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PhD thesis in Insect Science and Biotechnology

Molecular and functional characterization of the Odorant Receptor2 (OR2) in the tiger mosquito *Aedes albopictus*

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CONTENTS

Abstract

In mosquitoes, olfactory system plays a crucial role in many behaviors, including nectar feeding, host preference selection, searching for the right place to lay eggs. *A.albopicus*, known also as tiger mosquito, is an anthropophilic species which in the last years, due to a strong ecological plasticity, has spread throughout the world and all over Italy with a high abundance in man-made environments. Although long considered a secondary vector of viruses, the potentiality of its vectorial capacity is very dangerous and may constitute the foundation for a public health alert. Nevertheless, to date, for this mosquito nothing is known at molecular level. Based on the idea that an improved understanding of the olfactory system of mosquitoes may help in developing control methods that interfere with its behavior, recently we have undertaken a study aimed to characterize the *A. albopictus* Odorant Receptors. During my PhD work, I focused my attention on the identification, cloning and functional characterization of the *A. albopictus* OR2 ortholog. My data indicate that *A. albopictus* OR2 (AalOR2) shares a high degree of identity with the other mosquito OR2 orthologs characterized to date, confirming that OR2 is one of the most conserved mosquito ORs; furthermore, AalOR2 is expressed in the olfactory appendages of larvae and adults and its expression increases after a blood meal, as determined by a semi-quantitative RT-PCR. Interestingly, this is the first report of an up-regulation of an OR in response to a blood meal; this increase could suggest a role of AalOR2 in searching oviposition right places. AalOR2, such as the other orthologs, is narrowly tuned to indole, a ubiquitous volatile compound that has been linked to host seeking, and oviposition. The de-orphaning of AalOR2 has been obtained, with same results, through Ca^{2+} imaging assay in HEK293 cells, and “in vivo” experiments using the Single Sensillum Recording (SSR) in an engineered neuron of the fruitfly *Drosophila melanogaster* that express AalOR2. Furthermore, by using this technique, I was able to identify also a

molecule, (-)Menthone, that produced an inhibitory effect on this Odorant Receptor. In summary, this work led to the cloning and de-orphaning of the first Odorant Receptor in *A. albopictus*, that may be used as potential molecular target for developing environmentally friendly strategies to control mosquito populations.

Introduction

During the endless process of evolution, animals have specialized sophisticated sensory modality to interact with the external world, that today we know as eyesight, hearing, smell, taste and touch. Among all these senses, the smell is the oldest one and plays a key role for the life; all animals are embedded in a world of smells, olfactory molecules that function as signals able to trigger vital behaviors such as to eat, find mates, and avoid dangers. Furthermore, olfactory cues control many social and sexual interactions among individual of the same species. The olfactory system appears to be much more complex than visual or auditory system, which discriminate only between two simple parameters such as wavelength and frequency. The olfactory system performs the complex task of discriminating the quality and assessing the concentration of thousands of different odorants that differ in shape, size and electric charge. This complex identification is achieved through the interaction of volatile molecules with a large number of specialized Olfactory Receptors (ORs), that are expressed in the Olfactory Receptor Neurons (ORNs). ORNs respond to odorswith a sequence of action potentials that reflects the quality, intensity, and temporal structure of the odor stimulus. The signals generated by ORNs are transmitted from the peripheral olfactory system to the higher centers of the brain, where processing takes place. To date, many efforts have been made to understand anatomy, molecular processes and behavioral responses that underlie olfactory perception with particular attention to mammals and insects, animals in which new insight has recently been gained. In particular, insect olfaction has been a field of deep interest for two reasons. First of all, insect olfactory systems are simple relative to vertebrate olfactory systems but well conserved across phylogeny (Hildebrand JG and Shepherd GM, 1997). Moreover, given that insects that cause enormous losses to agriculture and carry devastating diseases, localize their plant and hosts via olfactory cues, understanding the

molecular basis of insect olfaction may lead to develop novel approaches to control pest insects.

Olfactory systems

The functional organization of the olfactory system is very similar in organisms ranging from insects to mammals. Briefly, in both kinds of animals, odorsbind to receptors in the cilia or dendrites of the olfactory receptor neurons (ORNs), each of which expresses one or a small number of Odorant Receptor types. In both insects and mammals, ORNs that express a given OR send axons to the same glomerulus, a spheroidal structure that consist of the ORN axon terminals and of the dendrites of second order neurons. The glomeruli form the antennal lobe (AL) of the insect brain, or its mammalian equivalent, the olfactory bulb (OB). In both of these centers, the olfactory signals are processed and relayed to higher centers of the brain. A growing body of works is providing new understanding of how the identity and intensity of odorsare first encoded in the olfactory organs and how they are subsequently decoded in the central nervous system.

Mammals olfactory system

In mammals, there are multiple olfactory organs, which differ in location, numerical complexity, receptors expressed, and in the targets of their neurons within the central nervous system. General odorsare mainly detected in the main olfactory epithelium (MOE), lied in the dorsal nasal cavity, that contains the Epithelium Olfactory Neurons (OENs or ORNs). ORNs have a bipolar architecture with a basal axonal pole and an apical dendritic pole. In these neurons the recognition of the olfactory molecules and the subsequent

conversion of the chemical message to an electrical signal takes place. The dendrite ends in a swelling provided of numerous cilia that innervate the lining of the nasal cavity and carry the Olfactory Receptors (ORs) (Menco BP and Jackson JE, 1997). On the other side of the neuron, a single axon projects to the Olfactory Bulb, a specialization of the forebrain that serves as the first relay station of the odorant information. In the OB, these axons synapse with the dendrites of projection neurons within the glomeruli (Fig.1). In the mouse, there are 5-10 million of ORNs in the epithilium and about 2000 glomeruli in each OB. This ratio leads to an about 1000-fold convergence of ORNs axons into each glomerulus (Firestein S, 2001). This convergence lies at the heart of the coding strategy for olfactory information (rewieved in DeMaria S. and Ngai J., 2010), that is based on two principles. Each ORN in the olfactory epithelium expresses only one allele of a single member of the OR gene family (Chess A *et al.*, 1994; Serizawa S *et al.*, 2003; Lewcock JW and Reed RR, 2004). This phenomenon is known as the "one receptor, one neuron" rule, and assume that the array of odorsto which a given ORN can respond, called "receptive field", is directly correlated to the properties of its expressed OR. Second, although neurons that express a given OR are randomly distributed throughout the olfactory epithelium, they converge their axons into 1-3 glomeruli in the olfactory bulb (Mombaerts P *et al.*, 1996; Buck LB, 2005-2006), in a spatially invariant pattern (Ressler KJ *et al.*, 1994; Vassar R *et al.*, 1994; Mombaerts P *et al.*, 1996; Mori K *et al.*, 1999) showing a mirror simmetry. Thus, neuronal activity in a given glomerulus reflects the stimulation of on specific type of OR in the nose. As an odor molecule can be recognized by different receptors, it is thought to be the combination of activated glomeruli that defines the unique neuronal representation of an odor (Ache BW and Young JM, 2005). In turn, each glomerulus is linked to a single mitral cell (second-order olfactory neuron) that transmit the signal to the cortex (Fig.1) (Menini A. *et al.*, 2004). In summary, it is now clear that the olfactory system is elegantly organized in the olfactory epithelium, and that

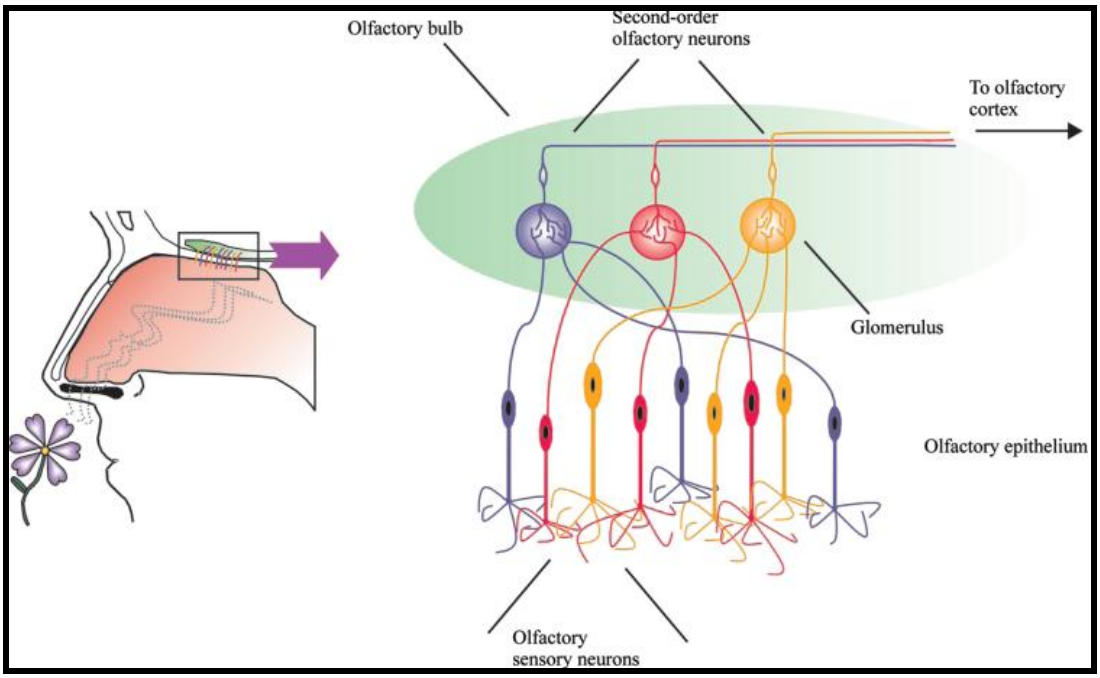

Fig.1: **Organization of the mammals olfactory system**. In the olfactory epithelium (small box on the left side), the olfactory sensory neurons (ORNs) (on the right side) expressing a given odorant receptor, project their axons to the same glomerulus. In turn, each glomerulus is connected to a second-order olfactory neuron that transmits the signal to the cortex. The olfactory neurons that express the same receptor are represented by the same color (Menini A *et al.*, 2004).

the convergence of the olfactory signals from several thousand of olfactory epithelium neurons to few glomeruli, permits to optimize the sensitivity and to recognize odorseven at low concentrations; the initial signal organization and processing take place in the olfactory bulb before information is transmitted to the olfactory cortex of the cerebrum, where odor perception takes place (Buck LB, 2000).

Mammals Olfactory Receptors

An important step forward in understanding olfactory information processing was the identification in the rat of a large multigene family of Olfactory Receptors by Linda Buck and Richard Axel (Buck L and Axel R, 1991) recognised by the 2004 Nobel Prize. This finding was later extended to all vertebrates studied. These Olfactory Receptors belong to the rhodopsin class of the G-protein coupled receptors (GPCRs) family, a group of transmembrane proteins that exhibit seven membrane-spanning regions, and an extracellular N terminus (Fig.2). GPCR gene families are the largest ones in the eukaryotic genome, comprising proteins involved in many important functions such as vision, olfactory identification, chemosensory pathway and the hormonal system (Brody T and Cravchik A, 2000; Hill *et al.*, 2002). In mammals, OR proteins are exposed to odors on the endings of ORNs dendrites in the olfactory epithelium in the nose and stimulate, upon olfactory molecules binding, the transformation of a chemical signal into an electrical response. The size of the OR gene family in mammals is enormous, ranging from about 400 genes in humans to over 1.200 genes in rodents (Mombaerts P, 2004; Nei M *et al.*, 2008). Such as GPCRs, Olfactory Receptors show a common seven-transmembrane domain architecture, in which seven transmembrane α-helices are joined to three extracellular and three intracellular loops. The ORs are highly divergent in sequence within their transmembrane domains that are

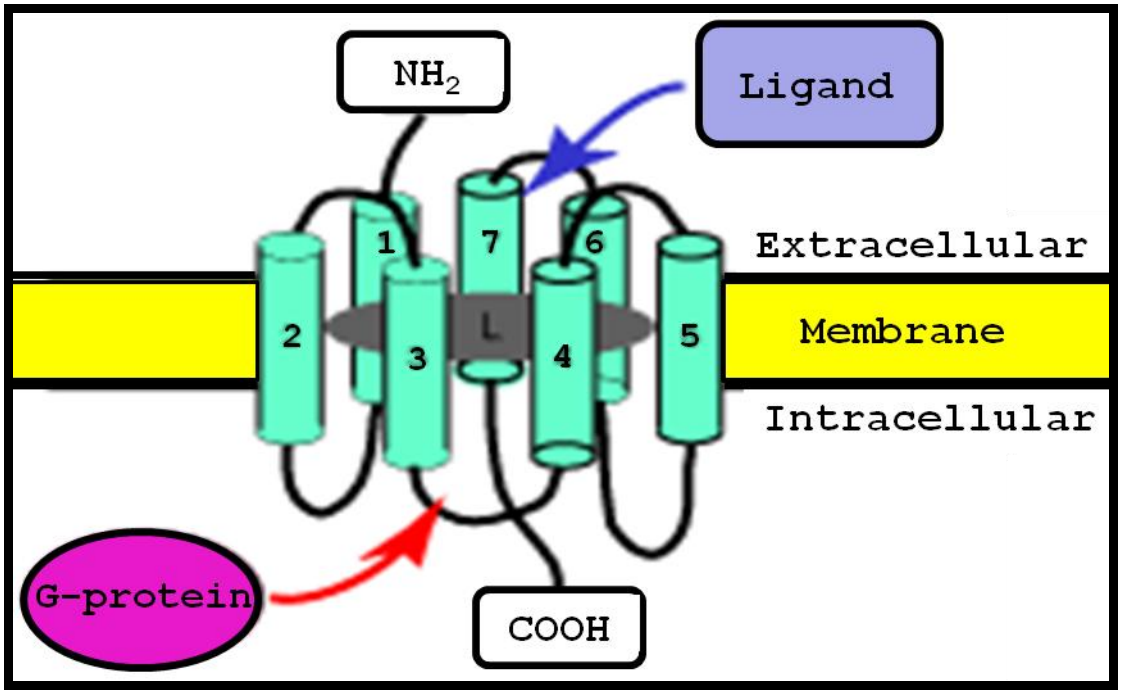

Fig.2: **Schematic structure of a typical GPCR**. GPCRs have an extracellular N-terminus (NH_2), seven transmembrane domains (light blue cylinders), three extracellular loops (black strings), three intracellular loops (black strings) and a C-terminus (COOH) (Chien EY *et al.*, 2010).

considered the sites of ligand binding. Recently, experimental and computational studies had provided evidence that the odour binds to a pocket surrounded by transmembrane domains 3, 5 and 6 of the OR (Katada S *et al.*, 2005; Saito H *et al.*, 2009), via loose interactions such as hydrophobic and van der Waals connections. The high degree of sequence diversity suggest that this gene family has evolved, and is still evolving, to detect a wide range of odors present in the animal's natural environment. Since their discovery, a body of works based on their numbers, sequence diversity and expression profiles in the ORNs has supported the idea that these receptors could play a key role in binding chemical compounds. Nevertheless, detecting the ligands for these receptors, process known as de-orphaning, has been very difficult. Initially, this de-orphaning found a major impediment in the difficulty to express the OR in the cell membrane of heterologous cells (Touhara K, 2007). An alternative strategy was an approach "in vivo", based on the study of virally transduced or endogenous ORs in native ORNs. (Zhao H *et al.*, 1998; Malnic B *et al.*, 1999; Touhara K *et al.*, 1999). These studies led Zhao and collaborators (Zhao H *et al.*, 1998) to de-orphanize the first OR. However, this strategy did not allow screening ORs against a large panel of odours. So, various alternative strategies to obtain surface expression in heterologous cells were used, finally allowing to functionally charactherize a large number of ORs. For example, Grosmaitre and collaborators (Grosmaitre X *et al.*, 2009) used patch-clamp on mice intact epithelial preparations and heterologous expression in Hana3A mammalian cells to assess that MOR256-3 was broadly tuned to many odors such as heptanol, octanol, hexanal, heptanal and octanal; Sanz and collaborators (Sanz G, 2005) used the VOFA technique (Volatile-Odorant Functional Assay) coupled with calcium imaging experiments in heterologous HEK293 cells to de-orphanize two human Odorant Receptors, OR52D1 and OR1G1, respectively; furthermore, electrophysiological experiments on Xenopus oocytes expressing heterologous olfactory receptors led to identify the agonists of all members of the mouse Odorant Receptor 42

(MOR42) subfamily (Abaffy T *et al.*, 2006). Taken together, these studies suggested a combinatorial code in which the identity of a given odorant was encoded by a particular subset of ORs that it activated. Changing molecular features of the ligand elicits a different subset of receptors originating the perception of a different smell (Zhao H *et al.*, 1998; Araneda RC *et al.*, 2000; Kajiya K *et al.*, 2001; Abaffy T *et al.*, 2006; Repicky SE and Luetje CW, 2009; Saito H *et al.*, 2009). As more mammalian ORs have been de-orphanized, it is appeared increasingly clear the existence of this combinatorial coding strategy in which subsets of ORs, specific for a given odorant, exist and recognize a given odor (Malnic B *et al.*, 1999). ORs that recognize several structurally different odorants are defined as "broadly tuned", or generalist, while ORs that recognize a given odorant with high specificity are known as "narrowly tuned" or specialist. Nowadays, it is believed that the first ones, based on their overlapping responses, can justify the high discriminatory power of the olfactory system, while the last ones may finally activate specialized circuits in the brain, leading to particular behaviours.

Transduction pathway of Olfactory Receptors in mammals

In mammals the olfactory signal transduction pathway starts with the activation of the G protein Gαolf, a Gαs isoform enriched in ORNs (Belluscio L *et al.*, 1998), by odorant-bound activated ORs. (Gether U and Kobilka BK, 1998). G proteins are membrane heterotrimeric GTPase formed by three subunits called α, β, γ. The β and γ subunits are covalently associated, while the α subunit, which has GTPase activity, is non-covalently associated. In absence of ligand, the receptor is linked to the inactive heterotrimeric G protein. When the olfactory molecules bind the receptor cause the phosphorylation of GDP to GTP, and the consequent separation of the Gα

subunit from β and γ subunits. The activated Gαolf in turn stimulates α adenylate cyclase III (Wong ST *et al.*, 2000), leading into a cAMP increase. This increase in intracellular cAMP in turn opens a cyclic-nucleotide-gated ion channel (CNG), allowing the entry of sodium and calcium ions into the neuron. This cation influx causes the depolarization of the ORN, which is further amplificated by an efflux of Cl^-, due a subsequent activation of a calcium-activated chloride channel, recently identified as Anoctamin2 (Stephan AB *et al.*, 2009) (Fig.3). The cAMP cascade seems to be the major pathway in transmitting the odorant signal in vertebrate olfactory neurons, although in mice at least other two pathways, able to detect a subset of odorants, have been proposed (Fülle HJ *et al.*, 1995; Juilfs DM, 1997; Meyer MR *et al.*, 2000). After the activation, olfactory neuron must return to the steady state during the desensitization process to prepare for the next odor stimulus. This phenomenon appears to be due to several Ca^{2+}-mediated negative feedback mechanisms (Reviewed in Touhara K and Vosshall LB, 2009).

Insect olfactory system

Insects represent an attractive model in which to study olfaction because they display several olfactory-driven behaviors under the control of a nervous system much simpler than that of mammals. The anatomical and physiological properties of the insect olfactory system have been studied in numerous species, as well as honeybees, moths, cockroachs (Benton R *et al.*, 2006). Despite this wealth of information, molecular analysis was limited to mammals until the discovery of ORs in the fruit fly *D. melanogaster* in 1999 (Clyne PJ *et al.*, 1999; Gao Q and Chess A, 1999; Vosshall LB *et al.*, 1999), nearly a decade after the discovery of mammalian ORs. Nevertheless, in few years, the small number of Drosophila ORs (62 versus 1.220 in mouse) and

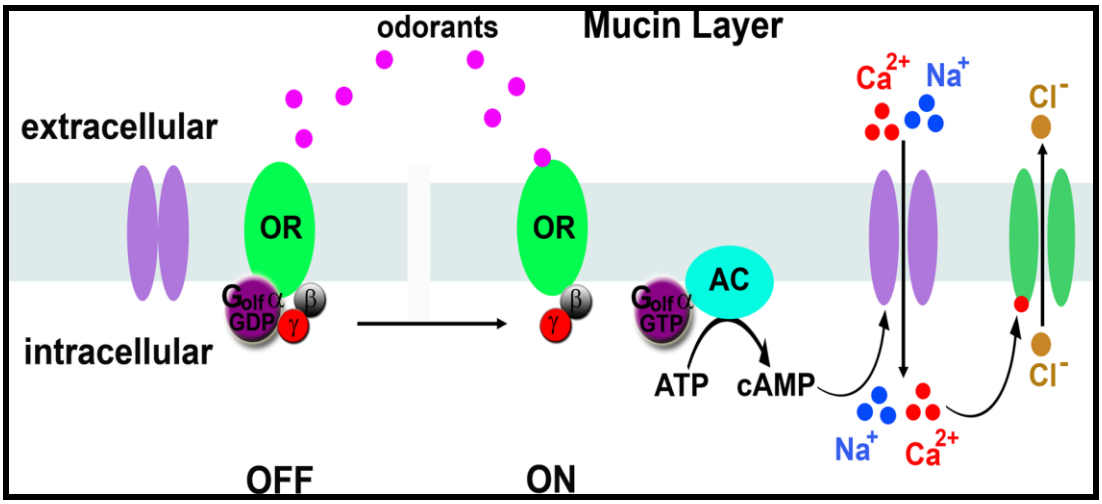

Fig.3: Olfactory signal transduction in mammals. In absence of odorant, the odorant receptor (OR) is bound to an inactive form of Gαolf (left site). Ligand (pink dots) binding to the OR causes the activation of the alpha subunit of Gαolf which, in turn, activates the adenylyl cyclase (AC) to produce cAMP from ATP. cAMP in turn binds a cyclic nucleotide-gated ion channel (CNG) that conduct sodium and calcium ions into the neuron. The calcium ions bind a calcium-activated chloride channel that allows an efflux of chloride ions, further depolarizing the neuron (Ha TS and Smith DP, 2009).

the powerful genetic tools of this organism, have permitted rapid and comprehensive descriptions of the role of ORs and their circuits in odor perception. Many common properties of insect and mammals olfactory systems have been revealed: in particular, individual ORNs express just a given type of OR, axons of ORNs expressing the same OR converge into defined glomeruli in the antennal lobe (AL), the insect equivalent of the olfactory bulb, and odorsare recognized by specific combination of ORs to create a spatial "code" of glomerular activation (Ache BW and Young JM, 2005). In this primary olfactory centers, the ORNs synapse with specific second-order neurons, known as insect projection neurones or mammalian mitral and tufted cells which, in turn, transmit information to higher brain centers, corresponding to the insect mushroom body and lateral protocerebrum or mammalian olfactory cortex.

Drosophila melanogaster peripheral olfactory system

Similarly to mammals, insects rely on multiple distinct organs for olfaction. In *D. melanogaster* adults, as well as in most insect, peripheral olfactory system is represented by two pairs of organs, the antennae and the maxillary palps (Fig.4). Both organs contain sensory hairs, named sensilla, which house the dendrites of up to four ORNs, although ORNs from the different organs project to glomeruli in different regions of the antennal lobe. Although these organs respond to overlapping sets of odors, maxillary palp lies close to the labellum that is involved in the taste sense, and seems that the olfactory input via maxillary palp enhances taste-mediated behaviors (Shiraiwa T *et al.*, 2008). Drosophila maxillary palp is a structure that protrudes from the mouth parts and it is covered by two types of sensilla, named s. basiconica and s. chaetica, of which only the s. basiconica have an olfactory function. Such as for all insects, the major "nose" of *D. melanogaster* is the antenna,

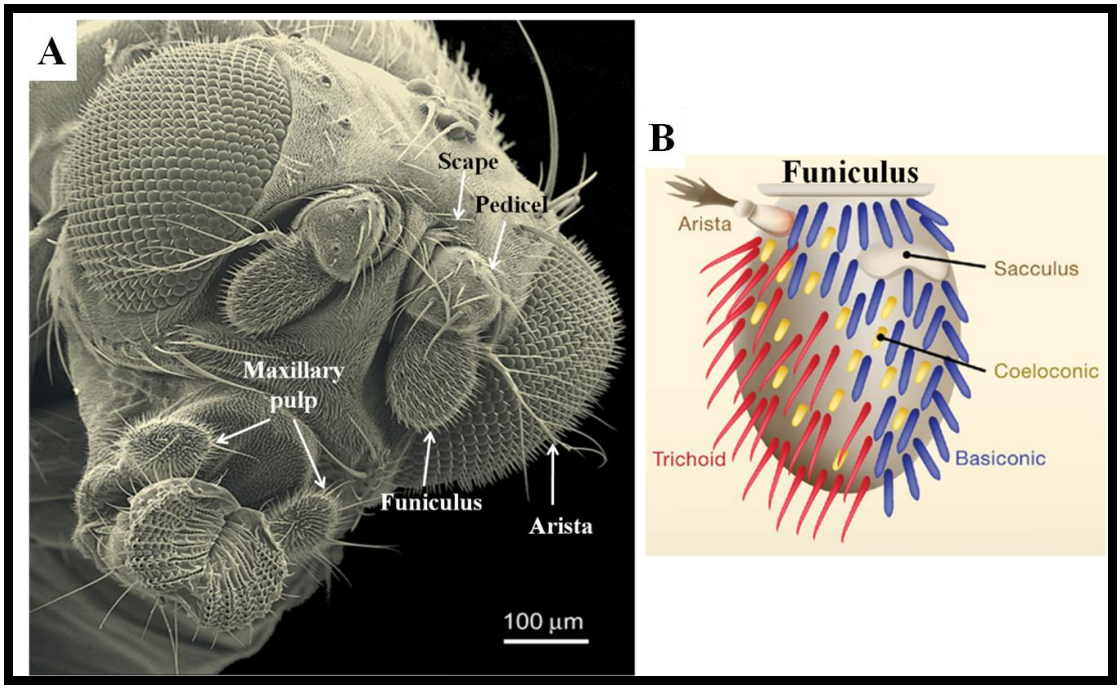

Fig.4: *Drosophila melanogaster* peripheral olfactory system. (A) Scanning electron micrograph of an adult *D. melanogaster* head. The two major olfactory organs of the fly are indicated: a pair of antennae, subdivided in scape, pedicel and funiculus, and a pair of maxillary palps close to proboscis (Laissue PP and Vosshall LB, 2008). (B) Schematic representation of the third antennal segment of an adult of *D. melanogaster*, or funiculus, that is densely covered by three different types of sensilla. Sensillar types and subtypes are distributed in a peculiar, non-homogenous pattern (Spletter ML and Luo L, 2009)..

a cuticle-covered appendage that can be subdivided in three segments called scape, pedicel and funiculus (Fig.4). To further increase the extent of anatomical diversity, the funiculus is densely covered by sensilla that fall into three morphologically distinct groups, known as sensilla trichodea, sensilla basiconica, sensilla coeloconica (rewieved in Stocker RF, 2001), briefly described below:

Sensilla Basiconica

There are about 200 sensilla basiconica which we can further distinguish in three subtypes depending on their shape and size: small (SB), thin (TB) and large (LB). Differently from s.trichodea, s. basiconica has pores arranged in rows and can house dendrites from 2 or 4 ORNs (Fig.5A-B).

Sensilla Coeloconica

There are about 60 sensilla coeloconica on the funiculus unevenly distributed; they can be divided into two subtypes depending on the number of innervating neurons: C-2 (two neurons), C-3 (three neurons). As for s.trichodea, also s.coeloconica show a sexual dimorphism because in males the subtype C-3 is much more common of the subtype C-2 (33 vs. 24), while in females the relationship is the opposite (22 vs. 32) (Fig. 5 C-D).

Sensilla trichodea

Sensilla trichodea are present in different number in male and in female, being about 166 in males, and about 144 in females. These sensilla are distributed diagonally on both sides of the funiculus showing an thick cuticular apparatus and pores that are not uniformly distributed on the sensilla wall. The sensilla trichodea can be divided into three subtypes depending on the number of the ORNs: T-1 (one neuron), T-2 (two neurons), T-3 (three neurons) (Fig. 5D-E).

Each sensillum can house from 1 to four ORNs, whose dendrites are dipped into the “sensillum lymph”, a fluid consisting of potassium and proteins. The sensilla, on their surface, present microscopic pores that lead into a system of tubules that ramify the sensilla walls. These tubules provide the access route of odorant molecules from environment into sensilla lumen where the sensory

endings of ORNs dendrites are located (Steinbrecht RA, 1996). Basiconic sensilla are located on both the antenna and maxillary palp, while trichoid and coeloconic sensilla are found only on the antenna and assolve distinct sensory functions. Basiconic ORNs respond to general odors, while trichoid neurons respond to pheromones (Clyne P *et al.*, 1997; Hallem and Carlson, 2006). This functional division among sensilla types seems to be philogenetically conserved, as other insects detect pheromones with trichoid sensilla (de Bruyne M and Baker TC, 2008). Further, each sensilla type can be subdivided in several classes based on the numbers and identities of the ORNs contained. To study response of single ORNs to odors, in Drosophila as well as in other insects, an extracellular recording technique or single-unit electrophysiology has been extensively used. These studies allowed establish that different ORNs respond to different odors, differing also in properties of the response, as well as dynamics and signaling mode (excitatory or inhibitory response) (rewieved in Hallem EA *et al.*, 2006). In *D. melanogaster*, the antenna contains 18 different functional classes of ORNs, which are found within eight types of basiconic sensilla, designed ab1 through ab8 (de Bruyne M *et al.*, 2001). The ab1 sensillum contains four ORNs, while the other ones each contain two ORNs. As in mammals, in these ORNs, the olfactory input and the subsequent transformation of a chemical signal into an electrical message takes place. In Drosophila, on the funiculus and on the maxillary palp, there are about 1200 and 120 ORNs, respectively; the ORNs are bipolar neurons that, from as well as in mammals their apical part, project the dendrites innervating the sensilla, while on the other side, send their axons to one of about 50 glomeruli located in the antennal lobe (AL). Each glomerulus, then send the olfactory information to the higher centers of the brain. This organization is very similar to that seen in mammals, where the signal is transmitted from olfactory epithelium neurons to the glomeruli in the olfactory bulb and from there to the cortex in the brain.

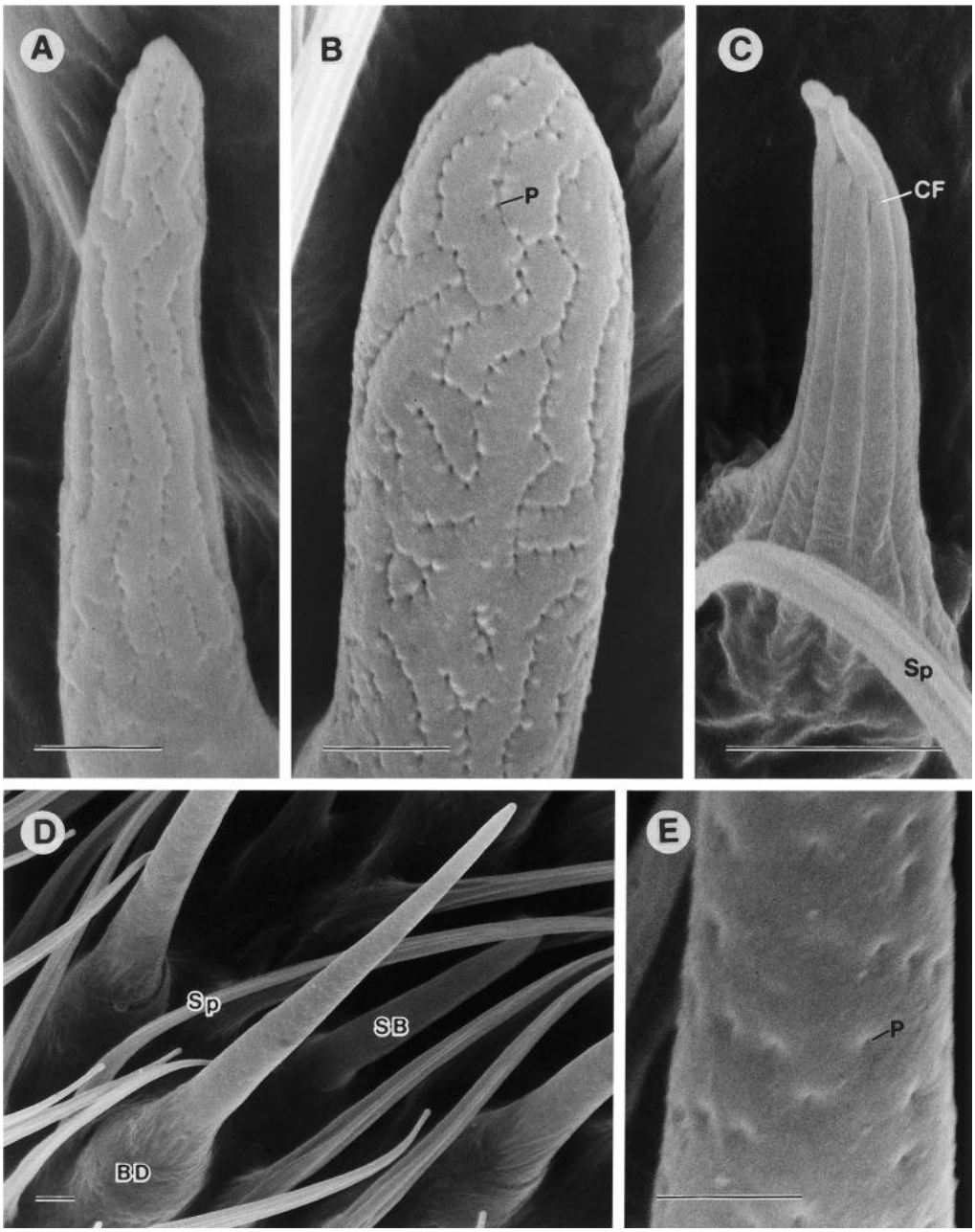

Fig.5: Morphological characteristics of the *Drosophila melanogaster* antennal olfactory sensilla. Scanning electron micrographs of the sensilla covering the funiculus of *D. melanogaster*. **(A)** Thin s. basiconicum, **(B)** large s. basiconicum, **(C)** s. coeloconicum **(D, E)** s. trichodeum. S. basiconica and s. trichodea show different structure and arrangement of wall pores -**P**-, while in the s. coeloconicum are present cuticular fingers -**CF**-. **BD**, basal drum; **SB**, small s. basiconicum; **Sp**, spinule (Shanbhag *SR et al.*, 1999).

Drosophila melanogaster Odorant Receptors

Odorant Receptors had been sought in insects for many years with a wide variety of genetic, biochemical and molecular approaches. Finally, in 1999, three groups identified a large gene family encoding candidate ORs in *D. melanogaster* (Clyne PJ *et al.*, 1999; Gao Q and Chess A, 1999; Vosshall LB *et al.*, 1999) by using a novel computer search algorithm. The *D. melanogaster* OR gene family, containing 62 members, encodes a novel family of seven-transmembrane-domain proteins selectively expressed in subsets of olfactory neurons in the antennae and maxillary palps. Subsequent to the discovery of the *D. melanogaster* ORs, genomic analysis has led to identification of 79 ORs in the malaria vector mosquito *A. gambiae* (Hill CA *et al.*, 2002), 170 ORs in the honeybee *Apis mellifera* (Robertson HM and Wanner KW, 2006), 131 in the yellow fever and dengue virus vector *Aedes aegypti* (Kent LB *et al.*, 2008), 341 in the beetle *Tribolium castaneum* (Engsontia P *et al.*, 2008), 66 in the silk moth *Bombyx mori* (Wanner KW *et al.*, 2007; Xia Q *et al.*, 2008), and, most recently, 301 in *Nasonia vitripennis* (Robertson HM *et al.*, 2010) and 180 in *C. pipiens quienquiefasciatus* (Arensburger P *et al.*, 2010). All these studies revealed that the number of ORs differs from insect to insect, suggesting that socio-sexual behavior and lifestyle may have positively influenced these gene families during the insect evolution (rewieved in Touhara K and Vosshall LB *et al.*, 2009). In *D. melanogaster*, the OR genes are widely distributed throughout the genome, and some members exist in small tandem arrays (Robertson HM *et al.*, 2003). The presence of these small clusters could suggest a mechanism of gene duplication that would determine the mechanism of expansion of this family (Ramdya P and Benton R, 2010). There are indeed some tandem arrays consisting of two or three genes that often, as is the case for OR22a/b, OR33a-c, OR59b/c, OR65a-c, OR85b/d and OR94a/b, share a higher degree of sequence similarity with each other than with the rest of the other OR genes. To obtain information on the molecular evolution of OR

genes on a timescale of 2-50 million of years (Myr), McBride CS (2007) carried out a phylogenetic analysis on the OR gene families of 11 Drosophila species. This study suggested that the insect OR genes seem to be more stable than the mammalian OR genes with fewer pseudogenes, relative constancy of overall gene number, but considerable gene duplication and loss. A further comparison of the Drosophila ORs with the three available mosquito ORs, further revealed the difficulty to identify orthologs pairs on this about 250 Myr timescale, with significative gene loss and expansion (Hill CA *et al.*, 2002; Bohbot J *et al.*, 2007; Kent LB, 2008; Arensburger P *et al.*, 2010). This evolutionary dynamics of insect ORs is different from what has happened in mammals, in which it is clear that ORs have evolved in part through both expansion and pseudogenization. (Glusman G, 2001; Robertson HM *et al.*, 2003; Touhara K and Vosshall LB, 2009). In insects, the trend towards gene subfamily expansion or loss seems to suggest a more complicated or simplified chemical ecology than was anticipated.

In general, insect ORs are highly different in sequence both within and between species (for example, the *D.melanogaster* ORs share less than 20% amino acid identity) and do not show any primary sequence similarity to either mammalian ORs or any other known GPCR. Although originally thought to be highly divergent G-protein-coupled receptors (GPCRs), which is the class of proteins that mammalian ORs belong to (Keller A and Vosshall LB, 2008; Thomas JH and Robertson HM, 2008), two major lines of evidence now suggest that this is not the case. First of all, insect ORs possess a transmembrane topology that is the reverse of typical GPCRs, with the N-terminal located intracellularly and the C-terminus located extracellularly (Benton R *et al.*, 2006; Wistrand M *et al.*, 2006; Lundin C *et al.*, 2007; Smart R *et al.*, 2008). Second, although insect olfactory transduction mechanisms are still controversial, in contrast to mammals, the evidence for the involment of G protein-mediated second messangers remains equivocal. A further key difference in OR biology reflects the existence of a highly conserved member

of this family, called OR83b after its name in *D.melanogaster*, that is co-expressed with other conventional ORs in most, if not all, olfactory neurons (Krieger J, 2003; Larsson MC, 2004). OR83b does not appear to be directly involved in the recognition of odor molecules and its role today is not entirely clear yet. Many studies suggest that OR83b functions as a chaperone; it forms a heteromeric complex with conventional ORs and helps the receptor localization in the ORN membrane where persists in this complex, suggesting that could act as a co-receptor in olfactory signaling (Larsson MC *et al.*, 2004; Nakagawa T *et al.*, 2005; Neuhaus EM *et al.*, 2005; Benton R *et al.*, 2006). In the last ten years many works have been carried out to de-orphan insect ORs through use of strategies similar to those used for mammalian ORs. Initially, Wetzel and collaborators (Wetzel CH *et al.*, 2001) identified ligands for the Drosophila OR43a by measuring the "in vivo" response pattern in a homologous system (the antenna), or by patch-clamp electrophysiological assays in Xenopus oocytes (Störtkuhl KF and Kettler R, 2001). Later, several other heterologous systems have been used, including human embryonic kidney 293 (HEK293) cells (Sanz G *et al.*, 2005), *Cercopithecus aethiops* kidney (COS-7) cells (Levasseur G *et al.*, 2003), *Spodoptera frugiperda* 9 (Sf9) cells (Matarazzo V *et al.*, 2005; Kiely A *et al.*, 2007), performing assays based on the detection of intracellular Ca^{2+} levels. To date the most powerful tool to study the profile of expression of a single OR is the "in vivo" electrophysiological recording from a particular basiconic sensillum named ab3 into the antenna of the Delta halo Drosophila strain (Dobritsa AA *et al.*, 2003). In this sensillum is located the ab3A neuron that normally expresses the two highly similar OR22a and OR22b. In Delta halo mutant, due to a deletion of the genomic portion containing these receptors, the ab3A neuron is unresponsive to any tested odors, and, for this reason, is referred as "empty neuron". This mutant Delta halo has been successfully used to characterize 24 Drosophila antennal ORs (out of a total of 62 ORs), by genetically introducing individual ORs into the mutant neuron and recording the electrophysiological

response against a panel of 110 odorants (Dobritsa AA *et al.*, 2003; Hallem EA *et al.*, 2004; Hallem EA and Carlson JR, 2006). All these works reveal that most, if not all, antennal ORNs express only one functional OR and stress the existence of combinatorial receptor codes for odorants, similar to those in mammals. A comparison of ligand specificities of a given OR revealed that many ORs respond to common ligands, reason for which one odour typically can activate multiple receptors (Hallem EA *et al.*, 2006). Nevertheless, rather than to be narrowly and broadly tuned, the insect ORs presented a continuum of tuning breadths. Expression of individual OR in the "empty neuron" allowed also to determine that the OR is the primary determinant of the odor response spectrum, spontaneous firing rate, signaling mode, response dynamics of the ORN in which it is expressed. Thus, each OR determines multiple aspects of odor coding in Drosophila (Hallem EA *et al.*, 2006).

Transduction pathway of Olfactory Receptors in insects

To date is not clear yet how the insect Olfactory Receptors transduce the olfactory information, neither if there is a single transduction pathway or more than one. Several groups have used heterologous expression of insect ORs to provide some answers to this problem. Initial works suggested that the co-expression of a given insect OR with OR83b in heterologous systems was sufficient to obtain an increase in intracellular Ca^{2+} (rewieved in Benton R, 2008; Kaupp UB, 2010). This activity could suggest that these receptors activate endogenous transduction pathways. Anyway, Sato and colleagues and Smart and collaborators (Sato K *et al.*, 2008; Smart R *et al.*, 2008) showed that this Ca^{2+} influx persisted in the presence of general inhibitors of G protein signaling. Furthermore, Sato reported that the latency of current responses was much faster than that of the mammalian ORs. To justify these results, Sato and colleagues hyphotized that the ORx/OR83b complex itself possessed ligand-

channel activity (Fig.6, top). Besides this rapid, ionotropic current that was independent from G proteins, Wicher and colleagues (Wicher D. *et al.*, 2008) described a later metabotropic current mediated, at least partly, by heterotrimeric G protein function. On the basis of these data, Wicher and colleagues proposed that the ORx (in this case, Or22a) coupled to G proteins, and that the OR83b co-receptor functioned as an ion channel allowing the passage of calcium ions (Fig.6, bottom). These observations led to the proposal of a two-step signaling model. Upon odorant binding, the ligand gated ORx/OR83b channel complex would produce a fast, inward current, followed by a larger–slower metabotropic cyclic nucleotide-gated current (Su CY *et al.*, 2009) (Fig.6, bottom). Despite the differences between the two recent papers discussed above, taken together, these results provide evidence that insect ORs have the ability to act as ligand-gated ion channels. Nevertheless, it is not clear what these two currents corresponds in vivo. Most the electrophysiological "in vivo" analysis reported that an increase in neuronal action potential frequency occurs in much less than a second of odor presentation, confirming the rapid odor gated-ionotropic properties of insect ORs. Nevertheless, it is possible that G protein signaling can modulate OR function, as recently suggested by reduced odor responses in a Drosophila mutant lacking a G alpha subunit (Kain P *et al.*, 2008).

Mosquito peripheral olfactory system

In mosquitoes, olfactory system plays a crucial role in many behaviors, including nectar feeding, host preference selection, searching for the right place to lay eggs. Peripheral olfactory system of mosquitoes consists of three parts: antenna, proboscis and maxillary palp, (Fig.7) all of which are densely covered by several classes of sensilla containing the dendrites of up to four ORNs. Antenna, that is the main olfactory organ, shows different

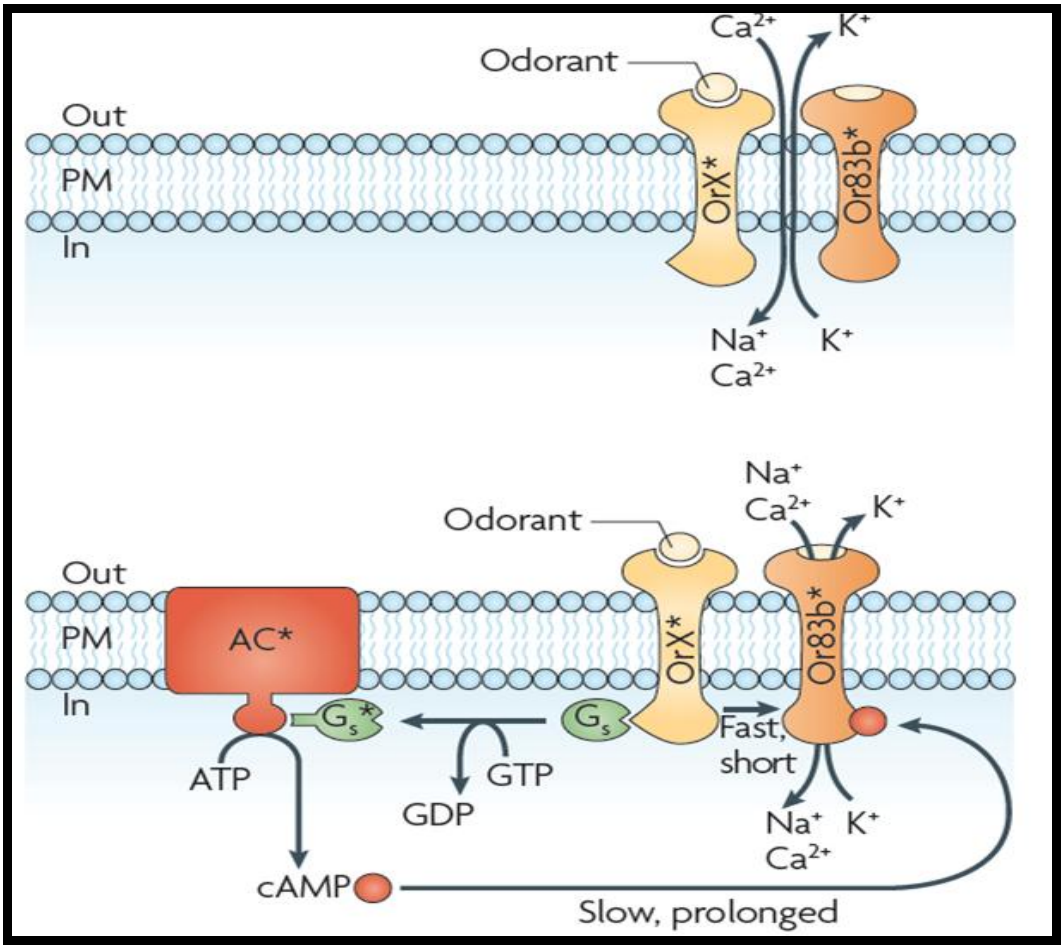

Fig.6: Insect olfactory signal transduction pathway. Scheme on the top: in the model proposed by Sato and colleagues and by Smart and colleagues the olfactory transduction pathway is independent from heterotrimeric G protein; a given Odorant receptor (ORx) form an ion channel along with OR83b that allows entry of calcium ions into the cell. Scheme at the bottom: Wicher and colleagues proposed a two-step signaling model: a fast-short pathway and a slow-prolonged pathway. The first one is activated by high concentrations of odorant and is independent from G proteins, while the second one could be activated at low concentrations of odorant and includes the involvement of heterotrimeric G protein that once phosphorylated in turn activates the OR83b ion channel. PM: Plasma membrane; AC: Adenilato cyclase (Kaupp UB, 2010).

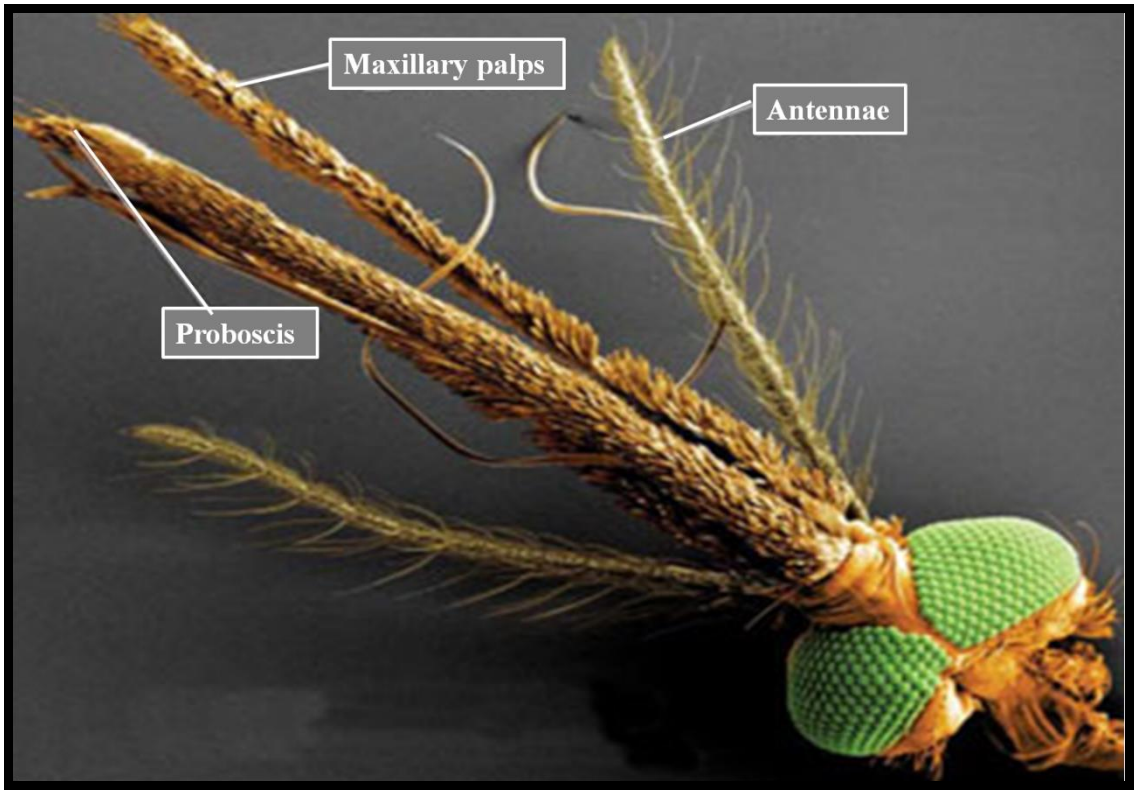

Fig.7: Mosquito peripheral olfactory system. Scanning electron micrograph of the head of a female *A. gambiae* mosquito, showing the parts of olfactory appendages: antennae, maxillary palps and proboscis (Image courtesy of Zwiebel LJ on web).

morphological characteristics in adult females and males, as well as a different length, 1.5 mm in female vs 2.2 mm in male, and a different distribution of sensilla. However, in both sexes the antenna shows the same basic organization, and is composed by three segments, named scape, pedicel, and flagellum. In turn, flagellum consists of 13 flagellar segments, and as the funiculus in *D. melanogaster*, is densely covered by sensilla (Fig.8A). Sensilla on the flagellum belong to five morphologically distinct classes: sensilla chaetica, sensilla ampullacea, sensilla coeloconica, sensilla trichodea and grooved peg sensilla. Among these, sensilla chaetica, ampullaceal and coeloconica respond to mechanical, thermal and hygro-stimuli, while grooved peg sensilla and sensilla trichoidea respond to olfactory stimuli, and represent 90% of all antennal sensilla (Fig. 8B).

Sensilla trichoidea (STr):

Sensilla trichodea are the major olfactory sensillum type found on mosquito antennae. These sensilla can be further subdivided into 5 classes morphologically distinct: LST (long sharp tipped), SST (short sharp tipped), SST-C (short blunt tipped curved), SBT-I (short blunt tipped-I), SBT-II (short blunt tipped-II). Each subtype show a different spatial location on the flagellum, with the LST sensilla that are more present and uniformly distributed from 2 to 13 flagellar segments, and the SBT-I sensilla and SBT-II that are rare and more present from 1 to 9 flagellar segments and from 1 to 8 flagellar segments, respectively (Fig. 8B).

Grooved peg sensilla (GPs):

Grooved peg sensilla are shorter than the other sensilla and are uniformly distributed on the flagellum. These sensilla can be subdivided into two types morphologically distinct: LGP sensilla (long gooved peg of about 9,5 µm in length) and SGP sensilla (long blunt tipped of about 4,9 µm in length) (Fig. 8B).

Sensilla trichoidea and Grooved peg sensilla show pores distributed on the wall that allow the entry of the olfactory molecules. These substances are

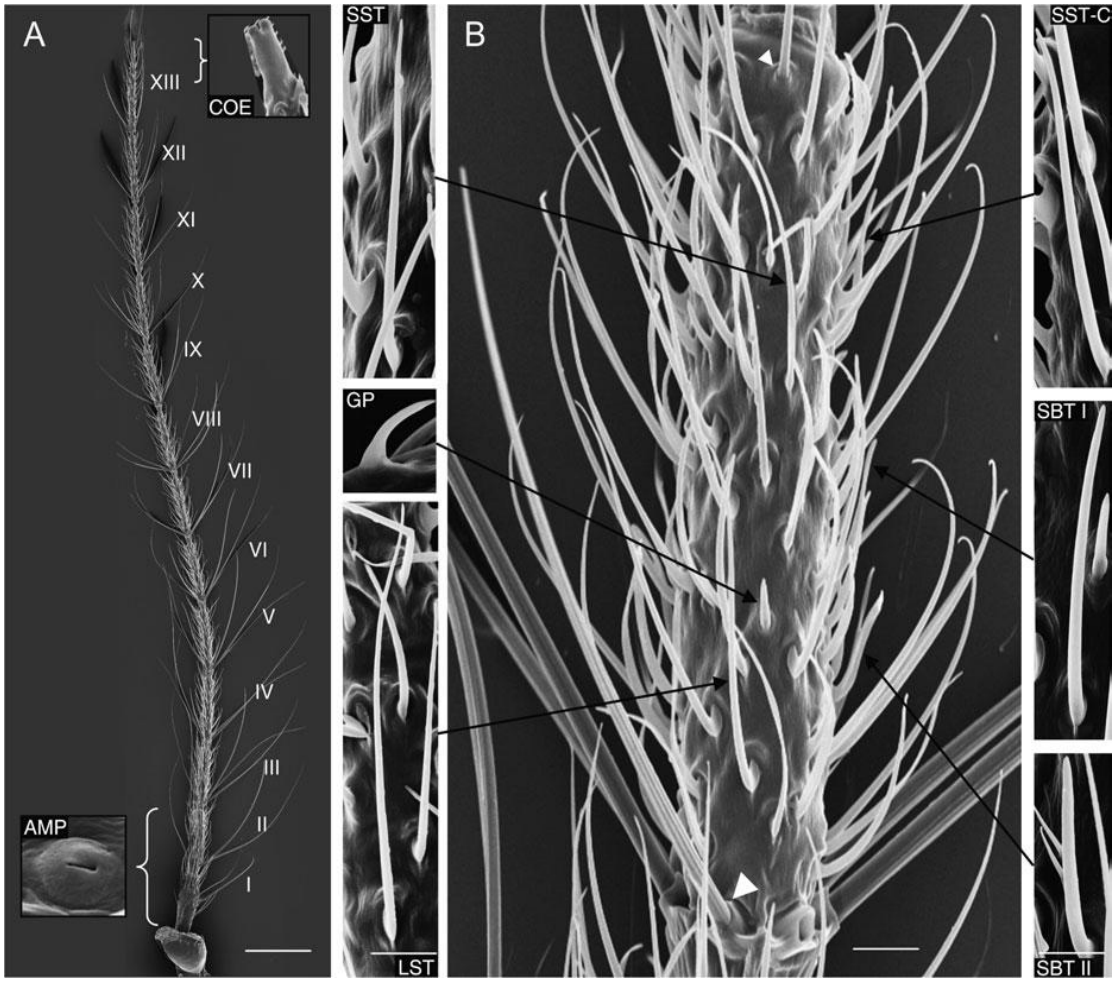

Fig.8:*Culex pipiens quinquefasciatus* antennae. (A) Scanning electron micrograph of the antennal flagellum of a *C. pipiens* adult, in which is indicated the subdivision in 13 flagellar segments (from I to XIII). AMP, non-olfactory sensillum ampullaceum. COE, non-olfactory sensilla coeloconica **(B)**, Micrograph of a single antennal flagellum displaying the 5 morphological subtypes of olfactory sensilla trichodea: SST; short sharp-tipped , SST-C; short sharp-tipped curved, SBT I; short blunt-tipped I, SBT II; short blunt-tipped II: LST; long sharp-tipped, GP; grooved pegs. Large and small white arrowheads indicate the non-olfactory long and short sensilla chaetica. (Hill SR *et al.*, 2009).

dissolved into “sensillum lymph”, where come into contact with the dendrites to the ORNs expressing the olfactory receptors. These sensilla are innervated by two –up to four ORNs, which, as in mammals and in *D. melanogaster*, have a bipolar structure: from one side extend dendrites that innervate the sensilla, and on the other side, send their axons to a glomerulus into the antennal lobe. Then, the olfactory information is transferred to the higher centers in the brain.

Mosquito Odorant Receptors

Mosquitoes are the most common vectors for malaria, dengue and yellow fever, diseases with catastrophic effects on global health. Due to their adaptability, these insects have been able to colonize all parts of the world and to resist at adverse conditions. For example, their eggs are capable to resist in a quiescent state during the winter, and to develop only when the environmental conditions become favorable. The most dangerous species are represented by *Anopheles gambiae,* the principal vector of malaria, *Aedes aegypti*, carrier of dengue and yellow fever, *Culex pipiens*, able to transmit lymphatic filariasis and encephalitis virus. Due their hazard, these mosquito species are also the most studied ones. The ability of these mosquitoes to identify host for a blood meal, or a correct site where to lay eggs is conferred by a rich repertoire of Olfactory Receptors that, during the evolution, have been increasingly specialized and seems to represent a gene family in expansion. *A. gambiae*, presents an olfactory receptors family of 79 members, that was identified on the basis of sequence similarity to the OR gene family (Holt RA *et al.*, 2002; Hill CA *et al.*, 2002). As in *D. melanogaster*, the OR proteins in *A. gambiae* are highly diverse. Differently from *D. melanogaster*, in which many OR genes are found in small genomic clusters of two or three genes, in *A. gambiae* the ORs are often found in larger clusters of up to nine

genes (Hill CA *et al.*, 2002). The first functional characterization of two *A.gambiae* ORs was obtained by Hallem and collaborators (Hallem EA *et al.*, 2004) by using the Single sensillum recording technique in the "empty neuron" of *D. melanogaster*. Precisely, the authors reported that AgOR1 responded strongly to 4-methylphenol, a known component of the human sweat (Cork and Park, 96) and AgOR2 responded to 2-methylphenol (Hallem EA *et al.*, 2004). Recently, it has been obtained significant insight into the sense of smell in *A. gambiae*, with the functional characterization of fifty AgOrs by using voltage-clamp in Xenopus oocytes (Wang G *et al.*, 2010) and the "empty neuron" system in *D. melanogaster* (Carey AF *et al.*, 2010). In particular, the results obtained by Carey and colleagues (2010) indicated that *A. gambiae*, such as *D.melanogaster*, used a combination of both narrowly tuned (specialists) and broad spectrum (generalists) ORs, and that each AgORs had a distinct odor-response profile and tuning breadth; further, certain odors activated some receptors while inhibited others, suggesting that responses to odors were regulated at the antennal level. However, differently from the *D.melanogaster* ORs that predominantly detect esters, rotting fruit signals, the *A.gambiae* ORs sense aromatic compounds, present in human volatiles. In this context, the narrowly tuned receptors are thought to transmit specialized information about high biological relevance smells (Wilson RI and Mainen ZF, 2006). Among the *A. gambie* narrowly tuned odorant receptors there were AgOR2, AgOr8, AgOR5 AgOR65. AgOR2 was narrowly tuned to indole, which was found to be 30% of the volatile headspace of human sweat (Meijerink J *et al.*, 2001). The successful identification of the *A. gambiae* OR genes on the basis of their sequence similarity to OR genes, opened the door to their identification from other insect species. So, Bohbot and collaborators (Bohbot J *et al.*, 2007) reported the characterization of the OR genes in *A. aegypti*. *A. aegypti* shows a phylogenetic distance of about 150 million years with *A. gambiae*, and even if they share similar behaviors, such as the human host preference, they show changes in morphology, mating behavior and

ovipositon preferences. In the genome of *A. aegypti* there are 131 putative ORs, 52 and 69 receptors more than *A.gambiae* and *D.melanogaster*, respectively. As is the case for other insects, AaOR proteins show a high level of divergence (Clyne PJ *et al.*, 1999, Robertson HM *et al.*, 2003), with most of them sharing less than 20% identity with each other and with those of *A.gambiae* and *D. melanogaster*. Genomic organization of the *A. aegypti* OR genes is similar to that previously reported for *A. gambiae*, whith many olfactory receptors that are organized in clusters of three to up to eleven genes (Bohbot J *et al.*, 2007). Based on a phylogenetic analysis, Bohbot reported the presence of 18 Aedes/Anopheles orthologous subgroups including the highly conserved OR7 gene that corresponds to OR83b of *D.melanogaster*. Very recently, Pelletier and colleagues (Pelletier J *et al.*, 2010), by an bioinformatics approach identified 158 putative ORs in the genome of the Southern house mosquito *C. pipiens quinquefasciatus*. Again, a phylogenetic analysis using OR proteins from these three mosquito species, revealed several species-specific lineages and subgroups of conserved ORs (Pelletier J *et al.*, 2010). Always last year, Pelletier and collaborators (Pelletier J *et al.*, 2010) and Bohbot and collaborators (Bohbot JD *et al.*, 2011) reported the functional characterization of a highly conserved subset of ORs among the three above reported mosquito species, by using voltage-clamp in Xenopus oocytes and Ca^{2+} fluorometry in heterologous cells. To answer to the question if protein homology correlated with odorant activation, Bohbot and collaborators carried out a comparative analysis between *A.gambiae* and *A. aegypti* OR2/OR10 and found that these ORs share a similar narrow response to indole. On the basis of their results, the authors deduced that this high sensitivity to indole could represent and ancient ecological adaption preserved because in some way important for the mosquito life cycle. In the same year, Pelletier and collaborators showed that also the OR2 ortholog in *C. pipiens* was strongly activated by indole, an oviposition attractant for this mosquito.

Introduction to experimental work

As previously said, in the last few years, increasing attention has been given to the functional characterization of mosquito Odorant Receptors with the aim to develop novel control strategies. Nowadays, ORs have been identified in three different mosquito species, such as *Anopheles gambiae*, *Aedes aegyti* and *Culex pipiens* which transmits pathogens causing severe human diseases, such as malaria, dengue, yellow fever and west Nile encephalitis. However, another mosquito specie, *A.albopicus*, has been recently (Reiter P *et al.*, 2006) reported as epidemic vector of dengue and chikungunya arboviruses in most of the Islands in the Indian Ocean, where the mosquito *A. aegypti*, usually implicated in such outbreaks, is virtually absent. *A. albopictus* is also known as tiger mosquito for the presence of white stripes on its legs and body. Originally, tiger mosquito was present only in the tropical forests of South-East Asia where layed eggs mainly in hollow trees containing small collections of water. Anyway, the proximity of urban areas to wooded areas, where this insect was endemic, has allowed the tiger mosquito to colonize anthropogenic areas, principally for two reason: the presence of tanks filled with water, useful as favorable breeding conditions, and the presence of human host, an easy meal of blood, necessary to bring the full development of eggs. Although this mosquito is not the major vector for the most devastating diseases, the potentiality of its vectorial capacity is very dangerous and may constitute the foundation for a public health alert. As a result of its strong ecological plasticity, *A. albopictus* has spread throughout the world and all over Italy with a high abundance in man-made environments. Since 2007, in Emilia-Romagna region (Italy), human diseases due to mosquito-borne viruses have been reported, such as the Chikungunya virus in 2007 and the West Nile virus in 2008. Recently, Calzolari and collaborators (Calzolari M *et al.*, 2010) and Dutto and Bertero (Dutto M and Bertero M, 2010) have connected the presence of this exotic mosquito in Italy to health risk, supporting the ability

of *A. albopictus* to serve as a bridge vector, capable of mediating the spillover of a virus from rural-cycle to urban-cycle. Despite its uninterrupted spread and its aggressivity, to date nothing is known at molecular level for this mosquito, and measures for the control of larvae and adults are obtained by use of repellents and larvicidals, and removing of breeding sites around house. Comprehensive behavioral studies have indicated that the most crucial cues regulating the main activities of mosquitoes, such as host-seeking, research of oviposition sites and feeding, are due to olfactory volatiles emitted from host or plants (Bowen MF, 1991; Takken W and Knols BG, 1999). These volatiles are subsequently analyzed by the mosquitoes' relatively simple, but highly sensitive, olfactory system. Therefore, it is expected that an improved understanding of the olfactory system of mosquitoes may help in developing control methods that interfere with its behavior. Recently, in the laboratory where I carried out my PhD thesis, a study aimed to characterize the *A. albopictus* Odorant Receptors has been undertaken. As a first step in this process, we think that the cloning and characterization of components of the Olfactory Receptors from *Aedes albopictus* will facilitate molecular and biochemical study of this mosquito's olfactory processes. Ultimately, these efforts may lead to identify novel biologically active compounds that could be used as chemo-attractants or chemo-repellents and then reduce the vectorial capacity of this insect. During my work I focused my attention on the identification, cloning and functional characterization of one of the most conserved mosquito ORs characterized to date, called OR2. This receptor has been cloned and functionally characterized in *A.gambiae* and very recently, in *C.pipiens* (Pelletier J *et al.*, 2010) and *A.aegypty* (Bohbot JD *et al.*, 2011). A first analysis performed on 2004 by Hallem against a panel of 23 odors(Hallem EA *et al.*, 2004) showed that 2-methylphenol was the best ligand of *A. gambiae* OR2 (AgOR2). Most recently, Carey (Carey AF *et al.*, 2010), by using the "empty neuron" system, has showed that the best ligand of AgOR2 was the indole. Furthermore, in the same year other two works

reported that *C. pipiens* OR2 (CpOR2) and *A. aegypti* OR2 (AaOR2) were narrowly tuned to indole (Pelletier J *et al.*, 2010; Bohbot JD *et al.*, 2011). In order to characterize the OR2 ortholog in *A. albopictus*, I used molecular and electrophysiological approaches, which allowed me to identify attractant and repellent molecules for this OR.

Results

To date there are no molecular information neither genomic or EST databases on the tiger mosquito *A. albopictus*. In order to identify genes expressed in the olfactory system of this species, first of all, I carried out an informatics-based conservation analysis of the most conserved Odorant Receptors belonging to the *A. gambiae*, *A. aegypti* and *C. pipiens* mosquito species. When I performed this analysis, Odorant Receptors had been identified only in *A. gambiae* (Fox AN *et al.*, 2001) and in *A aegypti* (Bohbot JD. *et al.*, 2007). To identify hypothetical orthologs in other species, based on the phylogenetic analysis performed by Bohbot and collaborators, I used individual members of his 18 *Aedes*/*Anopheles* orthologs subgroups as probes against mosquito specific transcribed sequences, by using the http://www.vectorbase.org/ database. This analysis suggested me that some olfactory receptors, such OR2, OR10, OR8 and OR49, shared a very high percentage of aminoacid identity (Tab.1), among these three mosquito species, as recently confirmed by Julien Pelletier (Pelletier J *et al.*, 2010). Since my starting hypothesis was that the Olfactory Receptors showing a high percentage of identity could be involved in important functions and play a key role in the mosquito life, among the ORs sharing the highest level of identity in these mosquito species, I choose to focus my work on the identification and functional characterization of the putative ortholog of the Olfactory Receptor 2 (OR2) in *A.albopictus.*

NAME	*SPECIES*	ID.GENE	AVERAGE PERCENTAGE OF IDENTITY
	A.gambiae	AGAP009519	
OR2	*A.aegypti*	AAEL005999	75%
	C.pipiens	CPIJ014392	
	A.gambiae	AGAP009520	
OR10	*A.aegypti*	AAEL006003	70%
	C.pipiens	CPIJ2479	
	A.gambiae	AGAP1912	
OR8	*A.aegypti*	AAEL012254	70%
	C.pipiens	CPIJO13944	
	A.gambiae	AGAP002558	
OR49	*A.aegypti*	AAEL005767	49%
	C.pipiens	CPIJ009579	
	A.gambiae	AGAP009640	
OR1	*A.aegypti*	AAEL016970	29%
	C.pipiens	CPIJ000986	

Tab.1: Comparative analysis of putative OR orthologs in three mosquito species. Putative Olfactory Receptors of *Anopheles gambiae*, *Aedes aegypti* and *Culex pipiens*, that share a high percentage of identity to each other. To calculate the percentage of amino-acidic identity, the ClustalW program was used (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Cloning of *Aedes albopictus* OR2

As revealed by my comparative analysis, showed in tab.1, the OR2 protein displays about 75% of identity among these three different mosquito species. In order to clone the corresponding transcript in *A.albopictus*, I used RT-PCR assays on total RNA with degenerated primers. First of all, I designed these primers on the best conserved regions of the aligned protein sequences of the OR2 orthologs, reported in Fig. 9, taking into account the mosquito codon bias (Isoe J and Hagedorn HH, 2007). The sequences of these primers were:

Fw1deg:TGGYTNTTYTGGWSNTAYYT

Fw2deg:GGNTAYTTACNGTNYTNTAYTT

Rw1deg:TGRAACATYTCNARNGTCAT

Rw2deg:CATRAADATRTANSWNCCDATCAT

Initially, in my experiments, I used total RNA extracted from about 30 manually dissected adult heads. At least 1µg of this RNA was retro-transcribed with an anchor primer-dT (the anchor is a sequence of 20 bases added to an oligo-dT made of 30T) by using the Reverse Transcriptase (Fermentas) in a final volume of 20µL. Then, 1µL of this synthetized cDNA was used as template in PCR reactions with degenerated primers. I carried out numerous attempts, changing several experimental conditions, such as the amount of template, magnesium and primer concentrations and the cycle conditions, but unfortunately, I obtained only faint, unspecific products of amplification. Based on the idea that this problem could be determined by the low representation of the OR2 transcript within the total RNA, I decided to use a different source of RNA. To this aim, I prepared enriched poly(A)$^+$ RNA from about twenty manually dissected adult antennae, and from about ten manually dissected heads of larvae. I decided to use also larval heads because in literature it has been reported that in *A.aegipty* OR2 and *A.gambiae* OR2 were expressed in larvae such as in antennae of adults (Bohbot JD *et al.*, 2007; Xia Y *et al.*, 2008). These RNA samples were obtained by using the

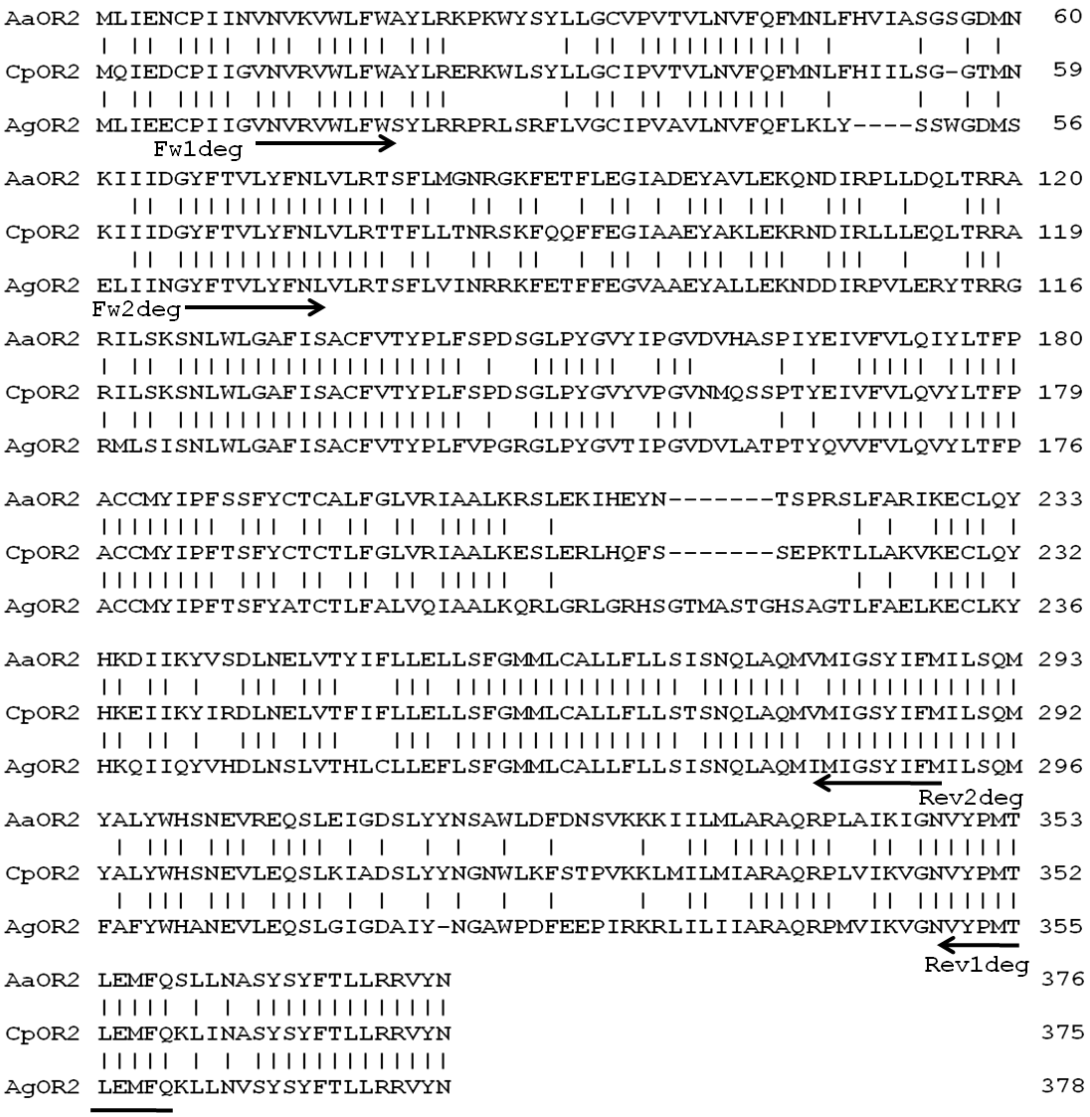

Fig.9: Alignment of the mosquito OR2 proteins. The aminoacidic sequences of *A. aegypti* OR2 (AaOR2), *C. pipiens* OR2 (CpOR2) and *A. gambiae* OR2 (AgOR2) were aligned by using the multi align program at http://www.ebi.ac.uk/Tools/msa/clustalw2/. Black arrows show the best conserved regions on which the degenerate primers were designed.

QuickPrep Micro mRNA Purification Kit (GE Healthcare). This kit allows to recover enriched poly(A)$^+$RNA, with at least 50% or more of the extracted RNA being poyadenilated; moreover, since the extraction starts directly with small quantities of tissues, it is bypassed the intermediate purification of total RNA. These RNA samples were retro-transcribed with the anchor primer-dT in a final volume of 20 µL. 1µL of each synthetized cDNA was used as template in PCR reactions with the following combinations of degenerate primers: Fw1deg/Rw1deg; Fw1deg/Rw2deg; Fw2deg/Rw1deg; Fw2deg/Rw2deg. Again, I carried out several attempts in order to obtain amplicons, changing the annealing temperature. In fact, the degenerate primers lack a specific annealing temperature, being a mixture of several different primers, each having different annealing temperatures. Finally, I obtained the better results by using the pair of Fw2deg/Rw2deg primers and the following cycle conditions: 98°C denaturation, 56°C annealing and 72°C extension for a maximum of 35 cycles. In these experiments, all the amplified fragments were eluted from agarose gels, cloned in the pGemT-easy vector and subsequently sequenced at PRIMM Biotech. The obtained sequences were virtually translated and compared with the OR2 proteins of *A.gambiae*, *A aegipty* and *C.pipens* mosquitoes. Beyond many unspecific amplification products, finally I obtained a fragment, 651 bp in length (Fig.10), encoding an hypothetical peptide fragment that showed an high level of identity with the AgOR2, AaOR2, CpOR2 proteins. I obtained this amplicon using as template RNA obtained from larvae heads, although later a similar result was obtained also with the antennal RNA of adults, indicating that also in *A. albopictus*, OR2 is expressed in larvae and adults. This fragment corresponds to the +72 to +295 region of the orthologues OR2 reported in Fig. 10, and unexpectedly presents a very high level of homology with the corresponding protein of *A.aegipty*, with 96% of identity. Based on this high degree of conservation, I was confident that this amplicon could correspond to a fragment of the *A.albopictus* OR2 transcript, that I called *A.albopictus* OR2 (AalOR2). In

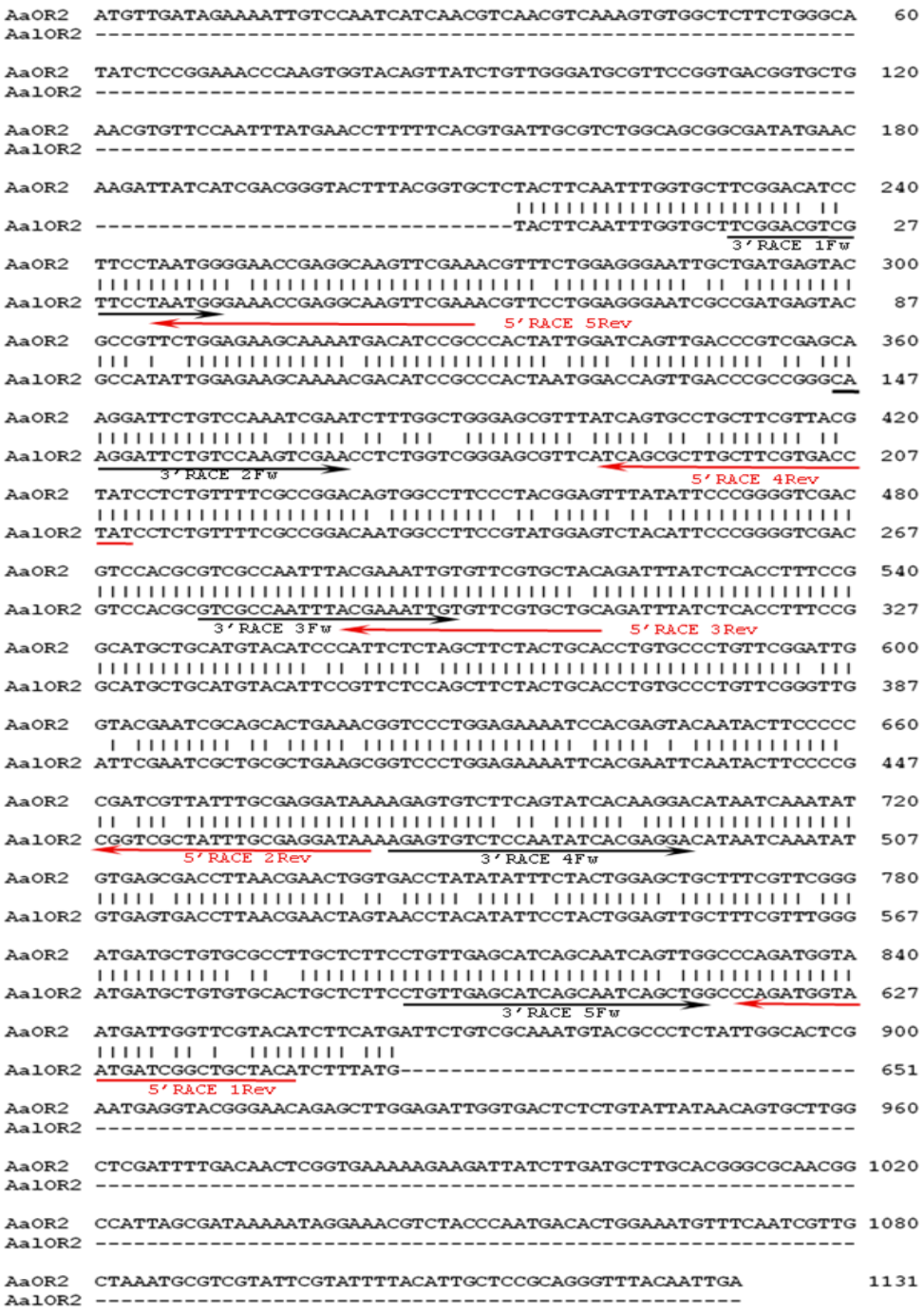

Fig.10: Comparison of the nucleotide sequence of AaOR2 and AalOR2 transcripts. The nucleotide sequence of the AalOR2 cDNA fragment, 651 bp long, (lower line) is aligned against the AaOR2 transcript (upper line). Black and red arrows indicate the specific primers designed to perform 5' and 3' RACE analyses.

order to obtain the whole coding sequence (CDS), I designed specific primers on this initial RT-PCR amplified region and used them in RACE 5' and 3´ analyses. The sequence of these primers, represented by black and red arrows in Fig.10, were:

3´RACE1Fw:TTCGGACGTCGTTCCTAATG

3´RACE2Fw:GCAAGGATTCTGTCCAAGTCGA

3´RACE3Fw:GCGTCGCCAATTTACGAAATTG

3´RACE4Fw:GAGTGTCTCCAATATCACGAGG

3´RACE5Fw:GTTGAGCATCAGCAATCAGCTG

5´RACE1Rv:AGCAGCCGATCATTACCATCTG

5´RACE2Rv:TTATCCTCGCAAATAGCGACCG

5´RACE3Rv:CTGCAGCACGAACACAATTTCG

5´RACE4Rv:CAGAGGATAGGTCACGAAGCAA

5´RACE 5Rv:TTCGAACTTGCCTCGGTTTCCCAT

To perform 3' RACE experiments, I reverse-transcribed 0,3-1µg of enriched poly(A)$^{+}$RNA extracted from manually dissected heads of 4th instar larvae with the anchor primer-dT in a volume of 20µL. Then, I used 1µL of this cDNA, in 3´ RACE PCR reactions with the following primer combinations: 3´RACE1Fw/anchor primer; 3´RACE2Fw/anchor primer; 3´RACE3Fw/anchor primer; 3´RACE4Fw/anchor primer; 3´RACE5Fw/anchor primer. For the general conditions of 3´ RACE PCR reactions for each primer combination, I used a denaturation temperature of 98°C, and an extension temperature of 72°C, for a maximum of 35 cycles. The amount of cDNA used varied from 1µL to 3 µL, and the annealing temperature was dependent for each primer pair: 56°C for 3´RACE1Fw/anchor primer; 58°C for 3´RACE2Fw/anchor primer; 60°C for 3´RACE3Fw/anchor primer; 60°C for 3´RACE4Fw/anchor primer; 58°C for 3´RACE5Fw/anchor primer. Again the amplified fragments obtained from each PCR reaction were eluted from agarose gels, cloned in pGemT-easy vector and subsequently sequenced at Primm Biotech. The obtained sequences

were translated and compared with the mosquito orthologues OR2. Finally, I was able to amplify a 440bp fragment by using the pair of 3´RACE4Fw/anchor primer (Fig.11). This fragment encodes a hyphotetical peptide ending with a stop codon in frame with the CDS of the first fragment cloned, and shares a 96% of identity with the corresponding region of AaOR2 protein. However, this fragment was lacking of a polyadenylation site. These results led me to conclude that, although the 3'end of the AalOR2 transcript was not entirely represented, lacking the 3'UTR, the CDS region was complete at the 3' end. In order to obtain the 5´ region, I carried out 5´ RACE experiments by using, as template, the same enriched poly(A)$^+$RNA of the 3´ RACE analysis. To performe 5´RACE analysis I used the 5´/3´ RACE Kit 2nd Generation (Roche). 0,3-1µg of enriched poly(A)$^+$RNA was reverse-transcribed with the specific primer 5´RACE1Rev corresponding to the coordinates +832bp to +856bp on the nucleotide sequence shown in Fig.10. At the 3´end of this first-strand cDNA, a homopolymeric A-tail was added, by using a recombinant Terminal Transferase and dATP. On this template, I performed a nested 5´RACE PCR using anchor primer-dT, that bound the A-tail added at the 5´end of resulting cDNA, in combination with each following primer: 5´RACE2Rev; 5´RACE3Rev; 5´RACE4Rev; 5´RACE5Rev. These primers were used in subsequent PCR reactions. Precisely, the outer specific primer was used in the first PCR reaction, obtaining a wide range of amplicons sharing the anchor primer-dT, which was in turn used as template for a second PCR reaction with another specific more internal primer (nested primers). I used cycle conditions in which denaturation and extension conditions were carried out in the same way of the 3'RACE experiments. The amount of cDNA used varied from 1µL to 3 µL and the annealing temperature was dependent for each primer pair: 62°C for 5´RACE2Rev/anchor primer-dT; 58°C for 5´RACE3Rev/anchor primer-dT; 58°C for 5´RACE4Rev/anchor primer-dT; 60°C for 5´RACE5Rev/anchor primer-dT. Again, the amplified fragments were treated as previously described. Unfortunately, despite

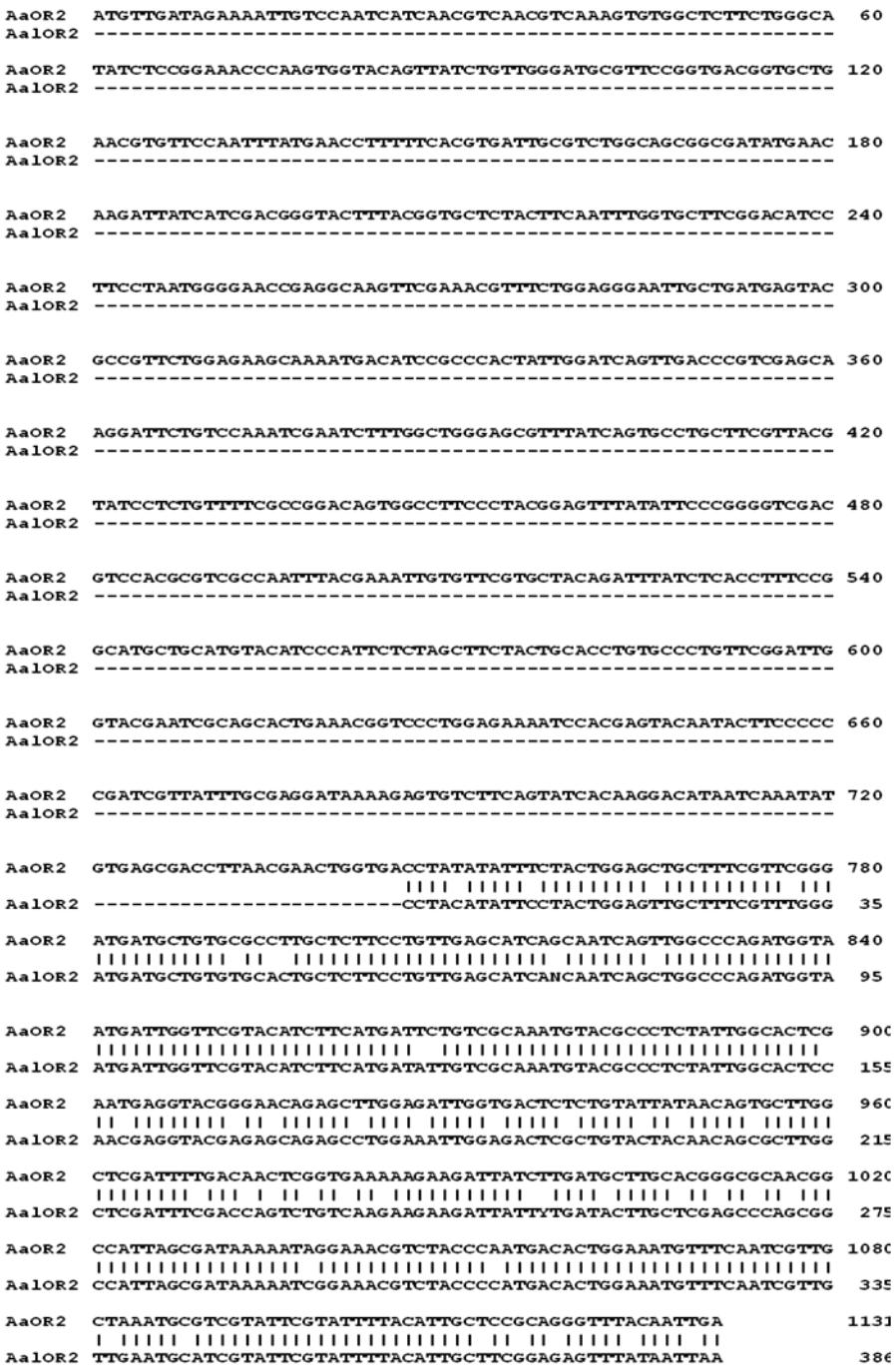

Fig.11: Comparison of the nucleotide sequences of the 3' ends of the AaOR2 and AalOR2 transcripts. The nucleotide sequence of the AalOR2 cDNA fragment, 440 long, (lower line) is aligned against the AaOR2 transcript (upper line). This fragment corresponds to the 3' end of the AalOR2 coding sequence.

numerous attempts, I failed to clone further the AalOR2 encoding region. At this point, I decided to use the following primer: AaOR2ATGTTGATAGAAAATTGTCCA. This primer was specifically designed on the 5´ region of the *A. aegipty* OR2 (AaOR2) CDS, and contained nucleotides corresponding to the first seven amino-acids of the AaOR2 protein, ATG included. I decided to design this primer since the comparative analysis of the so far sequenced regions of AalOR2 with the corresponding regions of AaOR2 showed a very high level of homology, corresponding to a 96% of identity over a 246 amino-acids long region. In this approach, I performed PCR reactions on the same cDNA used in the 3'RACE experiments with the following combinations of primers: 5´FwAaOR2/5´RACE1Rev; 5´FwAaOR2/5´RACE2Rev; 5´FwAaOR2/5´RACE3Rev; 5´FwAaOR2/5´RACE4Rev; 5´FwAaOR2/5´RACE5Rev. Again, denaturation, performed at 98°C, and extension, performed at 72°C, for a maximum of 35 cycles were the same for each primer combinations. The amount of cDNA used varied from 1µL to 3 µL, and the annealing temperature was dependent for each primer combination: 58°C for 5´FwAaOR2/5´RACE1Rev; 60°C for 5´FwAaOR2/5´RACE2Rev; 56°C for 5´FwAaOR2/5´RACE3Rev; 58°C for 5´FwAaOR2/5´RACE4Rev; 58°C for 5´FwAaOR2/5´RACE5Rev. The amplified fragments were eluted from agarose gels, cloned in pGem, sequenced and compared with the other OR2 orthologs. In this way, I was able to amplify a fragment, 680bp long, corresponding to the region +1 to +680 of the nucleotide sequence reported in Fig.12. The pair of primers and the cycling parameters were: 5´FwAaOR2/5´RACE2Rev primers at 98°C denaturation, 60°C annealing and 72°C extension for a maximum of 35 cycles. This fragment encoded a hypothetical peptide sharing 96% of identity with the corresponding region of the AaOR2 protein. At this point, I had three different cDNA clones, 651bp, 440bp and 680bp long, respectively, each containing different overlapping regions of the OR2 cDNA of *A. albopictus*. To obtain the corresponding single full length clone I carried out a further PCR reaction

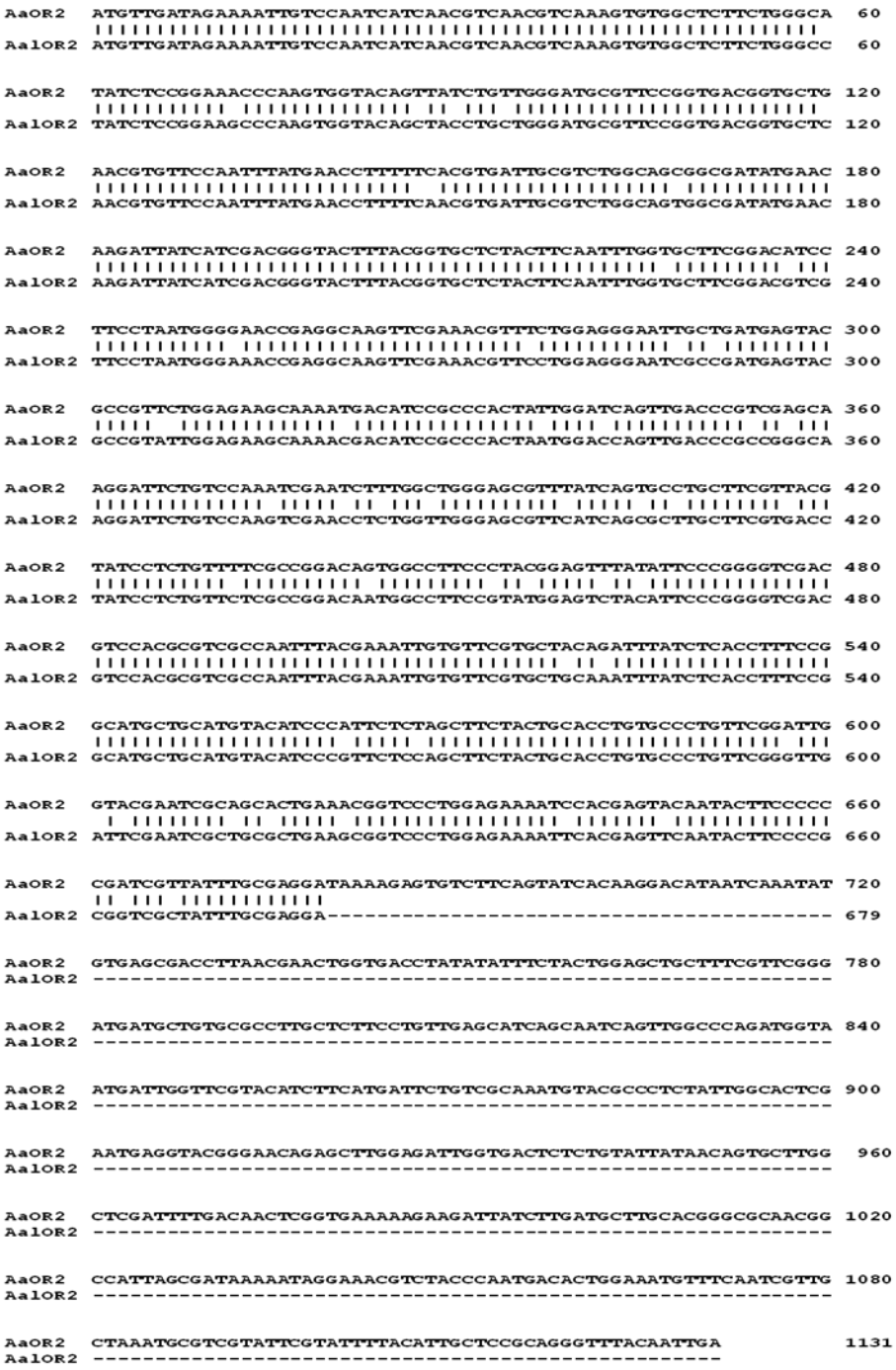

Fig.12: Comparison of the nucleotide sequences at the 5' ends of the AaOR2 and AalOR2 transcripts. The nucleotide sequence of the AalOR2 cDNA fragment, 680 nt long, (lower line) is aligned against the AaOR2 transcript (upper line). This fragment corresponds to the 5' end of the AalOR2 coding sequence.

with the AaOR2-5´ and 3´RevAalOR2 primers pair, by using the following cycle conditions: 98°C denaturation, 60°C annealing and 72°C extension for a maximum of 35 cycles. The amplicon obtained, 1.131 bp long, was elute from agarose gel, cloned in pGEMT-easy vector to obtain the pGEM/AalOR2 clone. As expected, the sequencing of this clone and its virtual translation revealed an Open Reading Frame (ORF) encoding a hyphotetical 376 amino acids polypeptide that shares 96% of identity with AaOR2 (Fig.13). In order to obtain informations on the genomic organization of AalOR2, I performed a PCR reaction using the primer combination 5'AaOR2/3'RevAalOR2 on genomic DNA extracted from about ten *A. albopictus* larvae by using Mammalian Genomic DNA Miniprep Kit (Sigma). PCR reaction was performed with the following cycle condition: 98°C denaturation, 60°C annealing and 72°C extension for a maximum of 35 cycles, on about 100 ng of genomic DNA. Again the amplified fragments obtained were eluted from agarose gels, cloned in pGemT-easy vector and subsequently sequenced at PRIMM Biotech. The genomic fragment, 1.556bp long, corresponded to the AalOR2 CDS. Its comparison with the corresponding genome region of the AaOR2 gene revealed the same genomic organization. Both CDS contain 6 exons highly conserved that are separated by 5 introns, conserved in length and in position and conforming the usual acceptor and donor splice sites (Fig.14).

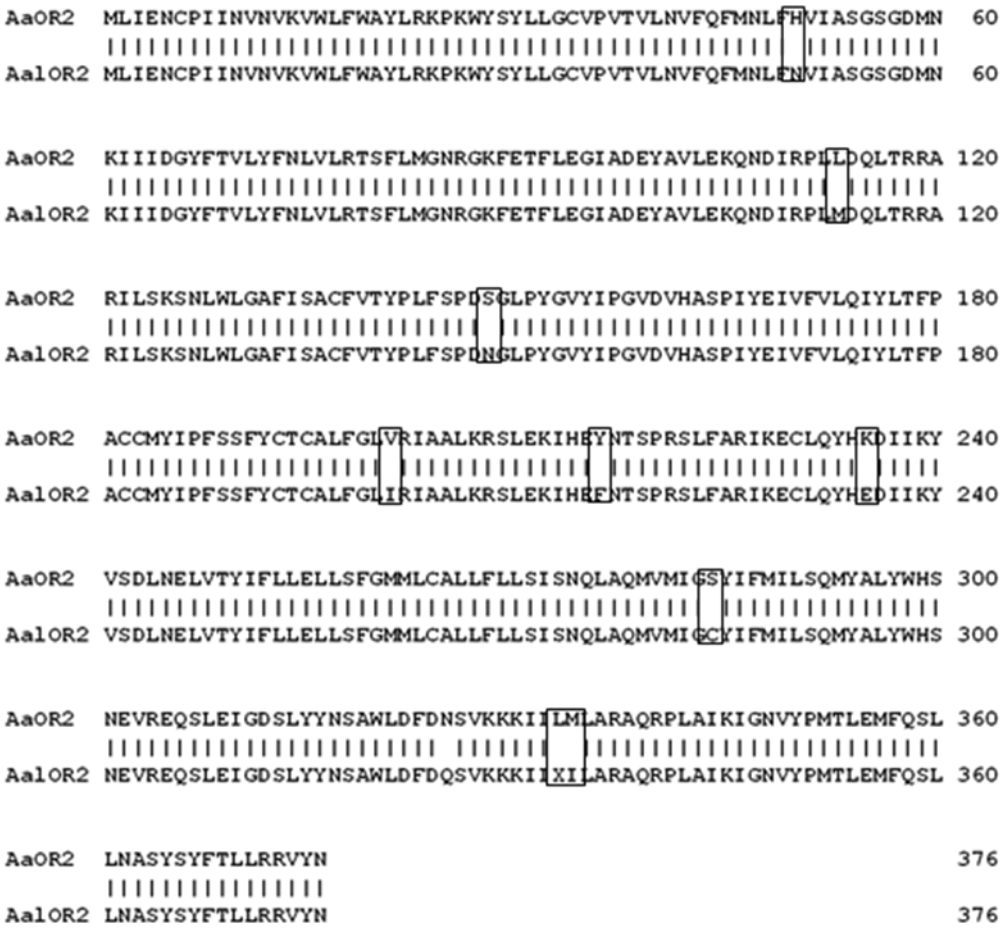

Fig.13: Aligment between the AaOR2 and AalOR2 hyphothetic proteins. The two proteins share same lenght and a very high degree of identity. The only aminoacidic nine differences are indicated with squared regions.

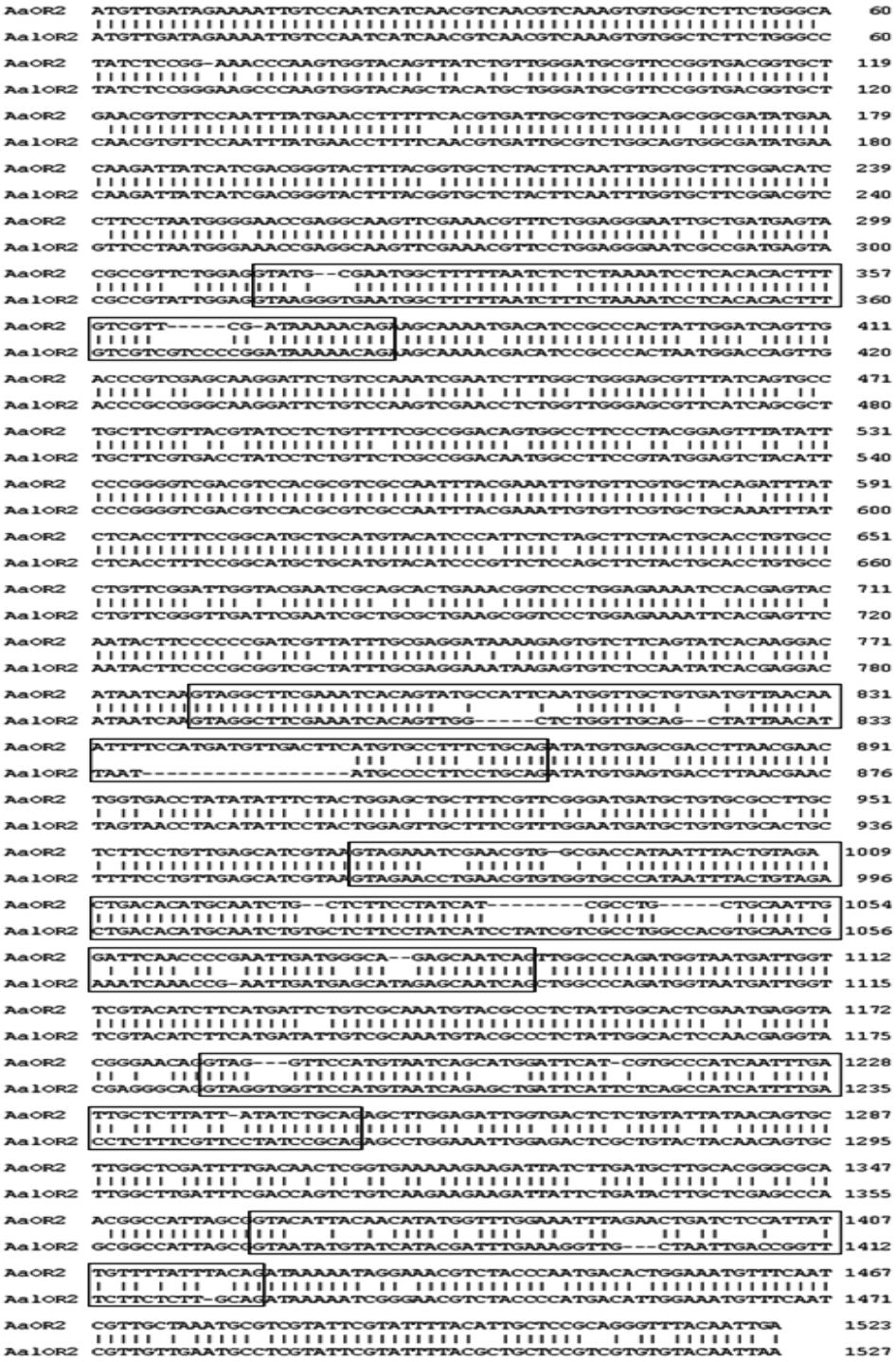

Fig.14: Comparison of the genomic sequences of AaOR2 and AalOR2. These two genes are highly conserved both in sequence and in genomic organization, being constituited by six exons, separated by 5 short introns (indicated by squared regions), conserved in length and in position.

Increased expression of *A.albopictus* OR2 in response to a blood meal

Based on the idea that several mosquito behaviors may be associated with a subset of olfactory genes displaying sex-specific and/or modulated expression, I studied the expression of AalOR2 before and after a blood meal, that represent a crucial point in the life cycle of mosquito. Previously, in *A.gambiae*, for example, it has been reported that the expression of AgOR1, expressed only in adult females, can change in response to blood feeding (Fox AN *et al.*, 2001). To address this question, I decided to perform a semi-quantitative RT-PCR. To this aim, I used QuantumRNA 18S Internal Standards (Ambion) on total RNA extracted from 30 manually dissected antenna pairs of *A.albopictus* male and female adults before and 12 hours after a blood meal. The RNA samples were diluited, quantized at nanodrop and brought to the same concentration. 300 ng of each RNA sample were reverse-transcribed to produce in vitro cDNAs. For this analysis, I used Reverse Transcriptase (Fermentas) and Random hexamers in a final volume of 20µL. PCR reactions were performed using 1µL of each template by using, as internal standards, the universal primer pair 18S primer/competimer (Ambion). Competimer technology can be used to modulate the amplification efficiency of a 18S template without affecting the performance of other targets. The 18S Competimers are modified at their 3' ends to block their extension by DNA polymerase. The goal is to find the right balance between 18Sprimer/competimer and gene-specific primer pairs to allow the amplification of the gene under examination without most of the PCR components, such as dNTPs, are not biased in favor of the ribosomal RNA amplification. Then, before performing the final experiment, I tested several ratios between 18S and competimer primers, along with an equimolar quantity of the specific AalOR2 primer pair, 3´RACE1Fw/5´RACE1Rev. In general, when the ratio biased in favor of 18S primer, I obtained the only amplification

of the 18S ribosomal RNA, while changing the ratio in favor of the18S competimer I obtained the only amplification of the gene under examination. Finally, I defined that the better ratio between these 18Sprimer/competimer, useful for a correct quantification of AalOR2, could be 4:6. Therefore, the final PCR reaction was performed by using a primer/competimer ratio of 4:6 and an equimolar quantity of 3´RACE1Fw/5´RACE1Rev primers. The cycling parameters were: 95°C denaturation, 56°C annealing and 72°C extension for a maximum of 30 cycles. As shown in Fig.15, I obtained an interesting result. In fact, I observed a modulation in the expression of AalOR2 in response to a blood meal, with an at least two-fold expression increase 12 hrs after a blood meal. This analysis shows also that the expression of AalOR2 is stronger in the antennae of adult females, because in the antennae of adult males it seems to be only a faint band. Taken together, these data suggest that AalOR2 is more expressed in antennae of adult females than in antennae of adult males and is up-regulated after a blood meal. I repeated this set of amplification for at least three times to avoid casual errors due to the preparation of the reactions, and always obtained the same result.

Study of the odorant response profile of AalOR2: “in vitro” and “in vivo” approaches

The high levels of divergence of the ORs within and between species do not allow predictive assignment of ligands based on extrapolation from empirical data of other ORs. The de-orphaning, or identification of ligand specificity of these receptors can therefore only be achieved through experimental means. Several approaches to de-orphaning ORs have previously been applied to insect ORs, and very recently AaOR2 and CpOR2 has been de-orphanized. To establish the response profile of AalOR2, I used an “in vitro” approach, performing Ca^{2+} imaging in a heterologous cell system, and an “in vivo”

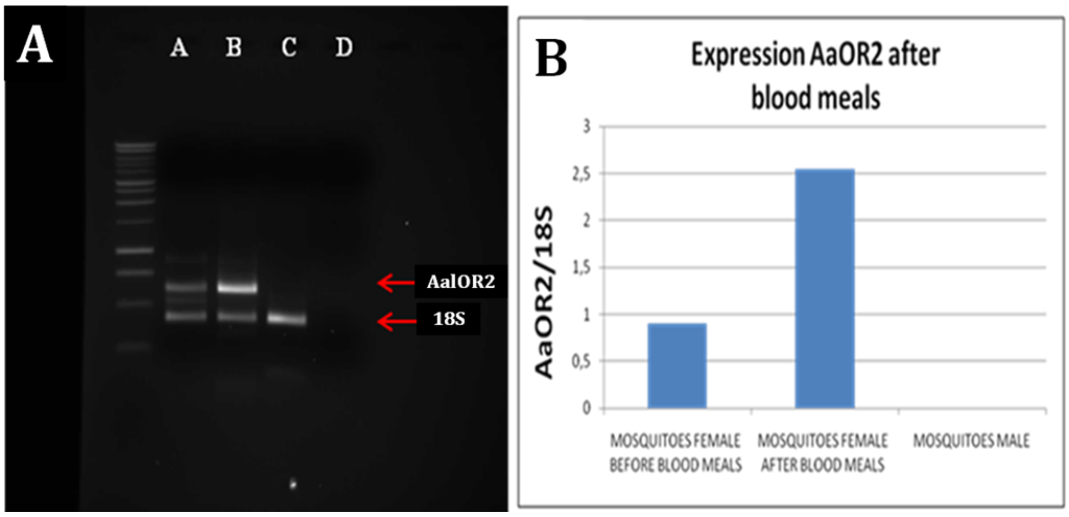

Fig 15: Expression of AalOR2 transcript in response to a blood meal. (A) Agarose gel of the products obtained through a semiquantitative RT-PCR reaction on total RNA of *Aedes albopictus* antennae of A, adult females, before a blood meal, B, adult females 12 hrs after a blood meal, C, adult males. On the first lane on the left was loaded 1 kb DNA marker (Fermentas). On the lane D was loaded a negative control obtained by using reaction mix with no cDNA **(B)** Blue columns indicate the AalOR2/18S ratio as calculated by densitometer Gene Tools software. It results evident that the AalOR2 expression is at least two-fold up-regulated in response to a blood meal.

experiment, by using Single Sensillum Recording (SSR) and Gas Cromatography-SSR (GC-SSR) in an engineered neuron of a transgenic *Drosophila melanogaster* strain that expressed AalOR2.

"in vitro" approach: odorant response profile of AalOR2 in CHO-K1 cells

Cloning of AalOR2 in pHM6/HA mammalian expression vector

The experiments I am going to describe in this section were performed at the Arterra Bioscience in Naples. In order to characterize the AalOR2 response profile to chemicals, I first analyzed its expression in a heterologous cell system. To this goal, I sub-cloned the AalOR2 coding sequence in the pHM6/HA mammalian expression vector in frame with the Hemoagglutinin (HA) sequence at the N-terminus. The CDS was amplified on the pGem/AalOR2 clone, by using the HindIII-AalOR2/AalOR2 3'end primer pair and the following cycle conditions: 98°C denaturation, 60°C annealing and 72°C extension for a maximum of 30 cycles. The HindIII-AalOR2 primer contained the sequence of the restriction enzyme HindIII and the first 22nt of the AalOR2 5'end missing of the ATG start codon. The lack of this initial codon was necessary to go in frame with the Hemoagglutinin (HA) sequence. The amplified fragment was cloned in pGEMT-easy vector to generate the pGEMT-easy/HindIIIAalOR2 clone. Subsequently, this clone and the pHM6/HA vector were digested with the HindIII and EcoRI restriction enzymes and run on agarose gel 1% to elute the HindIIIAalOR2 fragment and the linearized pHM6/HA vector, respectively. Their final ligation, by using T4 DNA ligase, allowed me to obtain the desired pHM6/HA-AalOR2 clone (Fig.16).

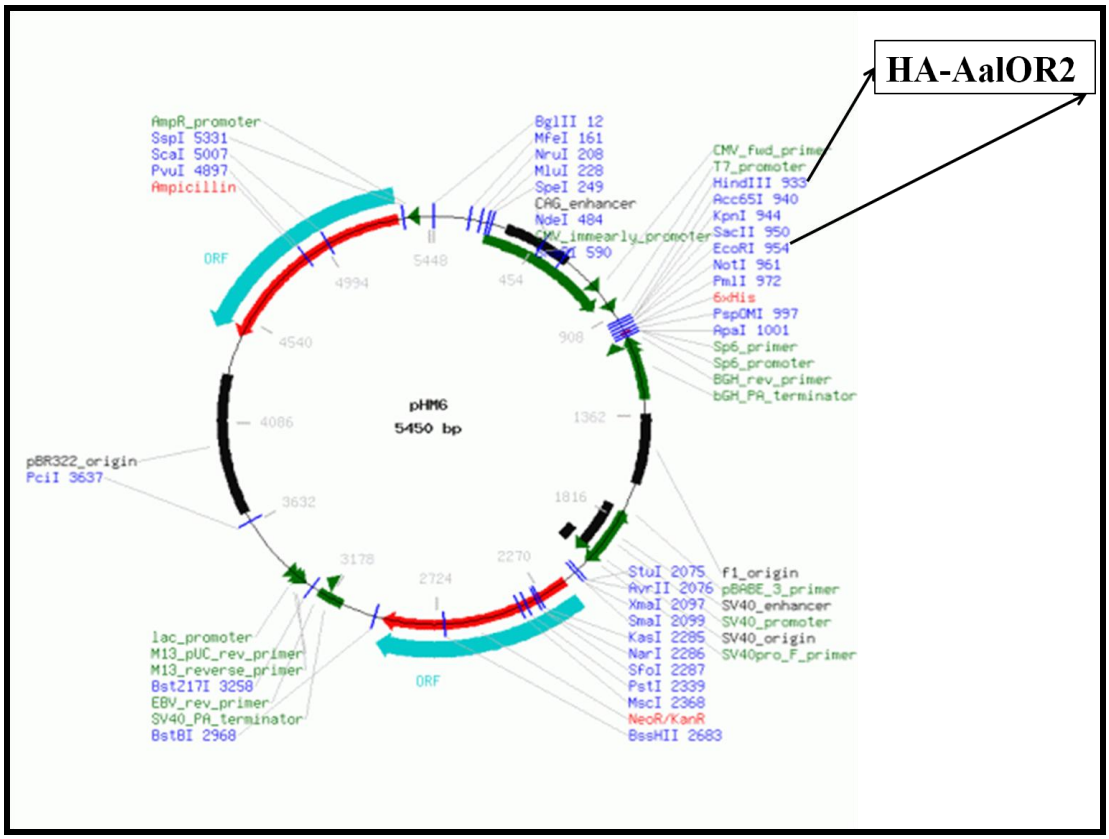

Fig.16: pHM6/HA-AalOR2 mammalian expression vector. pHM6/HA-AalOR2 is an eukaryotic expression vector for full-length A.albopictus OR2, in frame with a N-terminal HA (Haemagglutinin) epitope, under the control of the human cytomegalovirus immediate early promoter (CMV-IE) and enhancer. Since the termination codon of AalOR2 is included, the C-terminal His-tag is not expressed.

Determination of the HA-AalOR2 expression in a heterologous system by ELISA assay

The pHM6/HA-AalOR2 clone was used to transfect in transient CHO-K1 cells (Chinese Hamster Ovary Cells) to determine, by an ELISA assay, the right amount of DNA useful to obtain the maximum expression level of the recombinant protein. It is well known that the correct localization of insect ORs into the ORN dendrites is mediated by the highly conserved OR83b/OR7. Since at the Arterra Laboratories, where I was carrying out this part of my work, the *D.melanogaster* OR83b had been previously cloned in the mammalian expression vector pHM6 without HA tag (pHM6/DmOR83b), I decided to use this clone for my experiments. Unlikely of the other genes of the family, OR83b is expressed in almost, if not all, all antennal neurons of insects; furthermore, rather than having a direct role in the olfactory function, it interacts with the conventional OR members and is essential for their localization to the sensory cilia where interaction with the odorant molecules takes place. CHO-K1 cells were grown in the DMEM-F12 medium (Lonza) plus 10% FBS, at 37°C with 5% CO_2. To perform ELISA assays, 1.5 x 10^4 cells per well were seeded in a 96 well plate. After 24 hours cells were transfected with pHM6/HA-AalOR2 alone, and co-trasfected with pHM6/HA-AalOR2 plus pHM6/DmOR83b. The transfections were carried out by using Lipofectamine 2000 (Invitrogen), with a ratio Lipofectamine/DNA of 5:1. The amount of DNA used for each receptor ranged from 25 to 100ng. The ELISA assay is based on a specific immuno-recognition. In my hands, the N-terminus of the chimeric Odorant Receptor was recognized by an anti-HA primary antibody (*rabbit polyclonal* Santa Cruz Biotechnology), recognized in turn by a secondary antibody (*goat anti-rabbit* IgG beta-galactosidase) conjugated to an enzyme whose activity could be easily detected by a colorimetric reaction, based on the degradation of a chromogen substrate. This color reaction was detected by reading the absorbance of the samples at 550 nm about 5 hours

after the transfection through the Victor3 instrument (PerkinElmer). The color intensity is strictly related to the amount of the OR expressed. I obtained the maximum level of HA-AalOR2 expression by using 100ng of DNA on 1.5 x 10^4 cells. This assay was performed for five times at the right conditions, and values reported in the graph in Fig.17 are the average of the results obtained in each experiment. From the analysis of the ELISA data, it emerges that the expression of AalOR2 increases of about 20% when it is co-expressed with DmOR83b, confirming that OR83b acts as chaperon and helps the expression of AalOR2 (Fig.17).

Determination of AalOR2 localization in a heterologous system by an Immunofluorescence assay

To determine whether the receptor was correctly localized at the plasma membrane level, I performed an immuno-fluorescence experiment. 3 x 10^5 CHO-K1 cells per well were seeded in a 6 well plate, over a sterilized slide placed at the base of each well. After 24 hours, cells were transfected with pHM6/HA-AalOR2 alone, and co-trasfected with pHM6/HA-AalOR2 plus pHM6/DmOR83b, using the Lipofectamine 2000 (Invitrogen), in the same ratio with DNA plasmid previously determined by the ELISA assay. In this case, the amount of DNA was 1µg per well. 48 hours after transfection, cells were fixed in 4% paraformaldehyde in PBSCM for 15-20 minutes at RT (Room Temperature). After washing with PBSCM, fixed cells were incubated with HA primary antibody (*rabbit polyclonal* Santa Cruz Biotechnology) (used to recognized the HA-tag) diluted1:500 in Blocking buffer composed of PBSCM, 2% BSA and 0.1% Triton. In turn, a second reaction was carried out with a secondary antibody fused with FITC (*goat anti-rabbit* IgG FITC Santa Cruz Biotechnology) diluted 1:250 in Blocking buffer; this antibody was able to recognize the complex primary HA-antibody/HA-OR.

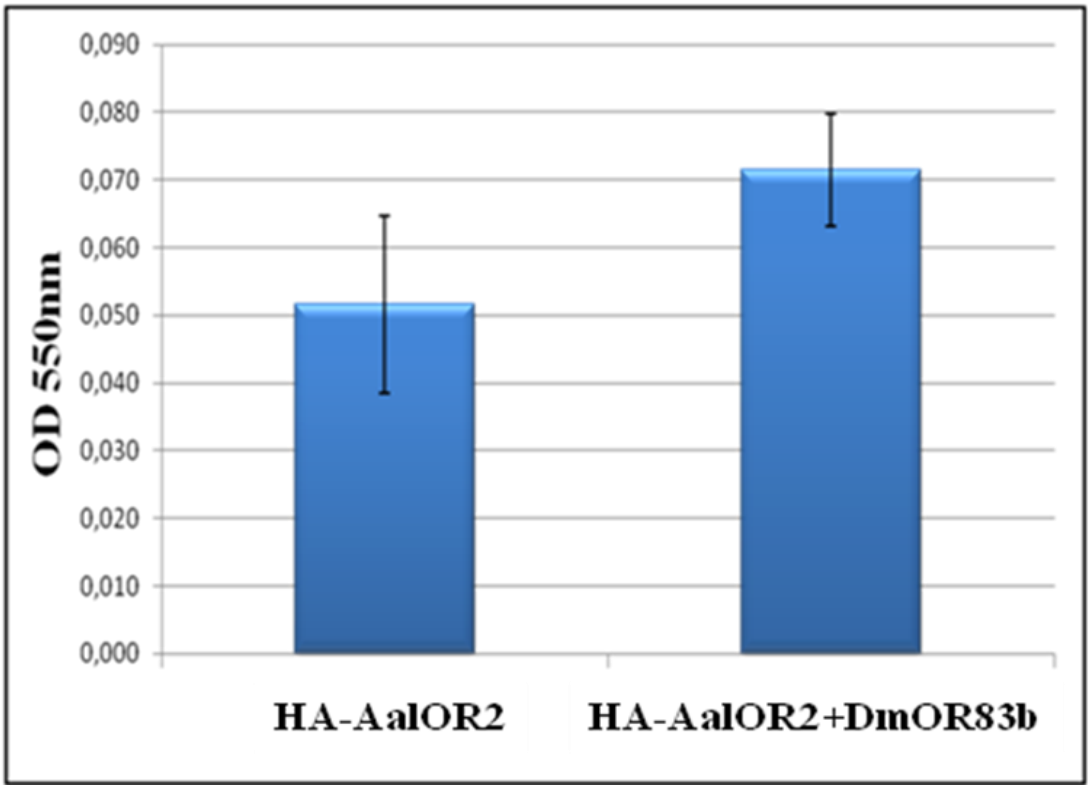

Fig 17: Elisa assay in CHO-K1 cells expressing pHM6/HA-AalOR2. Are reported the absorbance averages of the samples at 550 nm about 5 hours after the transfection. The expression of AalOR2 increases of about 20% when it is co-expressed with DmOR83b, suggesting that OR83b acts as chaperon and helps the expression of AalOR2. This assay was performed for five times at the right conditions, and values reported are the average of the results obtained in each experiment. Error Bars = SEM

FITC (Fluorescein-labeled Antibodies) is a small organic molecule conjugated to a secondary antibody, that is typically excited at 488 nm, while the emissions are collected at 530 nm. The observations of my samples were carried out at IGB-CNR with a Leica SP2-AOBS Confocal Microscope by using a 63X oil immersion objective equipped with a specific FITC filter. The expression pattern of HA-AalOR2 alone, shown in Fig.18, clearly indicates that AalOR2 alone fails to localize to the plasma membrane and it seems more likely internalized in vacuoles and/or vesicles in the cell bodies. By contrast, in presence of DmOR83b, AalOR2 was able to localize in membrane, indicating that in our system, such as in insect ORNs, the chaperone protein OR83b was indispensable for the correct expression and localization of AalOR2.

Ca^{2+} imaging measurement by using Fluo3/AM in CHO-K1 cells

Once I identified the right parameters of the AalOR2 expression in CHO-K1 cells I passed to perform Ca^{2+} imaging experiments to study the odorant response profile of AalOR2. To this aim, I decided to prepare a polyclonal cell line, to avoid to transfect cells continuously. CHO-k1 cells were seeded in a 6 well plate, with an average of 3 x 10^5 cells per well. After 24 hours, cells were co-transfected with pHM6/HA-AalOR2 plus pHM6/DmOR83b, using the conditions previously determined. 48 hours after the transfection, cells were treated with 600µg/mL of neomycin antibiotic. My chimeric expression vectors, pHM6/HA-AalOR2 and pHM6/DmOR83b, bring the resistance to the antibiotic neomycin; so, by treating cells after transfection with this antibiotic, the only cells that have incorporated the receptors will be able to survive. In this way, I was able to generate a polyclonal CHO-K1 line expressing AalOR2 along with OR83b. This polyclonal CHO-K1 cell line were grown in DMEM-F12 medium (Lonza) plus 10% FBS, at 37°C and 5% CO2 plus

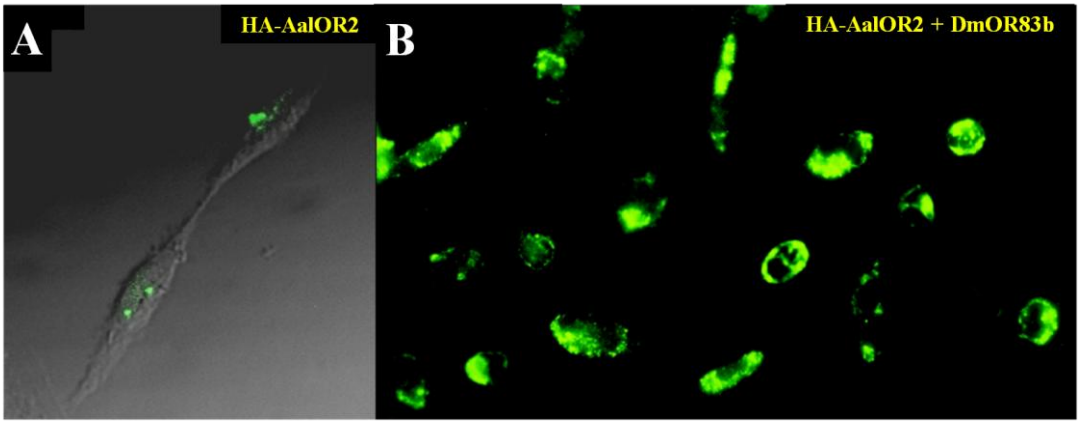

Fig 18:Localization of AalOR2 to the plasma membrane of heterologous CHO-K1 cells. Confocal images of FITC labeled AalOR2 indicate that **(A)** AalOR2 alone is not able to localize to the cell membrane, but remains internalized in the cell, **(B)** AalOR2, in presence of DmOR83b, is correctly localized to the plasma membrane.

600µg/mL of neomycin antibiotic. The expression of AalOR2 and DmOR83b in polyclonal cell line was constantly checked by RT-PCR on mRNA extracted from the cells using the GenElute™ mRNA Miniprep Kit (Sigma). At least 1µg of this RNA was retro-transcribed with primer-dT by using the Reverse Transcriptase (Fermentas) in a final volume of 20µL. 1µL of this cDNA was used in a PCR reaction by using a pair of specific primers for each receptor. In Ca^{2+} experiment, 1 x 10^6 cells were detached by using a non-enzymatic solution (Sigma), centrifugated and resuspended in 2mL HBSS solution. After adding 2.5 µM Fluo3/AM calcium dye, 0.02% pluronic and 2.5mM probenecid, cells were incubated in the dark at 37°C, 5% CO_2 for 45 min. $Ca2^+$ imaging experiment was executed by using EnVision Multilabel Plate Readers (PerkinElmer). This system consisted in a pump unit that, once immersed into cell suspension, automatically dispensed 100µL of cells in each well of a 96 well plate previously loaded with olfactory molecules, and read the fluorescence simultaneously. Therefore, during the incubation time, an empty 96 well plate was loaded with 1µM of each olfactory molecule in triplicate. To determine the odorant affinity of AalOR2, I tested compounds reported in Tab.2. After an incubation time of 45 min with the Fluo3/AM calcium dye, the 96 well plate was inserted into EnVision Multilabel Plate Readers (PerkinElmer) that automatically recorded the intracellular calcium variation in response to each tested odorant molecule. I performed several Ca^{2+} imaging experiments but unfortunately the results were non comparable. Probably, this problem could be due to a low level of sensitivity of this system, maybe related to my cell line. Alternatively, another possible reason for this failure could be related to the long time that the EnVision system spent to do all measurement (more than 30 minutes) that, in turn, caused a high mortality of cells. In Fig. 19 are displayed some graphs to show that the results of these Ca^{2+} imaging experiments were non-reproducible.

COMPOUNDS	CLASSIFICATION
HEXANAL	ALDHEYDE
VALERALDEHYDE	ALDHEYDE
TRANS-CINNAMALDEHYDE	ALDHEYDE
BENZALDEHYDE	ALDHEYDE
4-ISOPROPYL	ALDHEYDE
1-PHENYL ETHANOL	ALCOHOL
GERANIOL	ALCOHOL
1-HEXANOL	ALCOHOL
BENZYL ALCOHOL	ALCOHOL
3-METHYL CYCLO EXANOL	ALCOHOL
CHRYSANTHEMIC	ALCOHOL
CYCLOPENTANONE	KETONE
METHYL ACETO ACETATE	KETONE
ETHYLACETATE	ESTERE
2-METHYLPHENOL	PHENOL

Tab.2: Table of compounds used to perform Ca^{2+} imaging measurement by using Fluo3/AM in CHO-K1 cells.

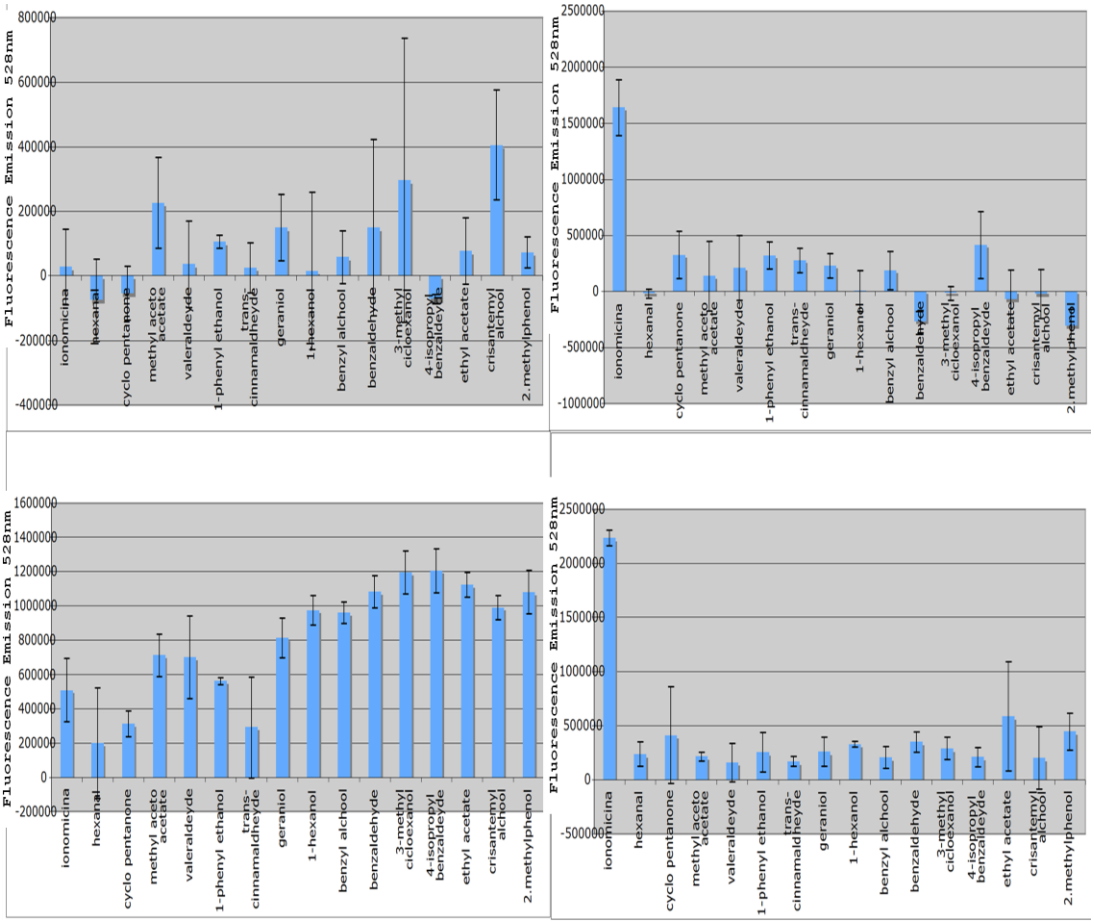

Fig.19: Ca^{2+} imaging measurement with Fluo3/AM in CHO-K1 cells. Some Ca^{2+} imaging measurements performed by using EnVision Multilabel Plate Readers (PerkinElmer) against the panel of odors indicated in Tab.2. For each experiment, ionomicin was used as positive control. In each experiment a given odor was used in triplicate, and the measures are the averages among these responses. The comparison among these different graphs, clearly indicate that the obtained results were not-reproducible or comparable, perhaps due to a low sensitivity of the used system or to cell death. Error bars= SEM

“in vivo” approach: Single Sensillum Recording (SSR) and Gas Cromatography-SSR (GC-SSR)

To overcome the difficulties in defining the odor response profile of AalOR2 in a heterologous system, I decided to use the “empty neuron” approach in *D. melanogaster*. To this aim, during the last months of my PhD, I had the opportunity to perform this electrophysiologal technique in the Professor Hansson’s Laboratory at the Max Plank Institute in Jena (Germany). Further, in this Laboratory, I could enlarge the panel of odorsto be tested, using several compounds kindly provided by Prof. Hansson’s group, and carry out a $Ca2^{+}$ imaging experiment in a different heterologous system.

Cloning of AalOR2 in the pUAST *D.melanogaster* expression vector

Concerning my studies *in vivo* I performed Single Sensillum Recording (SSR) and Gas Chromatography-SSR (GC-SSR) on a transgenic *D.melanogaster* strain that expressed the heterologous AalOR2 gene under the control of the binary system GAL4-UAS; this system utilizes the yeast transcription factor GAL4 and its target sequence UAS to which GAL4 binds in order to activate gene transcription. To this aim, I cloned the AalOR2 CDS in the pUAST expression vector, in frame with the Cavener sequence. pUAST vector is a modified P-element with five UAS upstream to a weak promote and multiple cloning sites. The recombinant UAST-AalOR2 vector was constructed by inserting the amplification product obtained using as template pGEMT-easy/AalOR2 with the pair of the Cavener-AalOR2 and the AalOR2 3’end primers by using the following cycle conditions: 98°C denaturation, 60°C annealing and 72°C extension for a maximum of 30 cycles. The Cavener-AalOR2 contains, immediately upstream to the start codon ATG, the Cavener sequence (CAAC), and the first 23nt of the AalOR2 5’end. In insects, the

Cavener sequence is the equivalent of the eukaryotic Kozak sequence and is necessary to promote translation of heterologous transcripts in *D.melanogaster*. Before to pass into the final vector, this amplified fragment was cloned in pGEMT-easy vector to obtain the pGEMT-easy/Cavener-AalOR2 clone. The pGEMT-easy/Cavener-AalOR2 and pUAST vectors were digested with the restriction enzyme EcoRI and, after an electrophoretic run on 1% agarose gel, the fragments corresponding to the Cavener-AalOR2 and to the linearized pUAST were recovered. Their subsequent ligation allowed me to construct the final UAST-AalOR2 clone, useful to transform *D. melanogaster* (Fig.20). To inject this construct into Drosophila w^{1118} embryos, I prepared plasmid DNA with the Qiagen maxiprep kit, that allows to obtain DNA of good quality. Then, I sent this DNA preparation to the Genetics Service, a company in Bloomington (Indiana University) that offers microinjection services. Few days after microinjection, the Company sent me about 100 II instar larvae, developed from the injected embryos, that were grown in my Laboratory. The transformation vector pUAST contains a miniwhite gene that is useful as marker gene to recover transformant flies since confers red eyes in the w^{1118} strain, that is phenotypically white eyes. So, among the flies that completed the development, I selected those showing red eyes, because they have integrated in their genome the UAST-AalOR2 plasmid. I obtained 15 lines deriving from independent insertion events of the construct in the genome. In order to determine the chromosome in which the costruct was inserted, I performed genetic crosses, using strains that carry balancer chromosomes. These chromosomes have many inverted repeats in order to prevent recombination among homologous chromosomes, and carry dominant characters to allow the identify the chromosome in which the pasmid insertion occurred. The final transgenic strain carries, integrated in the genome, the heterologous AalOR2 gene, under the control of the UAS (Upstream Activating Sequences) sequence. In this strain, gene remains transcriptionally silent because it needs GAL4 to be activated.

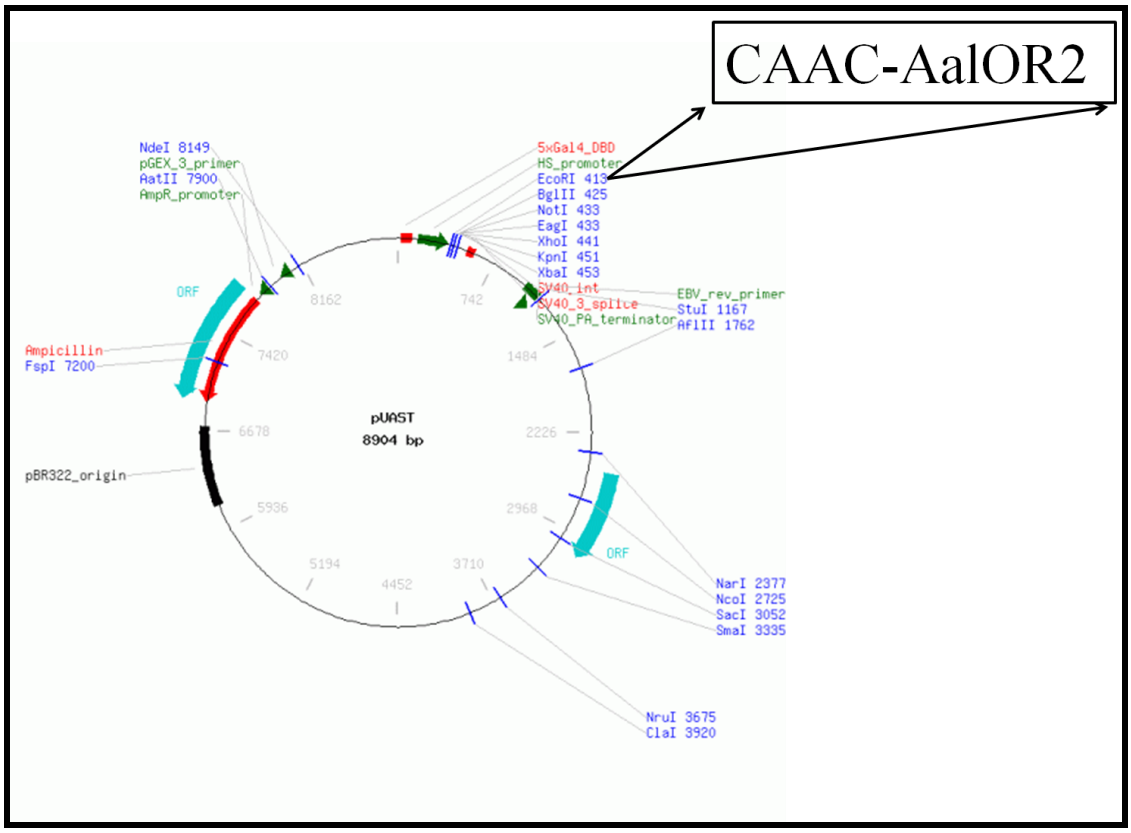

Fig.20: pUAST/CavenerAalOR2 *D.melanogaster* expression vector. This vector allows the expression of AalOR2 in the fruitfly *D. melanogaster*, in response to the activation with GAL4 protein.

So, to activate its transcription, the flies carrying the UAST-AalOR2 gene must be crossed with flies expressing GAL4 in particular tissues. A key advantage of this binary system is the separation of the GAL4 protein from its target gene in distinct transgenic lines, which ensures that the target gene is silent until the introduction of GAL4. So, it is possible to drive the expression of a given gene only in flies carrying both constructs. This is achieved by crossing a driver line (GAL4), with a UAS line, with the gene fused to UAS sequences being expressed in any time and location the driver gene would normally be expressed. In this sense, my goal was expressing the heterologous AalOR2 in the *D.melanogaster* ab3A "empty neuron", so that its electrophysiological responses to specific odors could be recorded without any interference from other receptors. To perform this, I used the Delta-halo Drosophila mutant (Dobritsa AA *et al.*, 2003; Hallem EA and Carlson JR, 2004). In this fly strain, the neuron ab3A, localized in a basiconic sensillum into the dorso-medial region of the antenna, fails to respond normally to odors, due to a genomic deletion of its endogenous Odorant Receptor genes, OR22a and OR22b. To obtain the expression of UAST-AalOR2 in the ab3A neuron, three indipendent transgenic lines carrying the heterologous gene inserted in different regions of the genome were individually crossed with a Delta-halo, 22a-Gal4, UAS(mCD8-GFP) Drosophila strain. In this way, I obtained three final UAST-AalOR2, Delta-halo, 22a-Gal4, UAS(mCD8-GFP) strains. I performed my further experiments on these three lines, for taking in account possible different expression levels of the heterologous gene, due to different insertion sites in the genome. In these flies, GAL4 is under the control of the OR22a promoter that works specifically only in the neuron ab3A. This ab3A specific production of GAL4 in turn activates the expression of both UAS transgenes, UAST-AalOR2 and UAS(mCD8-GFP), in this neuron. UAS(mCD8-GFP) localizes the GFP to the cell membranes because encodes the mouse lymphocyte surface marker CD8 fused in frame with the GFP protein (Fig.21). When activated by GAL4, the specifically GFP-labeled

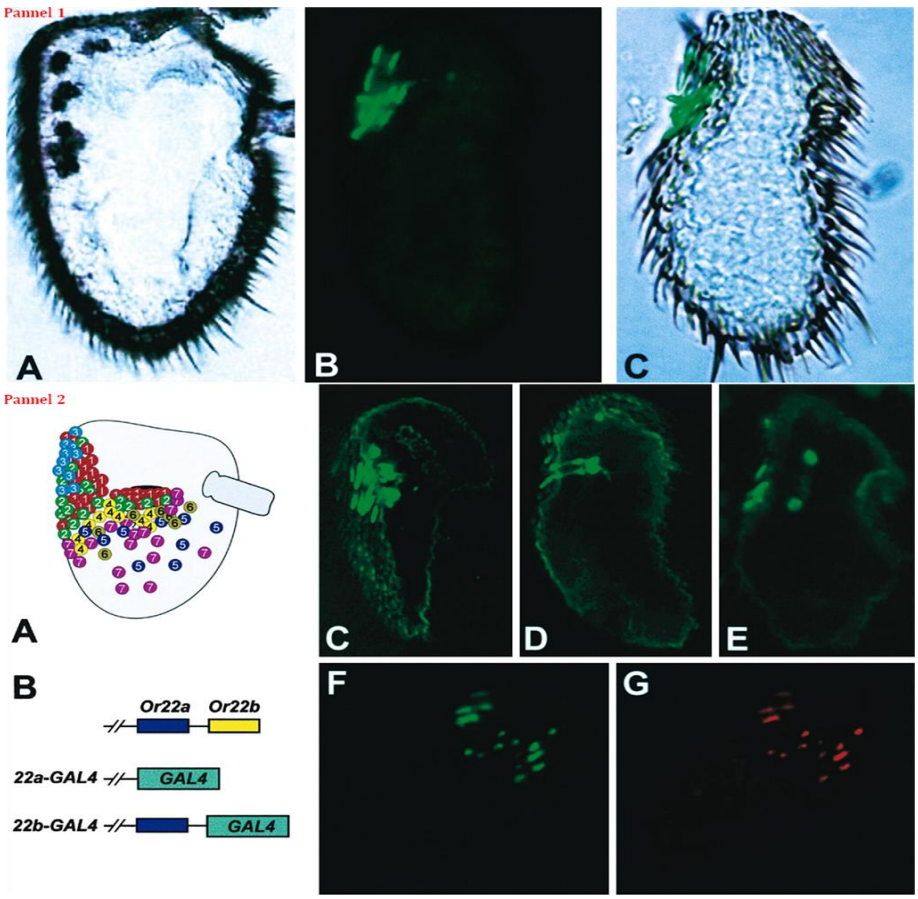

Fig 21: Localization of the ab3 sensillum. The neuron ab3A, expresses only OR22a and OR22b proteins, as determined by Dobritsa and collaborators (Dobritsa AA *et al.*, 2003), from which following images were taken: Panel 1 **(A)** In situ hybridization of OR22a/22b probe is revealed only in a subset of antennal cells, in the dorso-medial area, **(B)** fluorescent immunostaining of the antenna with the 22a/b antibody, **(C)** merge of B and its bright-field correspondent. Panel 2 indicates that OR22a and OR22b are expressed in ab3 sensilla. **(A)** Map of the functional types of basiconic sensilla on the antenna, where the number three indicates ab3 sensilla. **(B)** Structure of the 22a-GAL4 and 22b-GAL4 constructs. **(C,D)** Confocal images of GFP expression driven by 22a-GAL4 and **(E)** 22b-GAL4; **(F,G)** expression of GFP in UAS-GFP Drosophila lines, for control. Delta-halo Drosophila mutant has a synthetic deletion of these two genes.

sensilla are visible in live flies, thereby allowing to distinguish them and record from them electrophysiologically. However, this neuron is easily recognized also on the basis of the extracellular spike amplitude. In fact, it has been revealed that the extracellular spike amplitude is a property of the neuron that is independent of the OR it expresses (Hallem EA *et al.*, 2004). Instead, the OR is the primary determinant of the other ORN response properties, such as spontaneous firing rate, signaling mode, odor response spectrum and dynamics (Hallem EA *et al.*, 2004). Furthermore, it has been shown that a given OR confers to the ORN (in my case ab3A) a spontaneous firing rate without olfactory stimulation (de Bruyne M *et al.*, 2001), so allowing the detection of inhibitory and excitatory responses (Hallem EA *et al.*, 2004).

Single Sensillum Recording (SSR) of AalOR2 expressed in the Delta halo Drosophila mutant

In order to record action potentials of the AalOR2-"empty"ab3A ORN in its GFP-tagged sensillum, I placed an electrode through the sensillum wall into contact with the "sensillum limph" that bath the dendrites (Fig.22C). To this aim, a single, 5- to 15-day-old fly was mounted in a truncated pipette tip with the antenna protruding from the narrow end. The pipette tip was fixed with wax on a microscope slide, and the antenna gently placed on a cover-slip and stabilized with a glass electrode (Clyne P *et al.*, 1997; Stensmyr *et al.*, 2003) (Fig.22A,B). The antennal surface was observed at a 1000x magnification, which allowed individual sensilla to be clearly resolved, through an Olympus BX51 microscope fitted with fluorescence optics to view GFP. As recording electrode, I used a glass capillary with the tip drawn to 1µm diameter, filled with "sensillum lymph" Ringer (Kaissling KE and Thorson J 1980), and slipped over an AgCl-coated silver wire. Instead, the indifferent electrode was filled with Ephrussi and Beadle solution and was put into the eye. In this

system, I used a different panel of odorswith respect to that used at Arterra Bioscience in the Ca^{2+} imaging experiments (Tab.3). First of all, neat compounds were diluted in redistilled hexane down to a concentration of 100ng/µl. From these solutions, I pipetted 10µl onto a small piece of filter paper placed inside Pasteur pipettes. In this system, a glass tube, with its outlet at 5mm from the antenna, delivered a constant flow of humidified air at a velocity of $0.5ms^{-1}$ over the preparation. In order to perform stimulation, I inserted a Pasteur pipette into a hole in the glass tube. In turn, this Pasteur pipette was connected to a stimulus controller that generated air puffs (2.5ml for 0.5s) through the cartridge into a constant air stream in the glass tube. In my experiments, I always used a pulse duration of the olfactory stimulus of 0.5s (seconds). Finally, the signals originating from the ORNs were amplified 1000 times, digitally converted via Syntech IDAC-4 USB and visualized by Syntech Autospike 3.2. The signal was also fed to a loudspeaker for audio monitoring. Recording of action potentials were stored on the PC and all analysis was done with AUTOSPIKE software. The sensillum in which I performed my analysis, tagged with GFP, harbors two kinds of ORNs called ab3A and ab3B that present differences in the spike amplitude, with the spike generated from the ab3A neuron that is bigger than the ab3B spike (Fig.22D). This difference in the spike amplitude allowed separation of their activity in Single Sensillum Recording experiments. As control, in each experiment that I carried out on the three independent Drosophila transgenic lines, I used ethyl butyrate to stimulate the AalOR2-"empty-neuron". As reported by Dobritsa and collaborators (Dobritsa AA *et al.*, 2008), ethyl butyrate is the agonist of the OR22a and, when present, confers a strong excitatory response to ab3A neuron. In my experiments, as expected, this chemical failed to elicit a response, confirming the failure expression of the endogenous olfactory receptor OR22a in this neuron. In Fig. 23 are shown the responses of AalOR2 against the odorspanel tested. Firing rates were quantified from the number of spikes in one second of spontaneous activity although, to facilitate a

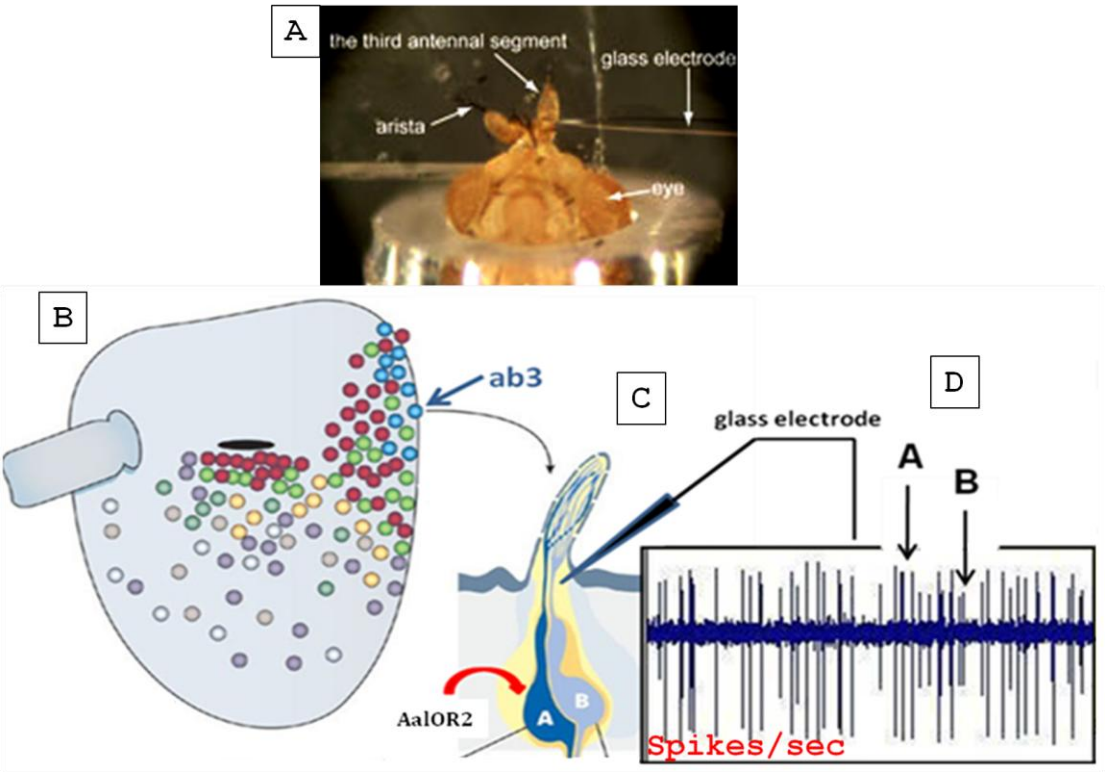

Fig 22: Single Sensillum Recording (SSR) of a given OR expressed in the Delta halo Drosophila mutant. In this cartoon is reported the "in vivo" technique I used to perform SSR. **(A)** A single, 5- to 15-day-old fly is mounted on a slide and the third segment of antenna is blocked with a glass electrode (Pellegrino M. et al., 2010), **(B)**, Schematic distribution of the functional types of basiconic sensilla on the antenna. The ab3 sensilla are indicated in blue color, **(C)** action potentials of the two ORNs in the ab3 sensillum are recorded by placing an electrode through the sensillum wall into contact with the lymph that bathes the dendrites of both neuron A and B haused in ab3 sensillum, **(D)** the extracellular analog signals originating from the ORNs clearly report the different spike amplitude of spontaneous activity from the larger, shorter A and B neuron, respectively.

COMPOUNDS	CLASSIFICATION
INDOLE	INDOLE
1-METHYLINDOLE	INDOLE
2-METHYLINDOLE	INDOLE
3-METHYLINDOLE	INDOLE
4-METHYLINDOLE	INDOLE
5-METHYLINDOLE	INDOLE
6-METHYLINDOLE	INDOLE
7-METHYLINDOLE	INDOLE
PHENOL	PHENOL
2-METHYLPHENOL	PHENOL
3-METHYLPHENOL	PHENOL
4-METHYLPHENOL	PHENOL
3-ETHYLPHENOL	PHENOL
4-ETHYLPHENOL	PHENOL
BENZALDEHYDE	ALDEHYDE
CINNAMALDEHYDE	ALDEHYDE
GAMMA NONALCTONE	ALDEHYDE
PHENYL ACETALDEHYDE DIMETHYL ACETAL	ALDEHYDE
4-ISOPROPYL BENZALDHEYDE	ALDEHYDE
HEXANAL	ALDEHYDE
1-HEPTANAL	ALDEHYDE
VALERALDEHYDE	ALDEHYDE
2-PHENYL ETHANOL	ALCOHOL
GERANIOL	ALCOHOL
1-HEXANOL	ALCOHOL
BENZYL ALCOHOL	ALCOHOL
3-METHYL CYCLO EXANOL (3MCE)	ALCOHOL
CYCLO PENTANONE	KETONE
ETHYL ACETATE	ESTERE

Tab. 3: Table of compounds used in Single Sensillum Recording and Ca^{2+} imaging in heterologous cells at the Max Plank Institute.

comparison, responses were normalized by defining the maximal odorant response, among all olfactory molecules, as 100% of the spike increasing. This normalization allowed me to assess responses among different chemical groups, defining that AalOR2 interacted with some aromatic compounds such as 3-methylindole, and benzaldehyde. However, the compound that triggered the strongest excitation of the neuron was indole, which induced more than 170 spikes/sec. To confirm that indole was a specific agonist of AalOR2 I performed an indole dose-response experiment. As described above, an adult transgenic fly expressing AalOR2 was treated with different concentrations of indole, ranging from 1ng to 2µg. My results clearly indicated that the neuronal spike frequency was directly proportional at the concentration of indole, as shown in Fig.24, confirming that AalOR2 was narrowly tuned to indole.

Gas Cromatography Single Sensillum Recording of AalOR2

The up-regulation of AalOR2 gene as determined by semi-quantitative RT-PCR experiments previously described, suggested that this receptor could have an important function for *A. albopictus* females that, after a blood meal, have to search the convenient place to lay eggs. Furthermore, indole, that I detected to be the strongest ligand of AalOR2, it has been shown to be an oviposition attractant for Culex mosquitoes (Clements AN, 1995). Taken together, these considerations led me to search for other ligands of AalOR2 naturally occurring in the oviposition sites. For this purpose, I performed Gas Chromatography-linked Single Sensillum Recordings (GC–SSRs) in the three transgenic fly lines expressing UASAalOR2 against a mixture of odors extracted from a plant locate in a putative oviposotion site. In this experiment, I used organic infusions originated by natural fermenting of *Acorus variegatus* leaves in water mixed to leaves, in order to extract volatile chemicals (Fig.25 A,B). This was obtained through a headspace volatile collection system that

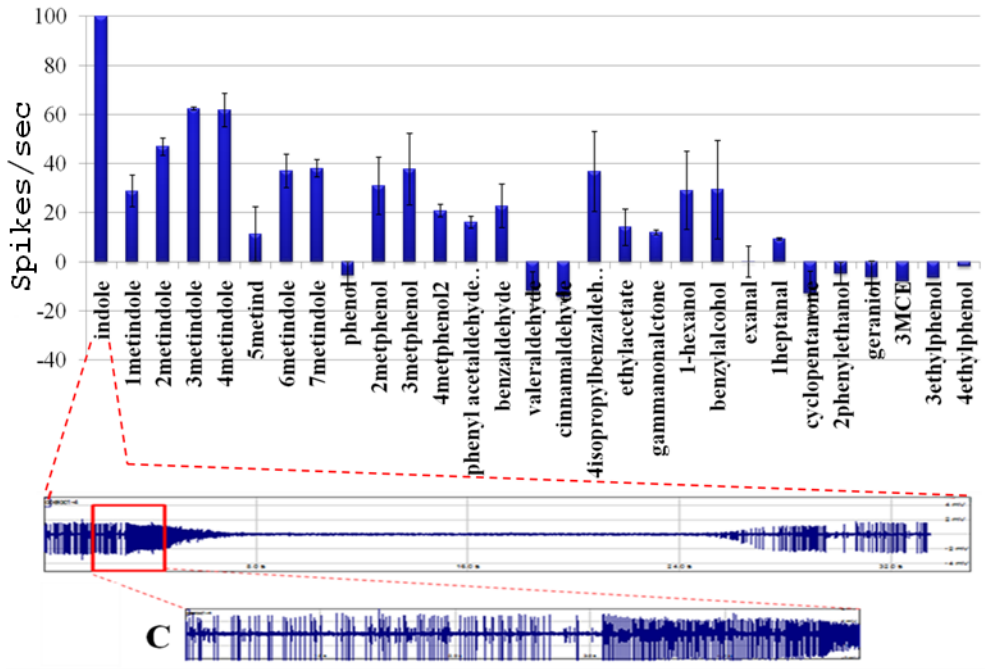

Fig.23: AalOR2 is narrowly tuned to indole (A) ab3A neuron in the Delta halo mutant was challenged with the panel of odors reported in Tab.2. Stimuli were presented by placing the tip of the pipette through a hole in a tube carrying a purified air stream directed at the fly and administering a pulse of charcoal-filtered air through the pipette containing the odorant. Each odor was applied at a 10^{-4} dilution with a pulse duration of 0,5 sec. Stimuli were used for a maximum of 3 presentation. Response were quantified by subtracting the number of impulses 0,5sec of unstimulated activity from the number of impulse in the 0,5sec following odorant stimulation, subsequently all responses were normalized to indole maximum response (>170 spikes/sec) and are presented as mean. This normalization allowed me to assess responses among different chemical groups, defining that AalOR2 interacts with some aromatic compounds, although indole triggered a narrow strong activation of AalOR2, **(B, C**) Firing rate of the AalOR2-"empty neuron" in response to indole; it is clearly evident that the excitatory response is very strong, producing an increasing in the number of spikes in one second. The spontaneous firing rates of the ab3B neuron, that resides in the same sensillum, is also visible as small spikes. n= 6; Error bars=SEM

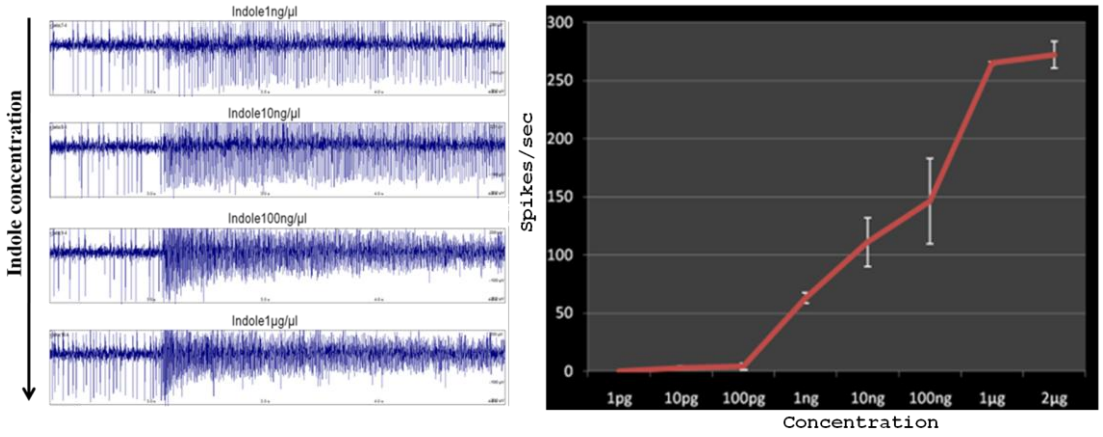

Fig.25: Dose dependent response to indole. On the left side are showed the firing rates of AalOR2-"empty neuron" in response to increasing concentrations of indole ranging from 1 ng to 2 µg. Indole led to an increase in spikes frequency directly proportional to its concentration, suggesting its specificity of action. On the right side, the same responses are reported in a graph. n=6. Error bars= SEM

consists of a pump connected to a plastic bag containing the organic sample described above. The pump evacuated the volatiles through a filter, where they were concentrated (Fig.25C). Volatile compounds absorbed on the filter were extracted with HPLC-grade dichloromethane and subsequently used in the gas chromatography-linked Single Sensillum Recordings followed by mass spectrometry (Fig.25D). The mass-spectra of each active component of the blends have been compared with a mass-spectra library of known molecules, with the aim to identify natural key ligands of AalOR2 (Fig.25E). As shown in Fig.25F, in the organic samples I used, were contained at least 3 molecules able to elicit as many responses of AalOR2-"empty-neuron", (red arrows in Fig.25E). Nevertheless, I did not identify any compounds contained in my sample extract, because their mass spectra did not show a high percentage of identity with any other compound present in the mass spectra databases. The only chemicals identified, as shown in Fig.25E, were undecane and (-)Menthone. Among these chemicals, by chance, but luckily, I choose to use pure (-)Menthone, due its availability in Laboratory (black arrow in Fig.25E), also if it did not correspond to any peak able to trigger a neuronal response. (-)Menthone (2*S*, 5*R*-*trans*-2-isopropyl-5-methylcyclohexanone), is an organic compound with a molecular formula $C_{10}H_{18}O$. Unexpectedly, when I used pure (-)Menthone, down to a concentration of 100ng/µl, in SSR, I obtained a strong inhibition of the neuronal activity (Fig.26), that was highly specific. Inhibition of olfactory receptors by odor molecules is widely documented. Many years of works have clearly indicated that each olfactory receptor can be activated or inhibited by several olfactory molecules and that a single olfactory molecule can activate or inhibit different odorant receptors (Carey AF *et al.*, 2010; Hallem EA and Carlson JR, 2006). Based on this consideration, my results clearly indicate that AalOR2 is tuned to indole and inhibited by (-)Menthone, in absence of other odorants.

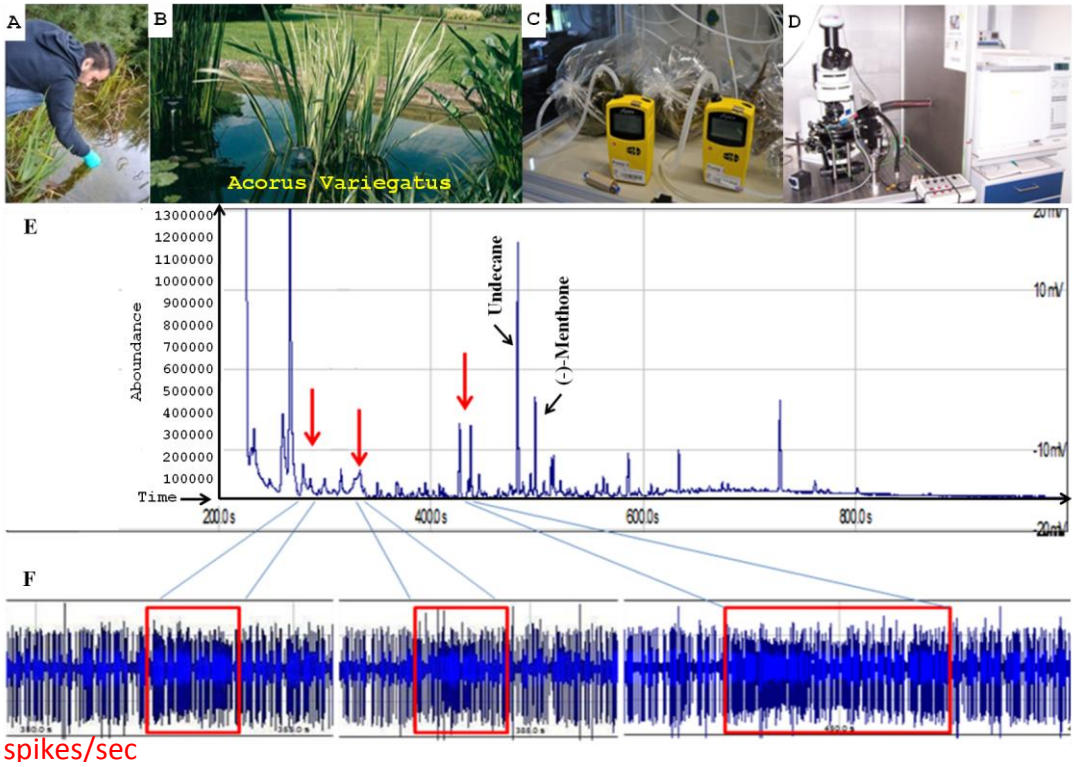

Fig.25: GC-SSR assay. (A,B) Images of *A. variegatus*, used in the experiments described in the text, **(C)** Headspace volatile collection system, used to collect odors from a given sample**.** Odor collection obtained in **(C)** is injected in the gas chromatography-linked single sensillum recordings **(D). (E)** Gas chromatography analysis of the odor collections with respect to **(F)** firing rates of AalOR2-"empty-neuron" obtained by single sensillum recording.

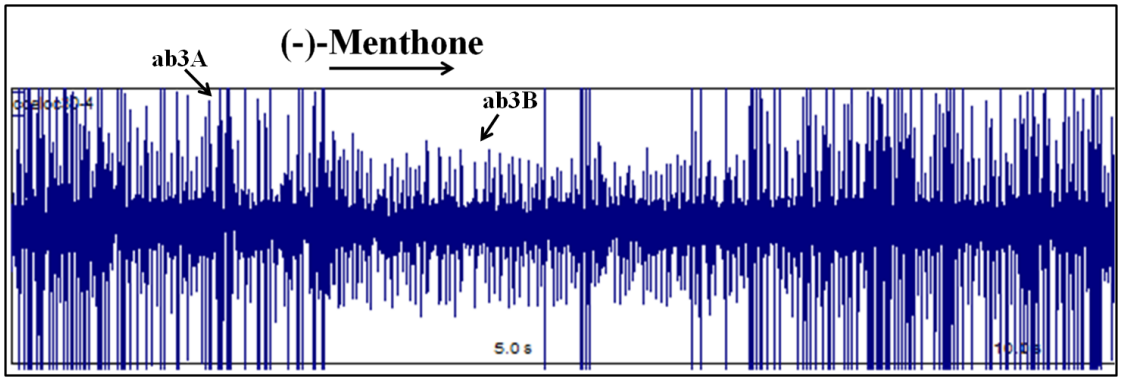

Fig. 26: Inhibitory effect of (-)Menthone on AalOR2-"empty-neuron". Firing rates in response to (-)Menthone indicate a decreasing in the number of large spikes- corresponding to ab3A neuron- in one second of activity. Small spikes, indicated by an arrow, belong to ab3B neuron.

Ca^{2+} imaging measurement with Fura2/AM in HEK293 cells

During the time I spent in the Prof Hansson‘s Lab, I had the opportunity to perform also Ca^{2+} imaging experiments on a heterologous system, by using a more sensitive technique with respect to that used at Arterra Biosciences. In these Ca^{2+} imaging experiments, 5 x 10^5 HEK293 cells (Human Embryonic Kidney 293 cells) were seeded in a single dish. After 24 hours, cells were co-trasfected with pHM6/HA-AalOR2 plus pHM6/DmOR83b in transient. Reagent used for transfection was Roti Fect Plus (Carl Roth), and the ratio used between Roti Fect Plus and DNA was 5:1. The amount of DNA used was 1µg for each receptor. 48 hours after transfection, when the production of the exogenous protein was highest, 2µM Fura2/acetomethylester (Invitrogen) were loaded in the dish containing cells and incubated for 20 minutes in the dark. After this time, medium containing Fura2 calcium dye was removed, and 2mL of SES solution (Standard External Solution containing -in mM- 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, pH 7.4) was added to the cells. To perform a comparative analysis with the results obtained through the SSR assay, I used the same panel of odorants reported in Tab.3. These were dissolved in DMSO and were applied into the dish using a microsyringe to a final concentration of 1µM/0,1% DMSO. Free intracellular Ca^{2+} concentration was determined using the 340/380 excitation ratio for Fura-2, and the fluorescence images were acquired using a cooled CCD camera controlled by TILL Vision software. Such as for SSR experiments, also for Ca^{2+} imaging, responses were normalized by defining the maximal odorant response, among all olfactory molecules, as 100% of the calcium increasing. Also in this assay, AalOR2 was able to interact with a set of aromatic compounds, such as 3-methylindole, benzaldehyde, 2-methylphenol, with indole producing the highest increase of intracellular Ca^{2+} concentration (Fig.27). After its use in SSR experiments, also in this assay I used (-)Menthone. I repeated the experiments three times and also in this case, as

expected, with this molecule I did not obtain any increment in the intracellular Ca^{2+} concentration.

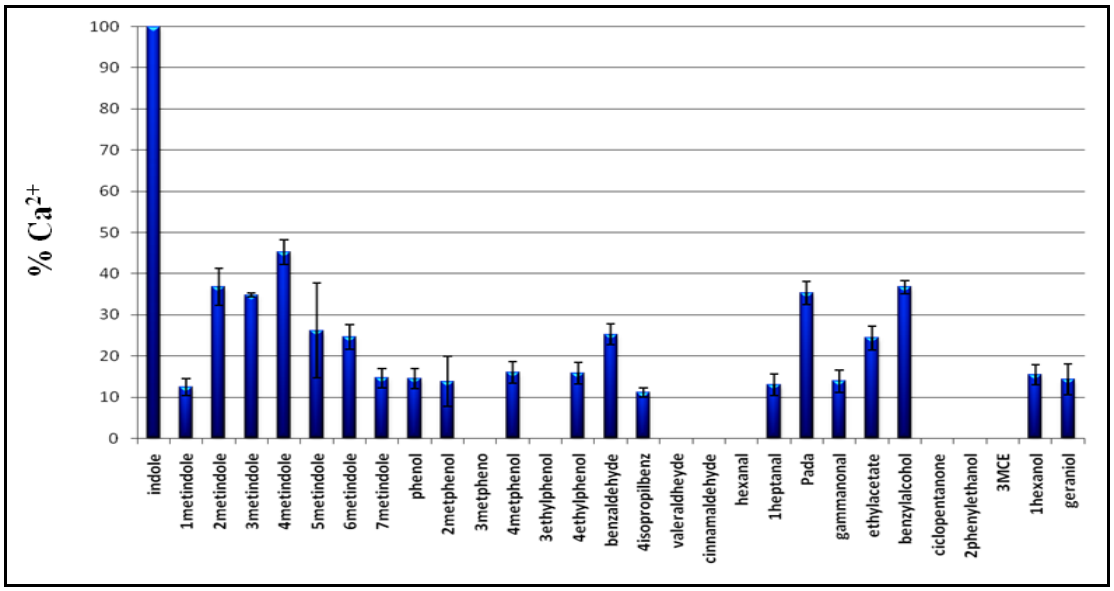

Fig.27: Quantification of the intracellular Calcium ($[Ca^{2+}]_i$) in HEK293 cells that expressing AalOR2 along with DmOR83b. Free intracellular Ca^{2+} concentration was determined using the 340/380 excitation ratio for Fura-2. For Ca^{2+} imaging, responses were normalized by defining the maximal odorant response, among all olfactory molecules, as 100% of the calcium increasing. This result clearly show that AalOR2 is narrowly tuned to indole, and that responds with lower sensitivity to other methylindoles, 2 methylphenol and benzaldhyde, as previously reported by SSR assay. Each measurement was repeated three time. Error bars= SEM

Discussion

To acquire nutrients for their eggs, female mosquitoes feed on human blood. During this process, the most dangerous mosquito species, such as *A. gambiae*, *C. pipiens* and *A. aegypti* unwittingly transmit parasites that cause serious diseases, so threatening most of the world's population. These diseases include malaria, dengue, yellow fever and some other. Nowadays, it is not clear how these insects find their victims, but a growing number of recent papers indicate that the Odorant Receptors play a key role in host selection as well as in other behaviors underlying the mosquito vectorial capacity. The functional characterization of ORs by these mosquito species becomes therefore very important, because it could lead to develop novel olfactory-based strategies for their management. Although long considered a secondary vector of viruses, another mosquito species has recently suggested to play a role in the arboviruses transmission on the Indian Ocean Islands. This species, *A. albopictus*, also known as tiger mosquito, belongs to the Culicinae subfamily, and represents an invasive species that can be found on all continents in the old and new world. This anthropophilous mosquito, originating from the tropical forest of the Southeast Asia, is able to adapt to most climates, and in the last few years has spread to all regions in Italy. Its rapid diffusion is due to its strong ecological plasticity; it is able to recolonize tree holes in forests and small water tanks in the urban areas after being transported to a new region, thus making it hard its control. In addition, the winter season induces its eggs to diapause, allowing them to survive at adverse conditions. The advent of *A. gambiae*, *A.aegypti* and *C. pipiens* mosquitoes' genome sequence led to the identification of ORs in these species and to functional characterization of many ORs. Differently from these well known mosquitoes, nothing is known at molecular level for *A.albopictus.* In order to search for molecular targets that could used for developing *A. albopictus* attractants or repellents, I cloned, by using a molecular approach, the first OR

for this mosquito, named AalOR2, and I functionally characterized it. To date, orthologs of this receptor have been reported for *A. gambiae, A aegipty* and *C. pipiens*. Despite the insect ORs display a high degree of divergence, the three OR2 horthologous chraracterized to date share an average of 75% amino acidic identity. Last year, several authors (Bohbot JD *et al.*, 2011; Pelletier J *et al.*, 2010) suggested that this sequece homology was strictly correlated to an odorant specificity. Infact, it has been reported that the highly conserved AgOR2, AaOR2 and CpOR2 orthologs share a similar narrow response to indole. On the basis of these results Bohbot and collaborators (Bohbot JD *et al.*, 2011) hyphotized that this high sensitivity to indole could represent an ancient ecological adaption preserved because in some way important for the mosquito life cycle. However, the same authors also reported the identification of OR2 orthologs from additional zoophilic and anthropophilic mosquito species, suggesting that the role of mosquito OR2 did not seem to be strictly associated with host selection. My results further stress this high degree of conservation previously reported, since AalOr2 shares with AaOr2 96% of amino acidic identity. In addition, as previously reported for AaOR2, my data show that in *A. albopictus* OR2 is expresed in antennae of larvae and of male and female adults. However, differenlty from that reported for *A. aegipty* (Bohbot J et al, 2007) my results indicate that this gene is more expressed in antennae of adult females and that is up-regulated after a blood meal, suggesting that AalOR2 could have an important role for the females in searching oviposition right places. Such as the other OR2 orthologs, also AalOR2 is narrowly tuned to indole, as detected by its expression in mammalian cells and in the *D. melanogaster* "empty neuron"; moreover, my data also show that, such as the other OR2 ortologs functionally characterized to date, AalOR2 responds with lower sensitivity to other methylindoles, 2 methylphenol and benzaldhyde, so confirming further a structural and funtional conservation of the mosquito OR2 orthologs. Indole, that constitute nearly 30% of the volatile headspace of human sweat (Meijerink J *et al.*,

2001), is a ubiquitous volatile compoud that has been linked to host seeking, and oviposition in aedine (Syed Z and Leal WS, 2009; Siju KP et al, 2010) as well as in anophelinne mosquitoes (Lindh JM 2008). Given that, in my experiments, AalOR2 resulted up-regulated 12 hrs after a blood meal, I speculated that this receptor was more likely specifically involved in the reception of oviposition attractants, doing mine the hyphothesis of Bohbot and colleagues (Bohbot J *et al.*, 2011) that indole reception can facilitate mosquito orientation toward key ecological resources using an ancient olfactory mechanism. Oviposition attractants are environmental cues that allow mosquito gravid females to locate suitable sites for egg-laying. For this reason, they could be used for environmetally friendly ”attract and kill” strategies to control mosquito populations. In order to detect more chemicals naturally occurring in *A. albopictus* oviposition sites, I performed a GC-SSR experiments on infusions of *Acorius variegates* leaves in water, based on the idea that the semi-aquatic habitat, where this plant grows, was a suitable area for laying eggs. However, this analysis did not give me the opportunity to detect novel natural ligands, because all molecules able to trigger a spike frequency increase did not fit any other compounds in mass spectra databases. Nonetheless, I obtained an unexpected, very interesting result; (-)Menthone, a component of my organic infusion, identified in Gas Chromatography, produced a inhibitory effect on the AalOR2 expressed in the “empty neuron” of *D. melanogaster*, in absence of applied odorants. (-)Menthone is an organic compound belonging to the ketone family and is a component of some essential oils such as *Mentha microphilla* that have insecticidal properties (Traboulsi AF *et al.*, 2002). The inhibition of odorant receptors by olfactory molecules has been widely reported; for example, Carlson and colleagues found that 6-MHO, that is a fly repellent produced by cows, inhibited AgOR1 and activated AgOR21 (Carey AF *et al.*, 2010). It is now well accepted that certain odorants activate some receptors but inhibit others, indicating that responses to odorsare regulated at the antenna level. This idea can lead to fresh

strategies in which mosquito attractants or repellents could be developed on the basis of the ability of test molecules to bind OR proteins. More studies aimed to better uderstand the way of action of (-)Menthone in the presence and in absence of applied odorants, such as of activating chemicals, its activity on the other OR2 orthologs and its function on other characterized ORs, as well as its effect on behaviors of *A. albopictus* larvae, are planned for the next future.

Materials and methods

Mosquito rearing and blood feeding

Aedes albopictus (Napoli strain) embryos were generated in-house and disinfected with 0,05% sodium hypocholorite prior to hatching in flat plastic pans with distilled water. Larvae were reared on a diet of ground Whiskas Original Recipe cat food (Kalkan Inc. USA) that was applied to the surface of the water. Pupae were transferred to plastic cups in one-deciliter plastic containers, where newly emerged adults were collected the following morning. Adult mosquitoes were maintained in one-deciliter plastic containers at 27°C with 75% relative humidity under a 12:12 h photoperiod and provided with a 10% destrose solution. 4-5-days-old adult females were blood-fed on human volunteers using standard protocols.

Conservation analysis of mosquito ORs

To identify hypothetical orthologous OR2 in mosquito species, individual members of the 18 Aedes/Anopheles orthologous subgroups identified by Bohbot and collaborators (Bohbot JD. *et al.*, 2007) were used as probes against mosquito specific transcribed sequences present at the http://www.vectorbase.org/ site. This analysis led to identify olfactory receptors, such OR2, OR10, OR8 and OR49, sharing a high percentage of aminoacid identity (Tab.1), among three mosquito species, as recently confirmed by Julien Pelletier (Pelletier J. *et al.*, 2010).

Identification and cloning of AalOR2

The following degenerate primers were designed using the better conserved region on the aligned protein sequences of the *A. gambiae*, *A. aegypti* and *C. pipiens* OR2 orthologs, obtained from http://www.vectorbase.org/ database:

Fw1deg: TGGYTNTTYTGGWSNTAYYT

Fw2deg: GGNTAYTTACNGTNYTNTAYTT

Rw1deg: TGRAACATYTCNARNGTCAT

Rw2deg: CATRAADATRTANSWNCCDATCAT

Molecular techniques were carried out according to general protocol reported in Sambrook *et al.*, 1989. Adult antennae and larval heads were manually dissected from animals anesthetized with ether, immediately frozen in dry ice and subsequently processed. Total RNA was extracted by using TRI Reagent (Sigma) according to the manufacture's instructions. Enriched poly(A)$^+$ RNA was prepared using QuickPrep Micro mRNA Purification Kit (GE Healthcare), following the manufacture's instructions. 2 µl of RNA were loaded on 1% agarose gel and quantized using a RNA marker (Fermentas) as reference, through the Gene tools (Perkin Elmer) software. 0.3 to 1 µg of RNA were retro-transcribed into cDNA using the enzyme Reverse Transcriptase (Fermentas) and 500 ng of anchor primer-dT (GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTT). The PCR reactions with degenerate primers were carried out in a 50µl final volume containing 0.2 mM deoxynucleotides (Fermentas), 1 unit of Phusion High-Fidelity DNA Polymerase (Finnzymes) and 2.5 mM primers in the correspondent buffers. Two percent of the synthesized cDNA was amplified by PCR with degenerate primers, with the following cycle conditions:

98°C for 2’ (initial denaturation)

98°C for 10”
from 56°C to 62°C for 30” } 35 cycles
72°C for 30”/1 kb

72°C for 10’ (final extension).

All amplicons were analysed by electrophoresis in agarose gels. The amplified fragments were cloned using the pGEMT-easy cloning vector (Promega) following the manufacturer’s instructions, and sequenced at PRIMM Biotech.

5'RACE and 3'RACE Analyses

5'RACE and 3'RACE analyses were performed using the 5´/3´ RACE Kit 2nd Generation (Roche) according to the manufacture’s instructions.

3’RACE

cDNA obtained from retro-transcription of the poly(A)$^{+}$ enriched RNA, extracted from manually dissected heads of 4th instar larvae using anchor primer-dT, was used for PCR reactions with anchor primer in combination with the following primers:

3’RACE 1Fw: TTCGGACGTCGTTCCTAATG;
3’RACE 2Fw: GCAAGGATTCTGTCCAAGTCGA;
3’RACE 3Fw: GCGTCGCCAATTTACGAAATTG;
3’RACE 4Fw: GAGTGTCTCCAATATCACGAGG;
3’RACE 5Fw: GTTGAGCATCAGCAATCAGCTG.

Cycles condition:

98°C for 2’ (initial denaturation)

98°C for 10”

56°C; 58°C; 60°C; for 30” (depending on the primer temperature) } 35 cycles

72°C for 30”/1 kb

72°C for 10’ (final extension).

Amplicons were cloned and analized as described above.

5’RACE

0,3-1µg of enriched poly(A)$^+$RNA was reverse-transcribed with the specific 5´RACE1Rev primer AGCAGCCGATCATTACCATCTG. A homopolymeric A-tail was added at the 3´end of this first-strand cDNA by using 1 µl of recombinant Terminal Transferase (80U/µl) and 2.5µl dATP (2mM). On this template, nested PCR reactions were performed using anchor primer-dT in combinations with the following primers:

5’RACE 1Rv: AGCAGCCGATCATTACCATCTG

5’RACE 2Rv: TTATCCTCGCAAATAGCGACCG

5’RACE 3Rv: CTGCAGCACGAACACAATTTCG

5’RACE 4Rv: CAGAGGATAGGTCACGAAGCAA

5’RACE 5Rv: TTCGAACTTGCCTCGGTTTCCCAT

Cycle conditions:

98°C for 2’ (initial denaturation)

98°C for 10”

58°C; 60°C: 62°C for 30”(depending on the primer temperature) } 35 cycles

72°C for 30”/1 kb

72°C for 10’ (final extension).

Amplicons were cloned and analized as described above.

The AalOR2 full length CDS was obtained by a PCR reaction on the same cDNA used in the 3'RACE analysis using the following primer combination:

5'AaOR2Fw: ATGTTGATAGAAAATTGTCCA

3'AalOR2Rv: TTAATTATAAACTCTCCGAAGC

Cycle conditions:

98°C for 2' (initial denaturation)

98°C for 10"
60°C for 30"
72°C for 30"/1 kb
} 35 cycles

72°C for 10' (final extension).

Amplicons were cloned and analized as described above.

Cloning of genomic sequence of AalOR2

Genomic DNA was prepared starting from about ten *A. albopictus* larvae through the Mammalian Genomic DNA Miniprep Kit (Sigma) following the manufacture's instructions. On 100 ng of this template a PCR reaction was performed with the following primer combination:

5'AaOR2Fw: ATGTTGATAGAAAATTGTCCA

3'AalOR2Rv: TTAATTATAAACTCTCCGAAGC

Cycle conditions:

98°C for 2' (initial denaturation)

98°C for 10"
60°C for 30"
72°C for 30"/1 kb
} 35 cycles

72°C for 10' (final extension).

Amplicons were cloned and analized as described above.

Semiquantitative RT-PCR analyses

To examine the AalOR2 modulated expression in response to a blood meal, a semiquantitative RT-PCR assay was performed by using QuantumRNA™ 18S Internal Standards (Ambion). Total RNA was extracted from about 20 manually dissected antennae of adult males, from about 20 manually dissected antennae of adult females before a blood meal and from about 20 manually dissected antennae of adult females 12hrs after a blood meal using SV Total RNA Isolation System. The RNA samples were subjected to treatment with DNase I and quantized according to the procedures described in the text. 300 ng of total RNA were reverse-transcribed by using the Reverse Transcriptase enzyme (Fermentas) with Random hexamers in a final volume of 20µL. RT-PCR reactions were performed using the universal primer pair 18S primer / competimer (Ambion) in a 4:6 ratio and an equimolar quantity of 3´RACE1Fw/5´RACE1Rev primers, with the Eurotaq polymerase enzyme (EUROCLONE) and the the following cycle parameters:

95°C for 2’ (initial denaturation)
95°C for 10”
56°C for 30” } 35 cycles
72°C for 1’/1 kb
72°C for 10’ (final extension).

The PCR products were separated on 1.5% agarose gel, visualized using the Geliance instrument (Perkin Elmer) and analyzed using a densitometer Gene Tools software.

Elisa assay

The ELISA assay is based on a specific immune recognition; in my hands, the N-terminus of the receptor is recognized by an anti-HA primary antibody,

recognized in turn by a secondary antibody conjugated to an enzyme whose activity can be easily detected by a colorimetric reaction.

The AalOR2 CDS region was cloned in pHM6/HA expression vector in frame with the HA (haemagglutinin) epitope at the N-terminus. The CDS was transcribed on the pGem/AalOR2 clone, by using the HindIII-AalOR2 (AAGCTTGTTGATAGAAAATTGTCCAATCA)/AalOR2 3'end primer pair and the following cycle conditions:

98°C for 2' (initial denaturation)

98°C for 10"
60°C for 30"
72°C for 1'/1 kb
} 30 cycles

72°C for 10' (final extension).

The amplified fragment was cloned in pGEMT-easy to generate the pGEMT-easy/HindIIIAalOR2 clone. Subsequently, this clone and the pHM6/HA vector were digested with the HindIII and EcoRI restriction enzymes and run on agarose gel 1% to elute the HindIIIAalOR2 fragment and the linearized pHM6/HA vector. Their final ligation, by using T4 DNA ligase, allowed to obtain the desired pHM6/HA-HindIIIAalOR2 clone (Fig.16).This plasmid was extracted with the Plasmid Midi Kit (Qiagen) and an amount from 25 to100 nanograms was used to transfect 1.5 x 10^4 CHO-K1 (Chinese hamster ovary) cells. Cells still attached to the surface of the 96 well plate were washed with a buffer containing 1x PBS, 0.5 mM $CaCl_2$, 1mM $MgCl_2$ for 20 minutes and fixed in 4% formaldehyde. After two washes with the same buffer, the fixed cells were incubated with a 1:500 dilution of primary antibody anti-HA (Santa Cruz rabbit polyclonal Biotechnology) in 1% BSA for two hours. At the end of the incubation period, cells were washed three times to remove excess unbound primary antibody, and incubated with secondary antibody conjugated with β-galactosidase (goat anti-rabbit IgG beta-GALACTOSIDASE) for about one hour. After this second incubation, cells were subjected to further series of washes and incubated with the substrate of β-galactosidase, the CPRG

(Chlorophenolred-B-Dgalactopyranoside) (Roche Diagnostics). The color reaction, that developed as a result of degradation of the substrate chromogen, was detected by reading absorbance at 550 nm of the samples after an incubation of about five hours through the instrument Victor3 (PerkinElmer). This assay was done by using the pHM6/HA-AalOR2 vector alone or by cotrasfecting the cells with pHM6/HA-AalOR2 along with pHM6/DmOR83b.

Immunofluorescence assay

Immunofluorescence is a technique allowing the visualization of a specific protein in cells by binding a specific antibody chemically conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC). In my experiments, 3 x 10^5 CHO-K1 cells were seeded in a 6 well plate over a sterilized slide placed at the base of each well. After 24 hours, cells were transfected with pHM6/HA-AalOR2 alone, and co-trasfected with pHM6/HA-AalOR2 plus pHM6/DmOR83b, using the Lipofectamine 2000 (Invitrogen). 48 after transfection cells were fixed in 4% paraformaldehyde in PBSCM (1X PBS + 0.5 mM Ca^{2+} + 1 mM $MgCl_2$) for 15-20 minutes at RT. Subsequently cells were washed with PBSCM to remove the excess of paraformaldehyde. After washing with PBSCM, fixed cells were incubated with HA primary antibody (*rabbit polyclonal* Santa Cruz Biotechnology) (used to recognized the HA-tag) diluted 1:500 in Blocking buffer composed of PBSCM, 2% BSA and 0.1% Triton. Subsequently cells were washed with PBSCM to remove the excess to primary antibody. In turn, a second reaction was carried out with a secondary antibody fused with FITC (*goat anti-rabbit* IgG FITC Santa Cruz Biotechnology) diluted 1:250 in Blocking buffer able to recognize the complex primary HA-antibody/HA-OR. The observations were done at IGB-CNR with a Leica SP2-AOBS Confocal Microscope by using a 63X oil immersion objective equipped with specific FITC filter.

Policlonal CHO-K1 cells line expressing AalOR2 plus DmOR83b

CHO-k1 cells were seeded in a 6 well plate (3 x 10^5 cells per well). After 24 hours cells were co-transfected with pHM6/HA-AalOR2 plus pHM6/DmOR83b, using 500ng for each receptor and Lipofectamine 2000 transfection reagent. 48 hours after the transfection, cells were treated with 600µg/mL of neomycin antibiotic. The chimeric expression vectors brought the resistance to the antibiotic neomycin; so, by treating cells after transfection with this antibiotic, the only cells that have incorporated the receptors were able to survive. This polyclonal CHO-K1 cell line was grown in DMM-F12 medium (Lonza) plus 10% FBS, at 37°C and 5% CO_2 plus 600µg/mL of neomycin antibiotic. The expression of AalOR2 and DmOR83b in this polyclonal cell line was constantly checked by RT-PCR on mRNA extracted from the cells using the GenElute™ mRNA Miniprep Kit (Sigma). At least 1µg of this RNA was retro-transcribed with primer-dT by using the Reverse Transcriptase (Fermentas) enzyme in a final volume of 20µL. 1µL of this cDNA was used in a PCR reaction with a pair of specific primers for each receptor.

Calcium imaging assay through Fluo3/AM.

In Fluo3/AM Ca^{2+} experiment, at least 1x10^6 policlonal cells were detached from flask by using anon-enzymatic solution (Sigma), centrifuged at 1.000 rpm, washed and resuspended in 2ml of HBSS solution (136 mM NaCl, 5.3mM KCl, 0.4mM $MgSO_4x7H_2O$, 0.5mM $MgCl_2x6H_2O$, 0.34mM $Na_2HPO_4x2H_2O$, 0.44mM KH_2PO_4, 5.5mM Glucose, 4.1mM $NaHCO_3$, 1.2mM $CaCl_2$, 2% FBS, 10mM Hepes, pH 7.4), in order to have about 500.000 cells/ml. After adding 2.5 µM Fluo3/AM calcium dye, 0.02% pluronic and 2.5mM probenecid, cells were incubated in the dark at 37°C, 5%

CO_2 for 45 min. Ca^{2+} imaging experiment was executed by using EnVision Multilabel Plate Readers (PerkinElmer). During the incubation time, an empty 96 well plate was loaded with 1µM of each olfactory molecule reported in Tab.2, in triplicate. After the incubation with the Fluo3/AM calcium dye, the 96 well plate was inserted into EnVision Multilabel Plate Readers (PerkinElmer) that automatically recorded the calcium variation for each odorant molecule.

Drosophila melanogaster **Stocks**

Drosophila stains were maintained on standard food. Flies used in my experiments were the following:

Δ*halo strain* (kindly provided by John R.Carlson Yale University): w; Δhalo/CyO;Dr/TM3,Sb

UAS-mCD8-GFP strain (Drosophila Stock Center -Bloomington, IN): P{w[+mC]=UAS-mCD8::GFP.L}LL4, y[1] w[*]; Pin[Yt]/CyO.

Or22a-Gal4 strain (Drosophila Stock Center -Bloomington, IN): w[*]; ;P{w[+mC]=Or22a-GAL4.7.717}14.2

UAS-AalOR2 strain (obtained in our laboratory): w; CyO/If; TM3,Sb/UAS-AalOR2.

To obtain the *UAS-AalOR2* transgenic strain, the entire ORF of AalOR2 was cloned into the pUAST vector (Brand and Perrimon, 1993), in frame with the Cavener sequence. This plasmid was extracted with Plasmid Midi Kit (Qiagen) and injected by Genetic Services (http://www.geneticservices.com/injectionservices.htm) into the w^{1118} strain. This transgenic construct was sequentially crossed with the Δhalo,UAS-mCD8-GFP and Or22a-Gal4 strains in order to obtain the final strain P{w[+mC]=UAS-mCD8::GFP.L}LL4, y[1] w[*];Δhalo /Δhalo;

P{w[+mC]=Or22a-GAL4.7.717}14.2/ UAS-AalOR2, that was used for the following analysis.

Electrophysiological recordings (Single Sensillum Recording)

A 5- to 15-day-old fly was mounted in a truncated pipette tip with the antenna protruding from the narrow end. The pipette tip was fixed with wax on a microscope slide, and the antenna gently placed on a cover-slip and stabilized with a glass electrode (Clyne *at al.*, 1997; Stensmyr *et al.*, 2002). The antennal surface was observed at a 1000x magnification, which allowed individual sensilla to be clearly resolved, through an Olympus BX51 microscope fitted with fluorescence optics to view GFP. Action potentials of the ORNs in the sensillum were recorded by placing an electrode through the sensillum wall into contact with the lymph that bathes the dendrites. For the recording electrode, a glass capillary with the tip drawn to 1µm diameter was filled with sensillum lymph ringer (Kaissling and Thorson, 1980) and slipped over an AgCl-coated silver wire. The indifferent electrode was filled with Ephrussi and Beadle solution (Ashburner, 1989) and was put into the eye. The extracellular analog signals originating from the OSNs were amplified 1000 times, digitally converted via Syntech IDAC-4 USB and visualized by Syntech Autospike 3.2. The signal was also fed to a loudspeaker for audio monitoring. Recording of action potential were stored on the PC and all analysis was done with AUTOSPIKE software. Separation of activity of collocated ORNs in single sensillum was based on differences in spike amplitude. The ORN with the largest spike amplitude corresponded to neuron A. The odor stimulation duration was 0.5 s.

Gas-Cromatography-SSR

This technique has been used to identify novel ligands of ORNs in a large number of insect species. An organic infusion of A variegates leaves was injected onto a GC-column. The column was located in an oven where it was possible to regulate its temperature. As the temperature of the column was increased the components of the extract were separated while traveling down the column and exited the GC set-up. The separated components of the extracts encountered the single sensillum from which a stable electrical contact was established. Responses of the ORNs housed in a single sensillum to the extract components were recorded. The chemical identity of the response eliciting component(s) was identified using mass spectrometry (MS) (*e.g.* Stensmyr *et al.*, 2003).

Calcium imaging assay through Fura2/AM in HEK293 cells.

In these Ca^{2+} imaging experiments, 5 x 10^5 HEK293 cells (Human Embryonic Kidney 293 cells) were seeded in a single dish. After 24 hours, cells were transiently co-transfected with pHM6/HA-AalOR2 along with pHM6/DmOR83b. The reagent used for this transfection was Roti Fect Plus (Carl Roth), and the ratio used between Roti Fect Plus and DNA was 5:1. The amount of DNA used was 1µg for each Odorant Receptor. 48 hours after transfection, when the production of the exogenous protein was highest, 2µM Fura2/acetomethylester (Invitrogen) were loaded in the dish containing cells and incubated for 20 minutes in the dark. After this time, medium containing Fura2 calcium dye was removed, and 2mL of SES solution (Standard External Solution containing -in mM- 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose, pH7.4) was added at the cells. Olfactory molecules were dissolved in DMSO and applied in the dish using a microsyringe to a final

concentration of 1µM/0,1% DMSO. Free intracellular Ca^{2+} concentration ($[Ca^{2+}]$) was determined by using the fluorescence ratio method (340/380), and the fluorescence images were acquired using a cooled CCD camera controlled by TILL Vision software

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