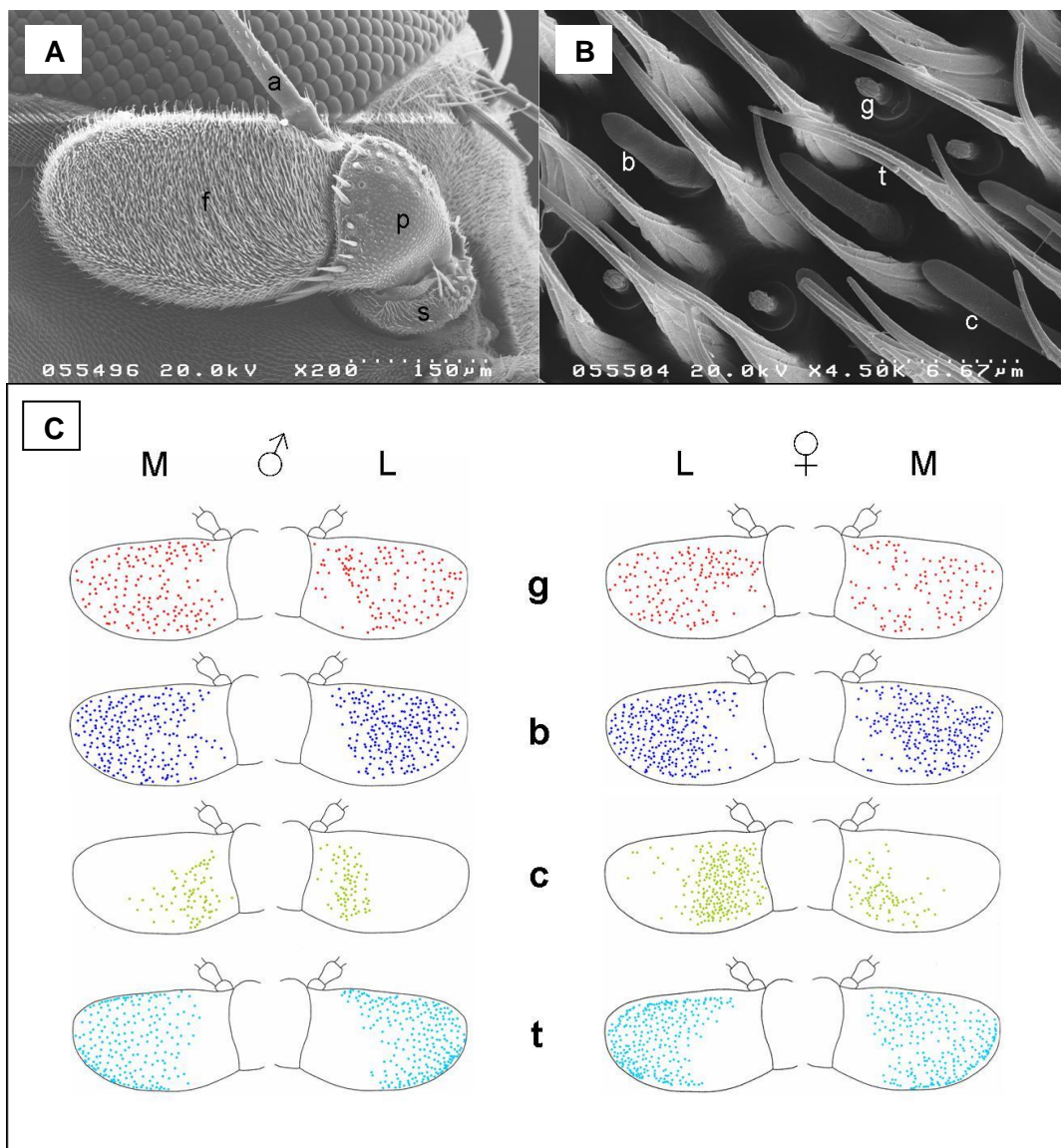


Figure 1. Morphological characterization of the antennae in *Ceratitis capitata*. (A) As other Diptera, the medfly presents aristate antennae, composed by scapus (s), pedicel (p), flagellum (f), and the arista (a). (B) The flagellar segment is covered by four types of olfactory sensilla: b = basiconic, c = clavate, t = trichoid, g = grooved. (C) Distribution of the diverse typology of sensilla in the medial (M) and lateral (L) pages of male and female antennae, with no difference between sexes except for clavate sensilla in the lateral surface of females antennae. (Images courtesy of Sollai et al., 2010)

Due to its central role in processing the olfactory information, the glomerular organization of the ALs have been largely studied and described in several insect species, such as *Drosophila* (Laissue et al., 1999; Stocker et al., 1990), moths (Berg et al., 2002; Kanzaki et al., 1989; Rospars and Hildebrand, 1992; Skiri et al., 2005), honeybees (Galizia et al., 1999), mosquitoes (Anton and Rospars, 2004; Ghaninia et al., 2007; Ignell et al., 2005), cockroaches (Chiang et al., 2001). The overall principle resulting from these studies is that this structural organization is conserved among species, presumably reflecting a common functional need (Galizia, 2008), but the number of glomeruli is variable depending on the species (Rospars, 1988).

In recent years, the improvement of staining techniques based on fluorescent dyes, combined with confocal reconstruction of entire ALs by image analysis, have allowed the creation of three-dimensional brain maps in several insects. Since no data are available for *C. capitata*, aim of this work was to give a contribution to the morphology of the ALs in adult medfly brains. For this purpose, unilateral antennal backfills in combination with immunohistochemical stainings were performed in adult males and females. A preliminary study was successfully conducted by means of the neuronal tracer neurobiotin, revealed by streptavidin-Cy3. Afterwards, further anterograde stainings were performed, and immunohistochemistry was applied in some brains. Confocal stacks acquired from whole-mount specimens were analyzed for the estimation of the number of glomeruli.

2. Materials and methods

2.1. Insects

All the experiments were performed on adult medflies of *C. capitata* of both sexes, kindly supplied by the Dept. of Animal Biology of the University of Pavia (Italy) at the pupal stage, and reared under controlled conditions ($22 \pm 1^\circ$ C, 60-70% relative humidity, 12:12 h light:dark cycle) in a climatic chamber. Adult flies were fed with a mixture of sugar and yeast (4:1). Fresh water was given twice a week.

2.2. Staining techniques

In order to obtain the morphological characterization of the antennal lobes in the Mediterranean fruit fly *Ceratitis capitata*, two different staining techniques were applied both in male and female flies. Preliminary unilateral antennal backfills were performed in 9 females, by means of the neuronal tracer neurobiotin (NB) (Vector Laboratories), revealed by streptavidin conjugated with the fluorochrome Cy3. For the further experiments, conducted both in males and females, backfills were performed using NB coupled with avidin-Alexa Fluor 488 as a one step process, or in association with an immunohistochemical procedure based on the application of a primary

antibody, specific for the visualization of brain areas with high synaptic density, such as the ALs. The 4',6-diamidino-2-phenylindole (DAPI) dye was used to visualize cell body nuclei. All chemicals were purchased from SIGMA-Aldrich, except otherwise stated.

2.2.1. Antennal backfills

Antennal backfills were performed with the same protocol both in the preliminary and in the further study. Living insects were inserted into 100 µl truncated plastic pipette tips, with the head protruding at the tip and immobilized with plastiline. Then, one of the antennae was placed vertically and stabilized with other plastiline. A small mould was built around the antenna with vaseline, filled with a drop of a NB solution (2% in distilled water), and the antenna was cut at the base of the flagellum. The mould was covered with more vaseline and the insects were kept at 4°C in a moistened chamber to prevent desiccation, for 5 hours or overnight to allow the complete migration of the tracer. Afterwards, heads were removed from the insect body, and placed in a Petri dish with 4% paraformaldehyde (PFA) dissolved in sodium phosphate buffer (PBS, 0.1 M, pH = 7.2). The brains were dissected out of the head capsule and post-fixed in new fresh PFA at room temperature, for a total fixation time of 3 hours. Subsequently, brains were washed several times (usually 8 x 30 min) in PBS with 0.2% Triton X-100 (PBST), in order to completely remove all the PFA from the tissues. Brains were then incubated in avidin-Alexa Fluor 488 (Invitrogen, Molecular Probes) at 1:400 dilution or in streptavidin-Cy3 (Jackson ImmunoResearch Europe) diluted 1:500 in volume, for three days. The nuclear marker DAPI (1:500) was added together with avidin or streptavidin for visualization of cell body nuclei. After incubation, brains were washed in PBST (8 times x 30 min), dehydrated in increasing ethanol solutions (50%, 75%, 95%, 2 times x 100%, 30 min each) and treated in xylene for 2-3 minutes. All the steps of fixation, washing and incubation were done on a shaker, in constant and gentle agitation. Finally, brains were mounted in DPX (a mixture of distyrene, a plasticizer, dissolved in toluene-xylene), using spacer rings to avoid pressure effects on the brain tissues by the coverslip.

2.2.2. Immunohistochemistry

In some specimens, after the application of the protocol used for the NB backfill stainings, and before incubation in avidin-Alexa Fluor 488 and anti-synapsin antibody, brains were treated with a blocking solution of PBST containing 0.2% bovine serum albumine (BSA) and 0.02% NaN₃. The blocking step was done at room temperature on a shaker, for 12 hours or overnight, in order to prevent unspecific binding of the antibody and to reduce the background noise. Then, brains were incubated in a solution with avidin-Alexa Fluor 488 (1:400) and the mouse monoclonal primary antibody anti-synapsin (1:250, Hybridoma, University of Iowa, IA, USA) in PBST with 0.2% BSA and 0.02% NaN₃. The specimens were subsequently washed (8 times x 30 min) in PBST and incubated in a goat anti-mouse secondary antibody conjugated with Alexa Fluor 546

(Invitrogen, Molecular Probes), diluted 1:200, and DAPI (1:500) in PBST with 0.2% BSA and 0.02% NaN₃. After incubation, brains were washed again in PBST (8 times x 30 min), dehydrated in increasing ethanol solutions (50 - 100%, 30 min each), treated with xylene (2-3 min), and finally mounted in DPX.

2.3. Image acquisition and analysis

Images from whole-mount specimens were obtained by means of two different confocal microscopes.

In particular, the image stacks of the brains treated with streptavidin-Cy3, used in the preliminary study, were scanned with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany), equipped with a 20x/1.0 water immersion objective for entire brains, and a 63x/1.4 oil objective for single ALs. Structures stained with Cy3 were excited with a HeNe laser at 543 nm, whereas the 2-photon excitation at 750 nm was employed to detect the cell body nuclei labeled with DAPI, with appropriate filters to detect the fluorescence. Improvement of the image quality was achieved adjusting the contrast and the brightness with the Zeiss LSM Image Browser.

Preparations treated with Alexa Fluor 488 and Alexa Fluor 546 were viewed with a Leica SP5 confocal microscope, by using a 20x/0.5 dry and a 63x/1.40 oil objective. Scans were made with a supercontinuum white light laser, with an excitation wavelength of 488 nm for Alexa Fluor 488, and 556 nm for Alexa Fluor 546. Fluorescence was detected in a range of 495-580 and 561-635 nm, respectively. Visualization of nuclei stained with DAPI was possible by means of a UV laser (405-diode) at 405 nm. Confocal image stacks were processed with the LAS.AF software before the computer analysis. Images of all specimens were obtained and stored in 12-bit resolution (1024 x 1024 pixels).

2.4. Glomerular identification and nomenclature

Among all the ALs scanned, 39 were selected for the analysis with the AMIRA 5.2.1 software (Visage Imaging, Berlin, Germany), by means of the Segmentation tool. Identification of glomeruli in the medfly ALs was made considering the location, the shape and the dimension, and the nomenclature used is based on that developed for *Drosophila* (Stocker et al., 1990). Each glomerulus was marked by one or two capital letters indicating the general position: A = anterior, P = posterior, D = dorsal, V = ventral, L = lateral, M = medial, C = central. Letters were followed by numbers to distinguish glomeruli in the same region.

2.5. Statistics

Two-way ANOVA was used to evaluate the interaction of gender and homologous ALs related to AL size (as diameter). Afterwards, one-way ANOVA was used to determine whether significant differences in AL size were present between sexes, and right and left ALs. Also the volumes of

matched glomeruli in males and females were analyzed to assess the existence of sexual dimorphism. For this purpose, three-way ANOVA was used to compare differences related to gender and glomerulus type in homologous or different ALs. Volumes of glomeruli were calculated after the segmentation procedure, corresponding to the number of voxels x size of a single voxel. The voxel size obtained from the scanning was 0.2405 x 0.2405 x 0.25177 μm (x, y, z). Post-hoc comparisons were conducted with the Tukey test. Statistical analyses were conducted using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK, USA). p values <0.05 were considered significant.

3. Results

3.1. Backfills and antibody stainings

Confocal acquisition of *C. capitata* brains allowed to visualize entire ALs in single image stacks. Among all the treated specimens, 39 ALs were successfully stained with backfills (**Fig. 2A**), and 5 preparations showed also a good immunostaining both in the left and the right ALs (**Fig. 2B**). Unilateral backfills with NB, subsequently labeled by avidin-Alexa Fluor 488 or streptavidin-Cy3, revealed the sensory afferents from the antennae into the ALs, which constitute the antennal nerve (AN). Inside the ALs, these afferents form more or less dense packed regions, which correspond to the glomeruli, more tightly associated in the anterior part than the posterior one. Fibers from ORNs project inside the ALs with an ipsilateral and contralateral innervation pattern of all the glomeruli visualized, in males as in females. Due to this contralateral innervation, left and right ALs result interconnected by the antennal commissure (AC) (**Fig. 2D**). Immunohistochemical staining with the anti-synapsin antibody allowed to visualize different brain regions with high synaptic density. The ALs appear clearly defined structures, with a major number of glomeruli than those revealed by backfills. Anti-synapsin antibody also allowed to visualize clusters of cell bodies surrounding the ALs, presumably representing somata of interneurons and projection neurons, the nuclei of which were stained by DAPI (**Fig. 2A-D**).

3.2. The ALs of *Ceratitis capitata*: general structure, identification of glomeruli and 3D reconstruction

As in other insects, medfly ALs are located in the anterior part of the brain, and possess an ellipsoid shape. For each AL, the diameter was measured (mean value = 105.857 μm in males, 120.8 μm in females). Two-way ANOVA revealed no significant interaction between gender and AL type ($F_{(1,16)} = 0.17680$, $p = 0.67973$); on the contrary, the one-way ANOVA analysis showed the existence of sexual dimorphism in size ($F_{(1,16)} = 5.7155$, $p = 0.02946$), with no differences between homologous ALs ($F_{(1,16)} = 0.00707$, $p = 0.93402$). Evaluation of glomeruli was achieved by the analysis of the confocal image stacks in both sexes. The comparison between the

anterograde and the antibody stained ALs (**Fig. 2C**) was necessary to better recognize single glomeruli, otherwise not individually identifiable in some cases.

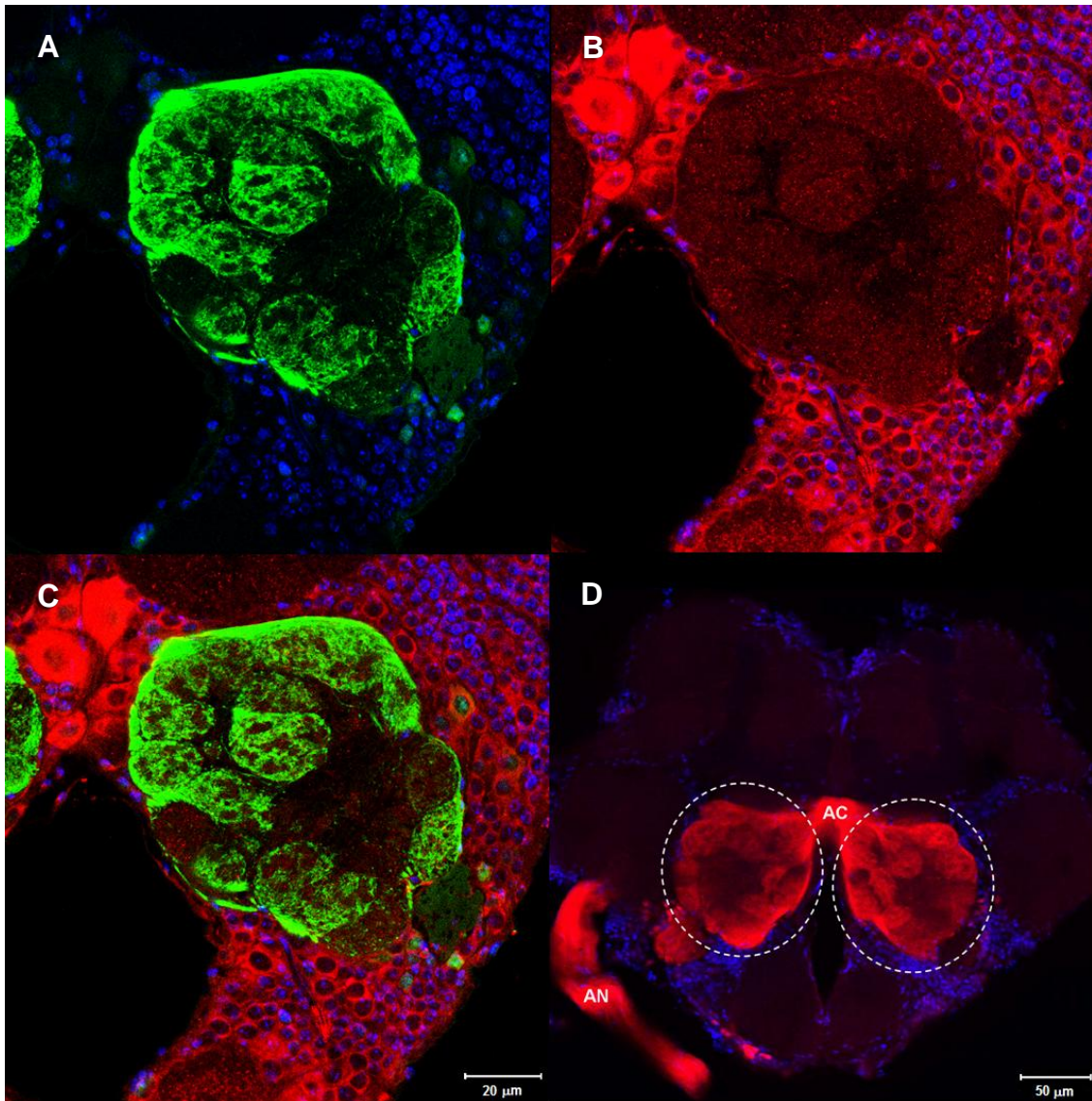


Figure 2. Staining results in the left AL of an adult medfly (A-C) and in a specimen treated in the preliminary study (D).

A. Anterograde backfill with NB, revealed by avidin-Alexa Fluor 488. Projection of ORNs enter the AL and form dense packed regions identifiable as glomeruli.

B. Immunohistochemical staining with anti-synapsin antibody allowed to highlight the presence of glomeruli inside the ALs, and clusters of cell bodies surrounding.

C. NB and anti-synapsin stainings merged together. Comparison of the two stainings was fundamental in some cases, in order to recognize single glomeruli otherwise not identifiable.

D. Preliminary anterograde backfill in a specimen treated with NB revealed by streptavidin-Cy3. Sensory afferents from ORNs enter the AL, forming the antennal nerve (AN). Due to a contralateral innervation, ALs are connected by the antennal commissure (AC).

The nuclear marker DAPI revealed the cell body nuclei (**A-D**).

The same 53 glomeruli were systematically counted in each AL independently of the sex, with some exception, and some supernumerary glomeruli resulted in few cases. A preliminary three-way ANOVA showed no significant interactions of glomerulus volume across gender, homologous ALs and glomerulus types ($F_{(52,844)} = 0.76321$, $p = 0.88958$), thus a two-way ANOVA was used to determine the existence of possible changes related to sexes. This analysis confirmed a sexual dimorphism ($F_{(52,844)} = 3.7331$, $p = 0.00000$), and post-hoc comparisons showed that only few glomeruli presented statistical changes in volume between males and females (**Fig. 3**). One of these larger glomeruli, named PD1 ($p = 0.000051$), is located dorso-medially, at the entrance of the AL. C3 ($p = 0.047801$) is located central, inside the AL. PV1 ($p = 0.00005$) is the most ventral glomerulus, oriented in the posterior area. A3 ($p = 0.004119$) is located in the anterior surface of the AL. Finally, the more consistent group of sexual dimorphic glomeruli, composed by PD4 ($p = 0.038628$), L1 ($p = 0.006187$), VL1 ($p = 0.00005$), AL1 ($p = 0.000051$), and AD1 ($p = 0.00005$), is distributed on the lateral region of the AL.

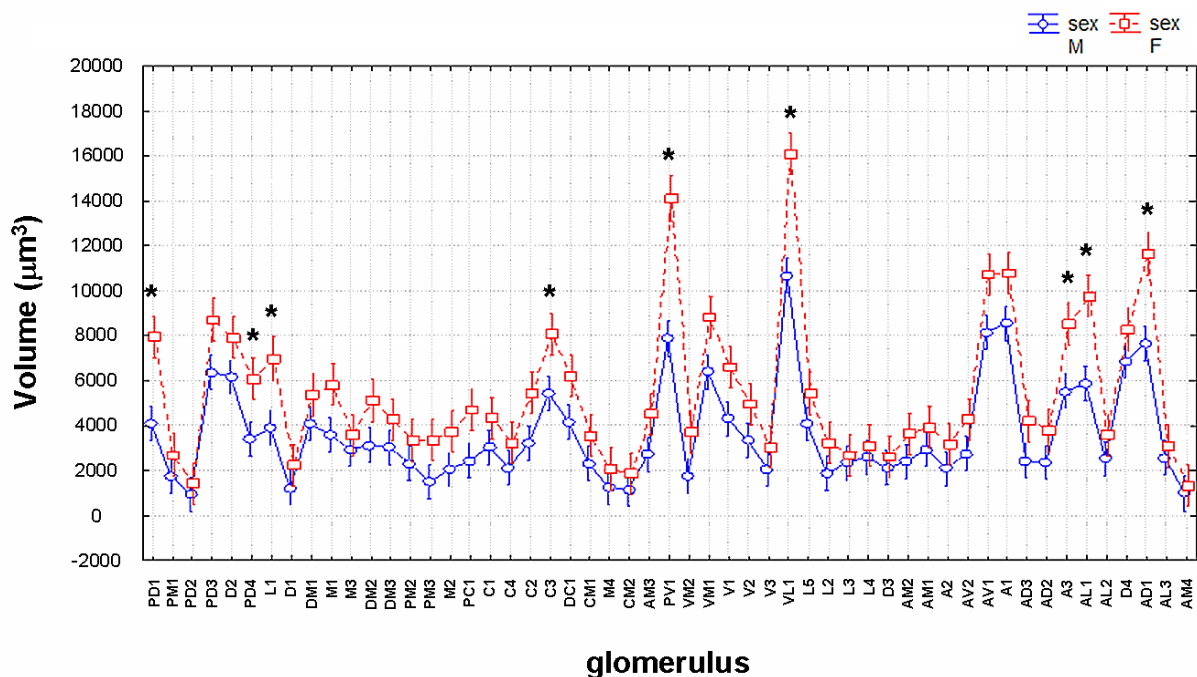


Figure 3. Mean volume of the 53 glomeruli counted in the medfly ALs analyzed. The post-hoc comparisons conducted with the Tuckey test revealed sexual dimorphism only in a few number of glomeruli. * = significant difference between males (in blue) and females (in red).

The identification of single glomeruli allowed the construction of 3D maps of the ALs, based on glomeruli segmentation by the AMIRA software, both in males and females (**Fig. 4-5**). In order to simplify recognition, given the presence of regions with a high density of glomeruli, each

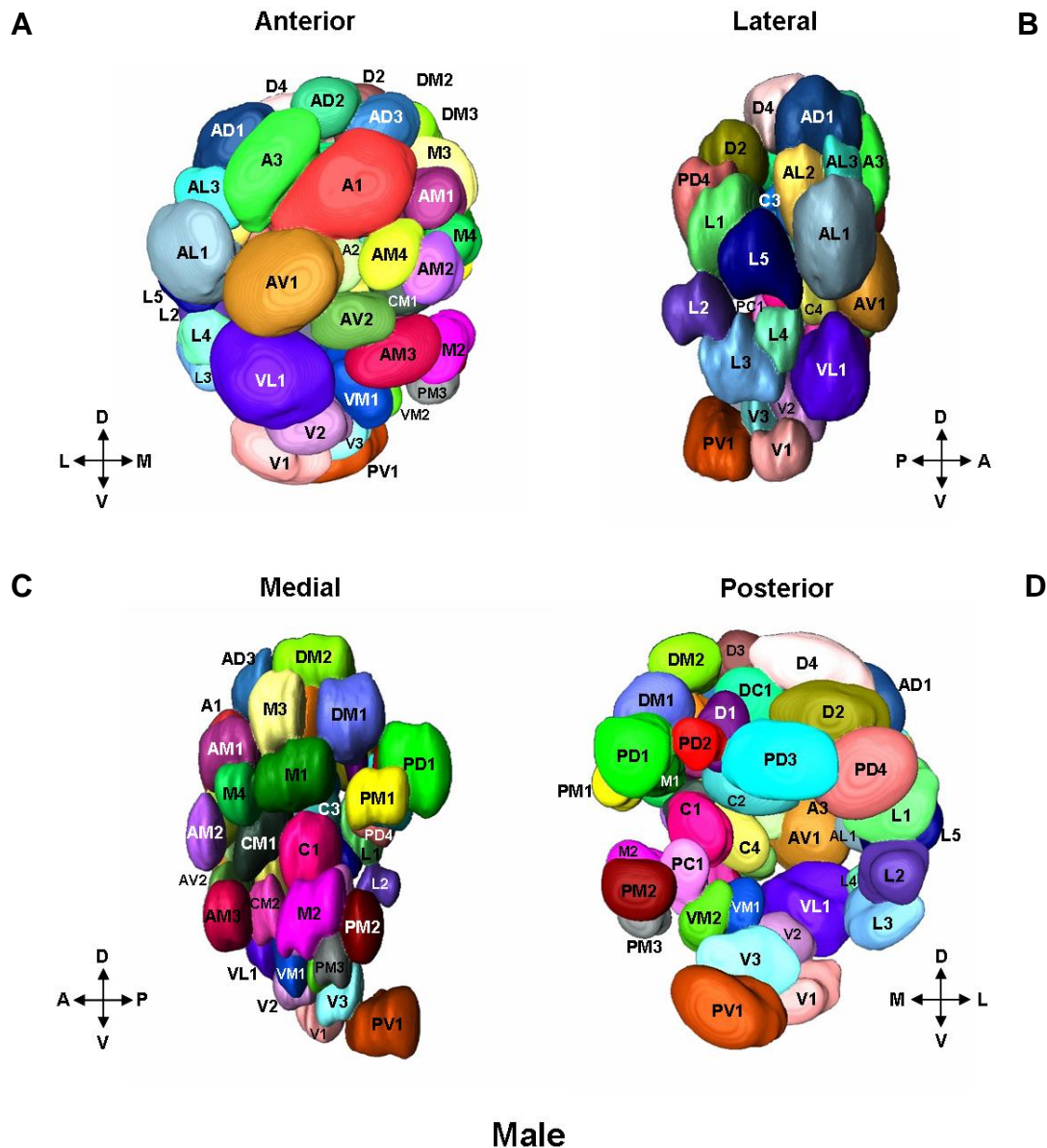


Figure 4. 3D reconstruction of the glomeruli in the right AL of a male medfly, shown in the anterior (A), lateral (B), medial (C) and posterior (D) part of the AL. Glomeruli have been segmented with different colors to distinguish single units, since areas of more packed glomeruli are present inside the ALs. 53 glomeruli have been counted, named with capital letters and numbers according to Stocker (1994). Capital letters indicate the general position of glomeruli inside the ALs, the numbers are required to distinguish glomeruli in the same region. The orientation of the AL is indicated by the cross: A = anterior, P = posterior, D = dorsal, V = ventral, L = lateral, M = medial.

glomerulus has been marked with a different color. Glomeruli have been named in the different specimens on the basis of the same principle, as the general position is constant in homologous ALs, and intra- and inter-individually.

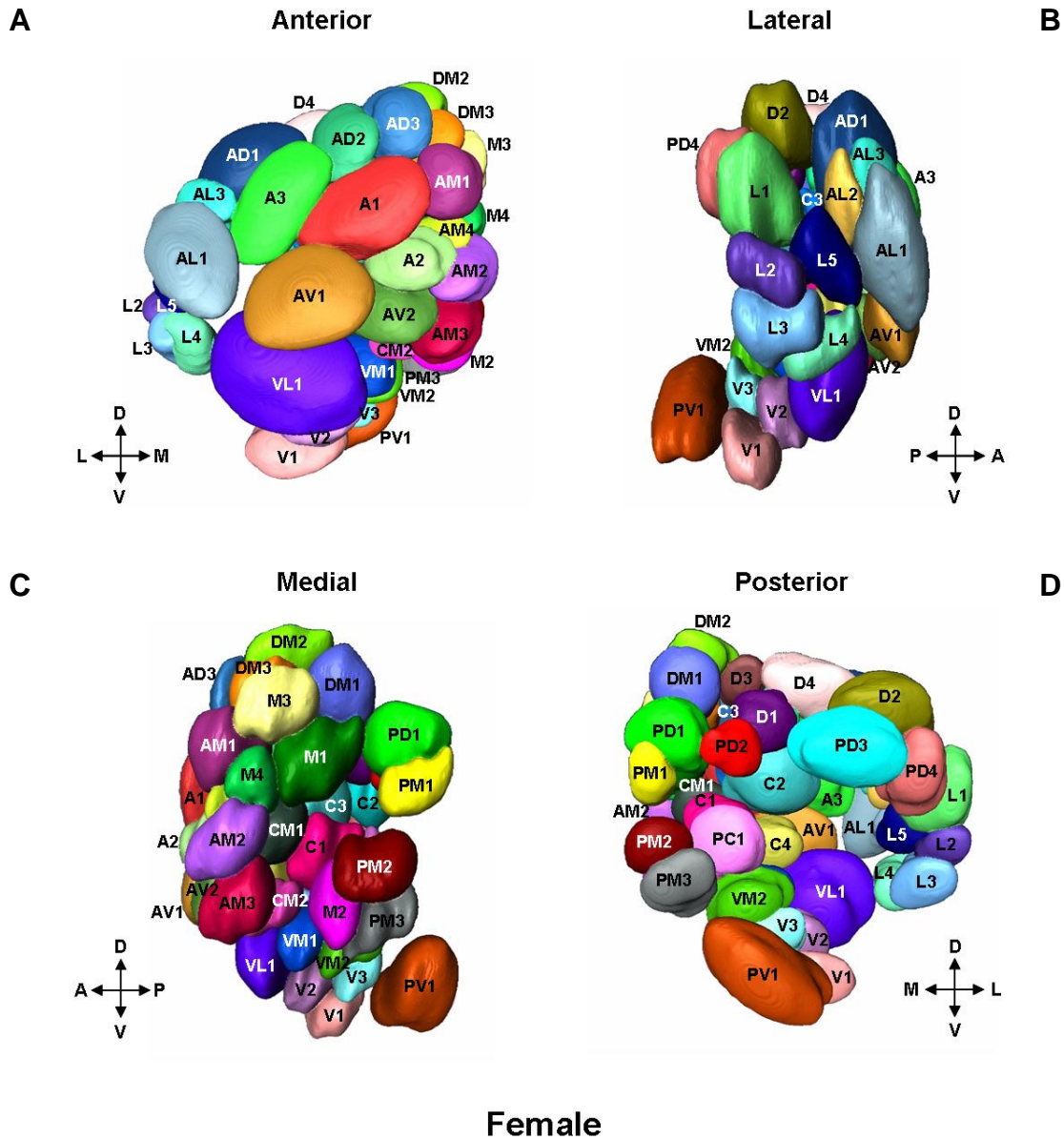


Figure 5. 3D reconstruction of the glomeruli in the right AL of a female medfly, shown in the anterior (A), lateral (B), medial (C) and posterior (D) part of the AL. As for the reconstruction in the male specimen, glomeruli have been segmented with different colors to distinguish single units, and also in this case 53 glomeruli have been counted, and named according to Stocker (1994). The orientation of the AL is indicated by the cross: A = anterior, P = posterior, D = dorsal, V = ventral, L = lateral, M = medial.

4. Discussion and conclusions

The aim of this study was to describe the morphology of the ALs in the Mediterranean fruit fly *Ceratitits capitata*, combining two different staining techniques, confocal microscopy and image analysis.

Both the anterograde backfills with the neuronal tracer NB and the immunohistochemical stainings with the primary antibody anti-synapsin successfully revealed the glomerular structure of the medfly ALs. Glomeruli represent the morpho-functional units of the ALs in many species of insects (Anton and Rospars, 2004; Berg et al., 2002; Chiang et al., 2001; Galizia et al., 1999; Ghaninia et al., 2007; Ignell et al., 2005; Kanzaki et al., 1989; Laissue et al., 1999; Rospars and Hildebrand, 1992; Skiri et al., 2005; Stocker et al., 1990), with a common general organization, but a variable number (Rospars, 1988). The NB backfills, in particular, allowed to highlight, in the medfly, the projection patterns of axons from the ORNs located at the antennal level: fibers converge in the antennal nerve and enter the antennal lobe to form the synaptic connections with the second order sensory neurons, which is consistent with the anatomical arrangement of olfactory pathways in other dipteran species (Poddighe et al., 2010; Stocker, 1994). Although NB was applied only unilaterally, both ALs were revealed: this is due to the contralateral innervation of homologous glomeruli by bilateral fibres projecting from ORNs, passing via the antennal commissure, which is a typical feature of Diptera (Poddighe et al., 2010; Stocker, 2001, 1994). Immunohistochemical stainings with the anti-synapsin antibody clearly showed the morphology of different brain areas, indicative of a high density of synaptic connections. Inside the ALs, more glomeruli than those highlighted by anterograde stainings were revealed, presumably because these glomeruli are not innervated by antennal ORNs, but receive projections originating from the sensory neurons located in the sensilla of maxillary palps (Anton and Rospars, 2004; Anton et al., 2003; Ghaninia et al., 2007; Stocker, 2001, 1994).

The analysis of confocal image stacks allowed the recognition of glomeruli into the ALs, but a comparison between images acquired from NB and anti-synapsin treated specimens was necessary to clearly distinguish single glomeruli. In *C. capitata* ALs, in fact, the differentiation of glomeruli in dense regions is not so evident, probably for the absence of a consistent glial sheath to separate them (Stocker, 1994). Interestingly, glomeruli of the medfly present different shapes, conserved in both sexes, and different volumes: these features facilitated the anatomical matching of glomeruli belonging to the same region in diverse ALs. The segmentation procedure in *C. capitata* allowed to systematically count 53 glomeruli inside the ALs analyzed, with absent or supernumerary units in some cases. From the literature it is known that the glomerular number may greatly differ depending on the species (Rospars, 1988), but in Diptera, such as flies and mosquitoes, ALs are usually characterized by a glomerular number ranging from around 50 to 60 (Ghaninia et al., 2007; Ignell et al., 2005; Laissue et al., 1999). In general, the count of missing or supernumerary glomeruli in different specimens during the segmentation step is not unusual, and this fact has been found in various species of insects studied so far (Couton et al., 2009; Kazawa

et al., 2009; Rospars and Hildebrand, 2000, 1992). The explanation for this may depend on a methodological error in the experimental and analysis procedures, as well as on individual variability. Although in *C. capitata* it is not possible to completely exclude the incorrect outline of glomeruli due to a methodological error, this should not be considered as the main source of differences, given the standardization of the staining protocols and the selection of confocal image stacks of a good quality for the count. On the contrary, individual variability may be largely responsible for the presence of anomalies in the number of glomeruli: this number, in fact, is directly related to the number of olfactory receptors expressed by sensory neurons (Couto et al., 2005), and perturbations during development may influence the expression of genes responsible for these olfactory receptors (Couton et al., 2009; Rospars and Hildebrand, 2000).

The results of this work highlight the presence of sexual dimorphism in the medfly: although glomeruli can be paired in homologous ALs and between sexes, females appear to possess some enlarged glomeruli as compared to males. Usually, species characterized by sexual dimorphism in the glomerular arrangement of the ALs may show differences in the number, dimensions and/or location of glomeruli (Rospars and Hildebrand, 2000). This dimorphism is generally present in species whose sexual behavior depends on pheromone communication. In male moths, for example, the detection of the sex pheromone emitted by females is assured by the presence of specific sensillum types on the antennal surface. Afferents from ORNs located in these sensilla project into the AL where they form, upon entering, the first glomerular station responsible of pheromone information processing, the macroglomerular complex (MGC, Berg et al., 1998; Hansson et al., 1995). Moreover, in *Manduca sexta*, in spite of its sexual dimorphism, three glomeruli have been found in females equivalent to the three glomeruli composing the MGC in males (Rospars and Hildebrand, 2000). In *C. capitata*, the comparison of the ALs between sexes showed the absence of a complex that resembles the MGC, however one of the glomeruli located at the entrance of the AL (PD1) results to be significantly larger in volume in females than in males. Even if functional studies should be conducted to verify the association of this glomerulus with the processing of pheromone information, the sexual dimorphism results coherent with the sexual behavior of medflies, in which males release the pheromone to attract females for mating (Arita and Kaneshiro, 1989; Flath et al., 1993; Jang et al., 1989).

In addition to PD1, other eight glomeruli resulted significantly larger in females than in males, probably due to the need of a sophisticated system for the detection of proper ovoposition sites (Varela et al., 2009).

The combination of two different staining techniques, confocal microscopy and image analysis allowed the construction of three-dimensional maps of the ALs of *C. capitata*, based on the manual segmentation of glomeruli, in both sexes. 3D reconstructions of the ALs have been made in several insect species so far (Berg et al., 2002; Couton et al., 2009; Galizia et al., 1999; Ghaninia et al., 2007; Greiner et al., 2004; Ignell et al., 2005; Laissue et al., 1999; Poddighe et al., 2010; Smid et al., 2003; Varela et al., 2009), since the availability of 3D maps of glomeruli is a

preliminary step to understand how the olfactory information is processed inside the brain, thus to obtain a chemotopic map of the ALs. In the medfly, the reconstruction of the ALs has been made in many specimens, based on confocal sections of single individuals. Glomeruli have been easily matched, thanks to the conserved shape and position inside the AL, and the number of segmented glomeruli was almost the same in all the specimens analyzed, with few variations only. This result is in accordance with studies conducted in various other insects, where number and position of glomeruli resulted conserved among individuals of the same species (Galizia et al., 1999; Rospars and Hildebrand, 2000, 1992).

In conclusion, the 3D maps of the glomerular structure in *Ceratitis capitata* ALs represent an necessary starting point for functional studies, in order to assess the relationship between the structure and the function of the olfactory pathways, and better understand how the olfactory information is processed. The results of this study provide a solid basis for future investigations on the interactions with host plants of this important agricultural pest.

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