Neuroethology of Olfaction in *Drosophila*

Evolution and Specialization

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Abstract

In insects olfaction is a primary sensory modality. As a result changes in the animal's ecology are often paralleled by modifications in the olfactory system.

Using a comparative approach between the generalist *Drosophila melanogaster* and its sibling specialist *D. sechellia*, I looked at the coding properties of the olfactory system.

Using electrophysiology, neuroanatomy, and behavioral assays we demonstrate how the olfactory system of adults of specialist fruitfly *Drosophila sechellia* has evolved to accommodate its unique preference for *Morinda citrifolia* fruit. We show that the fly has expanded the number of one antennal sensillum type inhabited by two neurons sensitive to *Morinda* volatiles. The numerical increased has caused the formation of a macro glomerular complex tuned to fruit volatiles. Accordingly, the olfactory preference of the species for these odors and combinations thereof has radically changed.

We subsequently show that also larvae of this species changed their olfactory preference. With such a simple olfactory circuitry, consisting of only 21 olfactory neurons, the identification of the factor underlying the switch is especially promising.

Finally, we looked what olfactory information is conveyed to the brain of the fruitfly via an evolutionarily old olfactory subsystem, that of coeloconic sensory neurons. These neurons express ionotropic receptors (Irs) instead of conventional olfactory receptors (Ors), and natural ligand for this set of receptors have been poorly investigated. We identified three new ligands biologically active for coeloconic neurons, and investigate the significance of these compounds in odor coding and in fly attraction.

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I   Dekker, T., Ibba, I., Siju, K.P., Stensmyr, M., and Hansson, B. S. 2006 Olfactory shifts parallel superspecialism for toxic fruit in *Drosophila melanogaster* sibling, *D. sechellia*. Current Biology 16, 101-109.

II Ibba, I., Angioy A.M., Hansson, B. S., Dekker, T. A macroglomerular complex for fruit odors radically changes blend preference in *Drosophila*. Submitted.

III Ibba I., Hansson B.S., Birgersson G., Dekker T. Angioy A.M. Identification of natural ligands for OSNs in the coeloconic sensilla of *Drosophila melanogaster*. Manuscript.

IV Ibba I., Hansson B. S., Hill S. R., Angioy A. M., Dekker T. Olfactory specialisation of *Drosophila sechellia* larvae. Manuscript.

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Objectives

The objective of this thesis was to investigate 1) how the olfactory system of a specialist species of *Drosophila* has evolved, 2) how the olfactory processing translates into appropriate behavioral responses, 3) how natural ligands for a specific class of odorant receptors are coded.

Introduction

Chemical senses are represented by the sense of taste and smell, depending on whether the stimulus is detected through contact or airborne. Taste and olfaction are the most ubiquitous sensory systems in the animal kingdom, being present in one form or another in nearly all air, water and land-dwelling creatures.

The sense of smell is probably the oldest sensory modality in the animal kingdom (Strausfeld & Hildebrand, 1999) and plays a central role in almost all tasks such as location of food, enemies and mates. Olfaction also serves as an important model system in neuroscience. In fact, the importance of olfaction to life and health was recognised by the award of the 2004 Nobel prize in Physiology or Medicine to Linda Buck and Richard Axel for their discovery of olfactory receptor genes and thus giving a vital contribution to the understanding of the olfactory organization (Buck & Axel, 1991).

In terms of biomass insects are the most important group of terrestrial animals. Since they arose 400–420 MY they adapted very well to

environmental changes, invading every niche except the benthic zone (Grimaldi & Engel, 2005). Insects are equipped with highly evolved sensory systems, which parallel in many ways those in vertebrates. The relative simple organization of insect neuronal circuits along with the short generation time and high reproductive rate of insects makes them excellent models to study olfaction. Comparisons between the olfactory pathways in vertebrates and insects have revealed striking similarities of functional organization, physiology and development, suggesting that olfactory information is processed through neural mechanisms more similar than different (Hildebrand & Shepherd, 1997; Strausfeld & Hildebrand, 1999; Ache & Young, 2005). Moreover research on the insect olfactory system has contributed to the control of insects that can be harmful to human health and agriculture. But also as it helps improving the positive functions that insects can have both for ecosystems as natural enemies and for humans as pollinators and honey producers (karg & Suckling, 1999).

Drosophilids, particularly *Drosophila melanogaster* was chosen as a genetic animal model at the beginning of 20th Century by Thomas Hunt Morgan (Sturtevant, 1965; Kohler, 1994). Since then it has been a very successful animal model for biological research.
An important reason for using *Drosophila* as experimental model is that has only 4 pairs of chromosomes (X/Y sex chromosome, 2, 3, 4 autosomes) and its genome, which encodes approximately 14.000 genes is fully sequenced (Adams et al., 2000). Moreover, it is cheap and easy to rear in laboratory. It has been calculated that a pair of flies can produce one hundred offspring in one week. Because its genetics became so well known, nowadays D. melanogaster is used for all sort of research from cell development, to physiology, to behaviour.
Drosophila melanogaster is considered also an excellent model for studying insect olfaction.
In the last decade, the combination of molecular genetic, neurophysiology and behaviour have been crucial for a substantial progress in understanding the circuitry of the olfactory system of *Drosophila* and providing a foundation for understanding insects olfaction (reviewed in Vosshall & Stocker, 2007; Masse et al., 2009; Hansson et al., 2009).

Drosophila and the melanogaster species complex

The over 2000 species of *Drosophila* so far described (Grimaldi, 1990; Powell, 1997; Ashburner, 2005; Bächli, 1999-2008) breed in a great variety of plants and other substrates; some species are highly polyphagous and cosmopolitan whereas others are specialized on a specific substrate and are endemic of a restricted areas of the globe. *Drosophila melanogaster*, is only one of the 174 species within the melanogaster group of the Sophophora subgenus (Schawaroch, 2002). *D. melanogaster* together with other relatives form the *D. melanogaster* subgroup: *Drosophila melanogaster* and *D. simulans* are cosmopolitan, whereas *D. yakuba*, *D. teissieri*, *D. erecta*, *D. orena*, *D. santomea*, *D. mauritiana*, and *D. sechellia* are endemic to the Afrotropical region; the last three having been found only on the islands of São Tomé, Mauritius and the Seychelles, respectively (Lemeunier et al., 1986; Jeffs et al., 1994; Lachaise at al., 2000). The melanogaster group is thought to have evolved 10-13 MY along the Cameroon Volcanic Line (CVL), thus, the distribution of *D. melanogaster* subgroup species can be related to the paleogeografic events of Africa (Lachaise et al., 1988).

Drosophila melanogaster subgroup species were split into three complexes on the basis of their male terminalia (genitalia), (Tsacas & Bocquet, 1976) of their polytene chromosome structures (Lemeunier & Ashburner 1984) and of their different ecological habits (Lachaise et al., 1988; 2000). The first of these complexes contains *D. orena* and *D. erecta***;** the second comprises *D. yakuba*, *D. teissieri* and *D. santomea*, and the third is represented by *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia* (Lachaise et al., 2003).

D. simulans, *D. sechellia and D. mauritiana* are the most closely related, based on DNA sequences (Kliman et al., 2000), their homosequential polytene chromosomes (there are no distinguishing inversions), and fertile F1 hybrid females (F1 males are sterile) (R'Kha et al., 1991; Lachaise et al., 2003). It has been estimated on the basis of molecular data that *D. sechellia* and *D. mauritiana* diverged from mainland *D. simulans* merely 250,000 years ago (McDermott & Klimann, 2008).

Drosophila sechellia

Within the four species of the *D. melanogaster* complex, two species, *D. melanogaster* and *D. simulans* are cosmopolitan and are found breeding on all kinds of food associated with human activities. The other two species, D. mauritiana and D. sechellia are island endemics in the Indian Ocean: the

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volcanic area of Mauritius and the granitic archipelago of the Seychelles, respectively. Although confined to an island *D. mauritiana* is now semi-domestic and polyphagous species (David et al., 1987), strongly differing to its sibling species from Seychelles.

Drosophila sechellia is certainly an interesting case. This fly species is the only specialist in the *D. melanogaster* complex breeding and ovipositing naturally only in the fruit of the rubiaceous evergreen shrub of Morinda citrifolia. This fruit contains toxins that the other melanogaster species subgroup, in all their developmental stages, can not tolerate (Jones, 1998; Amlou et al., 1998a,b Cariou, 2001; Jones, 2005).

M. citrifolia, known as noni, has been studied and many chemicals have been identified (Farine et al., 1996). The ripe *Morinda* fruit is characterized by middle chain aliphatic acids and esters which gives the fruit its peculiar smell, a mix of blue cheese and pineapple. It was demonstrated that among the acids, hexanoic and octanoic acid, are responsible for the toxicity (Amlou et al., 1997; Legal et al., 1994; Legal et al.,1999).

D. sechellia's adaptations to its sole breeding site have been compared with its relative *D. simulans* and their interspecific hybrids (R'Kha et al., 1991; R'Kha et al., 1997). *D. sechellia* adults not only breed preferentially on M. citrifolia, females prefer to oviposit on *Morinda* while *D. simulans* are repelled (Jones, 2004). Noni fruit stimulates oogenesis in *D. sechellia*, but inhibits it in *D. simulans*, which may be controlled by genes located on the second chromosome (Higa et al., 1993). However the diversity of the traits which are involved in adaptation of *D. sechellia* to *Morinda* are several, and it is not known if different sets of genes have diverged from the ancestral state or if it is a pleiotropic action of a few genes (Jones, 2005; Dworkin, & Jones, 2009).

Recent studies (Matsuo et al., 2007; Mc Bride et al., 2007; Mc Bride & Arguello 2007) explored chemosensory genetic factors underlying behavioural differences between *D. sechellia* and other *Drosophila* species. Matsuo and colleagues traced *D. sechellia* host-plant preferences to two genes, odorant-binding protein 57e (Obp57e) and Obp57d. They showed that the expression patterns of these genes changed in *D. sechellia*, so they suggested that these changes may result in the fly loss of gustatory avoidance behaviour of Morinda fruit. McBride and colleague compared rates of gene loss and substitution along the *D. sechellia* lineage and compared it with that of *D. simulans* lineage in the entire repertoire of 136 olfactory and gustatory receptor genes. They found that a high fraction of *D. sechellia*'s receptor genes are pseudogenes. Precisely, 6 of the 62 Or genes and 13 of the 73 Gr gene in *D. sechellia* show lack of function mutations, whereas in *D. simulans*

only 2 Gr are pseudogenes. These pseudogenes codify for receptors that respond mainly to bitter compounds. The authors have two explanations: 1) functional "bitter" receptors would have deterred ancestor flies from feeding from *Morinda* fruit 2)few functional bitter receptor are related to *D. sechellia* restricted niche, where the risk to encounter harmful compounds is less.

Drosophila sechellia's ecology

The Seychelles bank was completely emerged 16.000 year ago and was reduced to its present condition of scattered islands about 10.000 years ago. During the period of submergence, the Seychelles granitic micro continent was reduced from a continuous land mass of 130.000 km^2 to scattered islands with a total area of only 216 km^2 (Stoddart, 1984). This big reduction in land area was accompanied by massive extinction within all groups of plants and animals (Stoddart & Fosberg, 1984; Procter, 1984). Also the population of D. simulans and D. sechellia have suffered dramatic reduction of size (Lachaise, 2004). Population genetics of origin and divergence of the D. simulans subcomplex species (*D. simulans*, *D. sechellia*, *D. mauritiana*) were examined (Kliman et al., 2000; McDermott & Klimann, 2008) using patterns of DNA sequence variation found within and between species at 14 different genes. *D. sechellia* revelead low levels of polymorphism, and genes from *D. sechellia* have accumulated mutation at the rate that is nearly 50% higher that the same genes from *D. simulans*. *D. mauritiana* is presumed to have a long history of small population size, but surprisingly high genetic diversity was found. Both *D. mauritiana* and *D. simulans* are highly polymorphic and the two species shared many polymorphisms. One of the possible reasons why insular endemic species like *D. mauritiana* has retained genetic variation and *D. sechellia* has not, is related to the paleogeographic events in the Indian Ocean. Thus the submersion over the last 10,000 years is thought to have reduced D. sechellia genetic diversity through extinction and genetic bottleneck. Whereas as the overall paleogeography of Mauritius has remained unchanged during and after glacial period, it is most likely that D. mauritiana was not affected by a bottleneck as *D. sechellia* (Lachaise et al., 2003).

Morinda fruit ripens throughout the year and trees are patchily distributed. Each tree normally bears some ripening fruit. Once ripe, the fruit drops off in a few days and deteriorates. It is unknown whether *D. sechellia* oviposits on fresh fallen fruit or only on fruit still in the tree. However, the fruit most likely offers *D. sechellia* only a small window of opportunity for oviposition,

as ripe fruit quickly disintegrates, and the stronger competitor *D. simulans* can sometimes be found breeding on the less toxic, rotten fruit. (David et al., 1989). Lachaise (2004) proposed a more recent shift of D. sechellia to Morinda driven primarily to avoid competition from *D. simulans*. The question arises whether the genetic adaptations thought typical for its *Morinda* fruit are likely to take place in such a short time. However it is uncertain whether *Morinda citrifolia* fruit has always been the only preferential "life" source where *D. sechellia* could breed and have evolved its oligogenic resistance. As mentioned above the reduction in Seychelles' area was accompanied by massive extinction within all groups of plants and animals. Furthermore *Morinda citrifolia* originates from Southeast Asia and probably has been introduced by ancient French Polynesian people, into Seychelles, a long time after *D. sechellia* speciation (~250.000 years ago). A potential endemic host-plant that has been suggested to be suitable to the specialist fruit fly of Seychelles is the endemic screwpines, of which 4 endemic species of pandani have been found on the Seychelles: *Pandanus balfourii*, *P. hornei*, *P. multispicatus*, *P. sechellarum*. Those screwpines together with other endemic palms could be the most archaic constituent of the Seychelles archipelago flora. If this hypothesis is correct we could assume that *D. sechellia*'s primeval life source could have been Pandanus fruit (Lachaise 2004).

If so, are there similar or even the same compounds in Morinda fruit and Pandanus fruit that could have driven the shift of the specialist fly from one plant to another? And how is olfactory adaptation involved in either scenario? Unfortunately information regarding Pandani volatiles is very little (Vahirua-Lechat et al., 1996), and anything is known about the Pandani species   of   the   Seychelles

The fly's olfactory system

The olfactory system detects and process chemical volatiles from the environment. In the periphery chemical volatiles are detected and transformed into electrical signals, in the central nervous system these signals are processed.

Olfactory organs of adult fly

The olfactory organs of the adult *Drosophila* consist of two pairs of cephalic appendages: the third antennal segment, called flagellum, and the maxillary palps. The surface of both flagellum and maxillary palp is covered with hair like structures, called sensilla. The sensillum houses a complex of olfactory sensory neurons (OSNs) and auxiliary cells (Keil, 1999) protected from the insult of the external environment by the sensillum cuticular covering. Morphologically a sensillum can be either single walled or double-walled with numerous pores or slits, respectively, that allows the access of odor molecules into the lumen of the sensillum. Based on their characteristic size and shape *Drosophila* antennal sensilla are divided in three different types: club shaped basiconic sensilla, triangular trichoid sensilla, and grooved peg coeloconic sensilla (Shanbhag et al., 1999). Sensilla are distributed in a stereotyped pattern, with large basiconic sensilla clustered at the medial-proximal side of the antenna and trichoid sensilla clustered at the lateral-distal edge. Small basiconic and coeloconic sensilla are interspersed in the middle-distal region of the antenna. The most numerous sensilla are basiconic type (about 200), followed by trichoid (150) and finally coeloconic (60) types per antenna (rewieved in Stocker 1994). In *Drosophila* each sensillum houses up to four OSNs, which are surrounded by auxiliary cells,

which secrete a viscous medium (the sensillum lymph). In each *Drosophila* antenna there are in total between 1100-1250 OSNs (Stocker 2001). Fly's OSNs are morphologically similar to those of vertebrate (Ache & Young, 2005). Fly's olfactory neurons are bipolar, the sensory dendrite with cilia extending into the shaft of the sensillum, and the axon projecting (from the cellular body of the neuron) to the first olfactory station in the brain, the antennal lobe (AL), the functional homologue of the mammalian olfactory bulb.

OSNs along with auditory fibers from the second antennal segment and hygro and thermosensory neurons from the arista form the antennal nerve, that terminate in the AL. The maxillary palp contains 60 basiconic sensilla, each of which houses two OSNs. These neurons fascicultes together with gustatory sensory neurons from the labium and project through the suboesophageal ganglion (SOG) to the AL.

Odorant receptors

A very important element of the olfactory pathway are the olfactory receptors (Ors). At this level detection and discrimination of the distinct odorant molecule starts. The presence of genes encoding Ors was first reported from rodents (Buck and Axel 1991).The importance of the discovery was recognized in 2004 with the Nobel prize in Physiology or Medicine to the authors of the work. In 1996 olfactory receptor genes have been identified in Caenorhabditis elegans (Sengupta et al., 1996) and later in 1999 also in *Drosophila melanogaster* (Clyne et al., 1999b, Gao & Chess, 1999, Vosshall et al., 1999). *Drosophila* adult fly Or gene family comprise 62 olfactory receptors (Vosshall et al., 2000; Robertson et al., 2003). Nowadays, Or genes of other insects species have been identified e.g. Anopheles gambie (Fox et al., 2001), Aedes aegypti (Bohbot et al., 2007), Heliotis virescens (Krieger et al., 2002), Apis mellifera (Robertson et al., 2006).

In vertebrate the olfactory receptors belongs to a large superfamily of seven-transmembrane G-protein coupled receptors (GPCRs) (Ronnet et al., 2002). In insects olfactory receptors are, a seven membrane spanning domain but have no homology with the vertebrate GPCRs. Compared to vertebrates, insects Ors topology is inverted in the plasma membrane , the N-terminal faces the citosol and the C-terminal is located outside the cell (Benton et al., 2006 ; Wistrand et al., 2006).

Moreover, in insects, apart from the conventional ORs, and additional receptor gene was discovered, the Or83b gene family (Vosshall et al., 2000; Larsson et al., 2004). This Or gene is a highly conserved sequence among divergent insect species (Jones et al., 2005), but there is no mammalian orthologue of Or83b. It is co-expressed along with regular Ors in 70–80% of the antennal ORNs; it appears to be involved in proper localization and function of conventional Ors but does not influence ligand specificity (Larsson et al., 2004; Neuhaus et al., 2004; Benton et al., 2006).

The logic of Or gene expression in the larval olfactory system is similar to the adult and vertebrate design (Larsson et al., 2004; Fishilevich et al., 2005; Kreher et al., 2005). *Drosophila* larva Or gene family comprise 25 olfactory receptors, 13 are larval specific (Fishilevich et al., 2005; Kreher et al., 2005) whereas 12 Or genes are expressed in adults as well. As in the adult, the large majority of the neurons express one conventional Or along with Or83b (Larsson et al., 2004), whereas 2 OSNs were shown to express 2 conventional Ors together with Or83b (Fishilevich et al., 2005). In the adult stage there are also some cases in which multiple conventional Ors are coexpressed on a single OSN along with Or83b receptor (Dobritsa et al., 2003; Goldman et al., 2003). Unlike insects, in vertebrate each OSN expresses only one Or (Malnic et al., 1999; Serizawa et al., 2003).

A recent study from Benton and colleagues (2009) showed that in the adult *Drosophila* another family of olfactory receptor genes is expressed. These receptors are divergent member of the ionotropic glutamate receptor family, and are called Ionotropic receptor (Irs). Irs family comprise 61 members, 15 of them are expressed in the antennae. Precisely 9 Irs are expressed in a combinatorial manner on OSNs in *sensilla coeloconica* (Benton et al., 2009).

Apart of Ors and Irs there are also gustatory receptors (Gr) expressed in *Drosophila* antennae, e.g. Gr21a and Gr63a are known for detecting carbon dioxide (Jones et al., 2007).

The olfactory transduction pathway

Once the odour molecules enter through the pores of the sensillum or the olfactory dome bind the olfactory receptors. Most odorants are non-polar molecules and in order to bind Ors have to "float" in the aqueous sensillum lymph. In 1981 the study of Vogt and Riddiford showed the presence of water-soluble family of proteins in the olfactory tissue of the silk moth, Antheraea polyphemus. They called these hydrophilic proteins odorant binding proteins (OBPs) because of their possible role in guiding the odour molecules through the lymph to the Ors. OBPs have been described in a

number of insects (Vogt et al., 1999; Ishida et al., 2002; Bohbot & Vogt, 2005) and are also present in mammals (reviewed in Pelosi 2001).

In *Drosophila* there are 51 OBP genes (Hekmat-Scafe et al., 2002). The importance of OBPs for pheromone detection was demonstrated by Xu and colleagues (2005) with the construction in *Drosophila* of a mutant lacking the OBP LUSH. This protein is involved in guiding the fruitfly pheromone molecule cis-vaccenyl acetate to their putative Ors (Or67d). Flies without LUSH OBP were not able to respond neither physiologically nor behaviourally to their pheromone. Lauglin and colleagues (2008) recently showed that most likely Or67d does not detect the cis-vaccenyl acetate molecule itself but the altered conformation of LUSH OBP.

Once the odour molecules reach the Ors on the sensory dendrite, they interact with it. In vertebrate the olfactory signal transduction involves the G protein activation with the subsequent second-messenger production and dendritic membrane channel opening (Krieger & Breer 2003). Insects Ors have no homology with GPCRs and moreover each neuron express an Or along with Or83b receptor. This strongly suggests that the signal transduction mechanism in insects is distinct from that in vertebrates. Recently, the parallel work of two groups of scientists (Wicher et al., 2008; Sato et al., 2008) gave an insight into the olfactory transduction pathway in insects. Both groups demonstrated that the olfactory transduction mechanism involves a non selective cation channel conduction formed by the heterodimerization complex of the Or and the ubiquitous receptor Or83b. Sato and colleagues results indicate that the Or-Or83b complex function as a direct ligand gated ion channel without the need for a second messenger system. On the other hand Whicher and coworkers found that, especially at low concentration, the activation of the Or triggers a second messenger signaling cascade that activates Or83b receptor with subsequent inward flow of current. The outcome of these processes is that the increase in membrane conductance determines the membrane depolarization and the subsequent generation of action potentials along the OSNs axon membrane.

Olfactory coding

How is the information of volatile molecules in the environment coded in the insect's brain?

Recognition of odor molecules is performed by Ors and by the combinatorial activity pattern across OSNs. The functions such as filtering,

integrating and finally modification of the information into a new pattern take place in the AL and in the higher brain centers.

Two schemes of odor coding have been hypothesized at the peripheral level:
the labeled–line code
the across fiber patterning code

In the labeled line coding scheme Ors are extremely selective. A ligand can activate only one type of Or, the information goes directly from OSNs which express that receptor to the AL and with uniglomerular PNs directly to higher centers in the brain (reviewed in Hansson 1999 cap 5). A good example of this coding scheme is represented by the male moth pheromone system (Christensen and Hildebrand 1987), or the CO_2 sensitive neurons in *Drosophila* (Suh et al., 2004).

In the across fiber patterning OSNs respond more broadly to a range of compounds. More than one Or type responds to the same molecule, thus the activation of different OSNs varies and so does the number of glomeruli activated. Therefore discrimination of different odorants is facilitated. A good example of this coding scheme is volatiles emitted by plants or other food sources, which are detected by a broader number of Ors (Hallem & Carlson 2006).

Only the combination of the two coding scheme together can fully describe coding of olfactory information in the brain. Most Ors bind a range of odorant molecules. Depending on the physical and chemical properties of the molecule, the concentration, and the ligand affinity the activation of different OSNs vary and so the number of glomeruli activated (Malnic et al., 1999; Wang et al., 2003).

Central elements of olfactory processing

The primary olfactory center in insect's brain is the antennal lobe (AL) which corresponds to the olfactory bulb (OB) in vertebrates (Strausfeld & Hildebrand 1999; Ache &Young 2005). Here the olfactory information is processed and sent to higher brain centers, the mushroom bodies (MB) and the later horn (LH), for memory formation and the organization of behavior (Heisenberg, 2003; Strausfeld, 1998).

Primary olfactory center (the antennal lobe)

Adult

The antennal lobes are formed of a number of neuropilar shaped structures, called glomeruli. The number and the volume of glomeruli vary between species and sometimes also between sex (Anton & Homberg 1999; Hansson & Anton 2000).

Drosophila AL consists of 49 different glomeruli (Laissue et al., 1999). OSN expressing the same Or converge into the same glomerulus (Vosshall et al.,2000; Gao et al., 2000; Couto et al., 2005; Fishilevich & Vosshall 2005), therefore each glomerulus receives information about the molecule that activate its Or. Moreover in *Drosophila*, as in many Diptera species (Strausfeld, 1976), most OSNs send their axons to homologous glomeruli in the two lobes (Stocker et al., 1994). These target glomeruli have a stereotyped position across individuals.

OSNs axons arborizing in the same glomerulus synapse with two types of central neurons: local interneurons (LN) and projection neurons (PN) (Stocker, 1994; Anton & Homberg 1999). Local interneurons are restricted to the antennal lobe, can either ramify in a few or in many glomeruli,(Stocker et al., 1994; Hansson and Anton 2000). In *Drosophila* there are approximately 100 LNs, most of them are inhibitory, releasing GABA (Wilson & Laurent 2005). Recently in *Drosophila* has been found that there are also excitatory circuits (Shang et al., 2007; Olsen et al., 2007). All olfactory information from OSNs are first filtered at LNs level and then passed to PNs.

Projection neurons send information to high brain centers, the mushroom bodies and the lateral horn through a number of anatomically distinct fiber tracts (Anton & Homberg 1999). In *Drosophila* there are approximately 150 PNs (Stocker, 2001), and like LNs the cell bodies are found at the periphery of the AL (Anton and Homberg 1999). PNs innervate single glomeruli, therefore PNs arborizing in the same glomerulus receive imputs from OSNs expressing the same Or (Stocker 1990). Most of PNs are excitatory (Marin 2002; Okada et al., 2009).

Larvae

As in the adult fly, larval OSNs axons projects to the LAL, but diversely from the adult all projections are ipsilateral. In the larval AL, OSNs synapse with LNs and PNs (Python & Stocker 2002a; Marin et al., 2005). LNs are inhibitory and establish lateral connection within glomeruli whereas PNs are

excitatory and connect the LAL with the MB calyx and the lateral horn (Python and Stocker 2002b). In the *Drosophila* larval AL there are 21 glomeruli (Fishilevich et al., 2005, Kreher et al., 2005). Each glomerulus is the target of a single OSN expressing its proper Or, which synapse most of the cases with a single PN which project to approximately 21 calyx glomeruli (Ramaekers et al., 2005).

Unlike in the adult fly, the larval olfactory pathway exhibits no cell redundancy, ie each PN arborizes with a single OSN and vice versa. Moreover the similar number of OSNs, larval AL glomeruli, PNs and MB calyx glomeruli indicate that the system is organized in a 1:1:1:1 fashion with no convergent divergent connectivity (Ramaekers et al., 2005)

Olfactory information translated into behavior

One of the main questions in olfaction is how these neural olfactory components translate into appropriate behavioral responses e.g. attraction and repulsion to different odorants that are vital for finding food sources, mates, oviposition sites etc. Nowadays the olfactory map from Ors, to OSNs, to PNs and high brain centers in *Drosophila* is increasingly understood but still the neuronal computation for odor discrimination and the final behavior remain to be explained. Numerous studies are contributing in different ways to understanding behavioral responses elicited by olfactory stimuli (Suh et al., 2004; Stockinger et al., 2005; Billeter et al., 2006). However, for a better understanding of behavioral responses as result of olfactory processing it's crucial to consider both functional (i.e. internal state) and ecological forces involved.

Different *Drosophila* species have diverse lifestyles, therefore olfactory cues mediating behavior may largely differ between species. Moreover members of different species may even show opposite behavioral preference to the same olfactory stimuli depending on their ecological niches, and even individuals of the same species show strain specific differences in food odor preference (Reubenbauer et al., 2008). Nevertheless, it was shown they all must utilize evolutionary related and structurally similar olfactory systems (Stensmyr et al., 2003) to find their (species-related) food sources both as adults and larvae (Asahina et al., 2008; R'ka et al., 1991; David et al., 2004). For this reason the genus *Drosophila,* comprising over 2000 species with different ecology, is doubly a successful model for studying how olfactory information is translated into behavior.

Electrophysiological approaches (SSR, GC-SSR)

The single sensillum recording (SSR) technique is an electrophysiological approach that was first adapted for the insect system by Jürgen Boeckh in 1962, and modified to work in combination with gas chromatographic detection in 1982 by Wadhams. Since then it has been widely used in insect olfactory research.

SSR technique is very valuable because allows to study the functional properties of OSNs in single sensilla, enabling us to classify physiologically the different sensilla types and therefore generate a peripheral coding map.

SSR is an extracellular recording, it is performed by using two sharped tungsten electrode: the ground electrode is in contact with the haemolimph and the recording electrode is gently placed into the base of a single sensillum. The voltage difference generated between the two electrodes is amplified and the spikes recorded and analyzed via computer software. The different spikes size and waveform of the OSNs within a sensillum or between different sensilla allow us to distinguish them. When a biologically active odorant binds a receptor the chemical signal is transduced into an electrical signal that it is visualized in the computer screen as the increased or reduced spikes frequency of the OSN neuron.

The combination of this technique with gaschromatographic detection is very useful for identification of natural active compound.

An extract of collected volatiles is injected onto the GC column. The column is located into an oven, where it is possible to regulate its temperature depending on the chemical properties of the extract we are injecting. As the temperature of the column increase the different chemical compounds are separated, depending on their affinity to the column, and travelling down the column exit the GC. At the exit of the GC it is placed a glass tube with a humidified air stream flow. The compound(s) of our extract that exit the GC are mixed in this air stream that reach the head of the insect, from where we are recording OSNs activity. In this way it is possible to test the response of OSNs activity to natural compounds. The chemical identity of the physiologically active compound(s) can be further identified by combining GC with mass spettrometry (MS).

The use of SS, and GC-SSR technique in *Drosophila* allowed to generate an almost complete coding map of its peripheral olfactory organs (de Bruyne et al., 1999, 2001; Stensmyr et al., 2003; Hallem & Carlson 2006).

Summary of results

Evolution and ecological dynamics of *Drosophila sechellia*'s olfactory system (Papers I, II)

Insects use chemical volatiles to locate and identify food source, mates, and oviposition site. Geographic isolation, changes in the environment and in the natural sources of vital importance for the animals can determine changes in the olfactory system.

One of the model systems for studying olfaction is the fruit fly *Drosophila melanogaster*. Its olfactory system, from olfactory receptors expression and function to olfactory sensory neurons responses and odor representation in olfactory centers in the brain is nowadays increasingly studied and understood (reviewed in Vosshall & Stocker, 2007; Masse *et al.,* 2009; Hansson *et al.,* 2009). Moreover more than 2000 species of *Drosophila*, endemics to different areas of the globe and with different habits have been so far described (Bächli, 1999-2008). Therefore, fruit flies of the genus *Drosophila* offer an excellent opportunity to study evolution and ecological dynamics of the olfactory system.

In paper I and II we focused on the olfactory system of a *D. melanogaster* sibling species, *D. sechellia*. This fruit fly is geographically isolated and specialized on a single host, *Morinda* fruit, which is toxic for all other *Drosophilids*. Therefore *D. sechellia* is an excellent target for asking first how evolution acts in the olfactory system in mediating fruit fly specialization, and second how perturbation in the olfactory system affects behavioral preference to odors. We investigated the morphology and function of the peripheral olfactory system, the anatomical organization of the AL, and the

behavioral responses to *Morinda* fruit volatiles, singly and as blend, of *D. sechellia* and we compared it with that of the generalist *D. melanogaster*.

Periphery

D. sechellia antennae were morphologically distinct from that of *D. melanogaster*. Trichoid sensilla and small basiconic sensilla types were shorter in *D. sechellia* that in *D. melanogaster* and also hair-like structures were shorter in *D. sechellia*, the latter features is clearly visible under a microscope magnification 40x.

In order to understand if any difference occurred in the function of the peripheral olfactory system we collected volatiles from *Morinda* fruit headspace and we tested flies antennal responses to these compounds by combining gas chromatography with electro-antennographic detection (GC-EAD). *Morinda* fruit headspace is dominated by middle chain aliphatic acids and esters thereof, which give the fruit its characteristic smell reminiscent of blue cheese and pineapple. *D. sechellia* showed much stronger antennal responses to esters than to acids, with an increase sensitivity to methylhexanoate (MH) compared to *D. melanogaster*. Single sensillum recordings, from large basiconic sensilla types showed an increased number of ab3 sensilla (~80%), which house two neurons. The A neuron expressing receptor Or22a responding to ethyl (EH) and methylhexanoate (Stensmyr *et al.*, 2003; Hallem & Carlson, 2006). Overrepresentation of ab3 sensilla was paralled with the reduction of ab1 sensilla (~60%) and the near loss of ab2 sensilla (~95-100% depending on the fly strain) see also (Stensmyr et al., 2003, Dekker et al., 2006).

The ligand affinity to ab3A neuron was shifted in the two Drosophilids, *D. sechellia* was more sensitive to MH whereas *D. melanogaster* was more sensitive to EH. The increased sensitivity to MH in *D. sechellia* was possibly caused by Or22a up regulation as shown in other insects (Fox et al., 2001; Gatellier et al., 2004) or it could be caused by difference in amino acids sequence between *D. melanogaster* and *D. sechellia* Or22a homologs. Differently from esters we did not observe any difference in the physiological responses to acids between the two species.

In the second paper of this thesis (II) by using GC-EAD we found that in *D. sechellia* the second neuron of the overrepresented ab3 sensillum (ab3B) is tuned to another *Morinda* fruit volatile. This compound was missed in earlier studies as it co-eluted with methyl hexanoate. Gas chromatography coupled with single sensillum recording (GC-SS) showed that this compound elicits a strong response in spike frequency in the ab3B neuron. Gas

chromatography coupled with mass spectrometry (GC-MS) allowed us to identify this volatile as 2-heptanone (2HPT), key ligand of the ab3B neuron (Hallem *et al.*, 2006). The co-localization of two OSNs tuned to morinda volatiles possibly attenuated the pressure for more extensive OSN and, or Or rearrangements across the antenna.

Primary olfactory centers (AL)

As Ors are not directly involved in axonal guidance in *Drosophila* (Jhavery *et al.,* 2000; Komiyama *et al.,* 2004) we asked whether the axons of OSNs in the "new" ab3 sensilla (i.e. "replaced" ab1 and ab2 sensilla) still project to their "corresponding" glomeruli or rewire to arborize in "ab3 glomeruli". By using nc82 staining overview, we identified two glomeruli in the same dorso-medial region that were enlarged in *D. sechellia*. By using hybrid male *D. sechellia* x female *D. melanogaster* Or22a-nsyb-GFP, we demonstrated that the DM2 corresponding glomerulus of *D. melanogaster* received input from the ab3A neurons (paper I). By using anterograde backfills from ab3 sensilla under pulsed 2-heptanone presentations we demonstrated that the second enlarged glomerulus, located ventrally from DM2, receives input from ab3B neurons, irrespective of the position of the sensillum on the antenna (paper II). Volumetric calculation of the enlarged glomeruli showed that the *D. sechellia* DM2 glomerulus was 2.9x enlarged compared to the corresponding of *D. melanogaster*. Apparently, the antennal lobe of *D. sechellia* has formed a macroglomerular complex (MGC) tuned to volatiles of this species' sole host *Morinda* fruit. It's well documented that volume can be modulated over adult life span and in response to odor exposure (Devaud et al., 2001; Devaud et al., 2003, Sachse et al*.,* 2007) and that volume of glomeruli depends not only on the number of OSNs projecting to it, but also on the number and synaptic density of projection neurons (PNs) and local neurons (LNs) (Stocker et al., 1990; Stocker et al., 1994). However, in our study, the voluminar increase in *D. sechellia* approximates the increase in ORN input (Paper I). The MGC in antennal lobes of *D. sechellia* is to our knowledge, the first physiologically and behaviorally characterized MGC that is not tuned to pheromones.

Behavior

Behavior to single *Morinda* fruit volatiles

The abovementioned differences in the olfactory system of *D. sechellia* at the peripheral and central level are clearly reflected in its behavior. In stark contrast to *D. melanogaster*, *D. sechellia* was significantly more attracted to single *Morinda* volatiles MH and 2HPT, even at high concentrations, moreover the attraction to these compounds followed the same pattern in both species (Figure 2 paper II). Clearly 2-heptanone and methyl hexanoate are coded differently in the two sibling species, such that high concentrations attract uniquely *D. sechellia*. In addition, *D. sechellia* was attracted to both odors at concentrations to which *D. melanogaster* seemed indifferent. This could mean that the 3-fold increase in glomerular volume of *D. sechellia* may lower detection thresholds, although it is difficult to ascertain whether an increased behavioral sensitivity reflects an increased olfactory sensitivity of the olfactory circuitry. Compared to the generalist fly, *D. sechellia* was more attracted to all acids, even when presented pure. Hexanoic acid was strongly preferred over octanoic and ethanoic acid (Figure 2 paper I). The preference of *D. sechellia* to hexanoic acid is however most likely not caused by a change in the olfactory circuitry, but rather mediated through a change in the taste circuitry. A study of Matsuo and colleagues (Matsuo *et al.*, 2007) demonstrated that *D. sechellia* has a defective odor binding protein OBP57d, a deletion of which in *D. melanogaster* increased the attractiveness to hexanoic acid in a similar fashion as in *D. sechellia*.

Behavioral response to binary and ternary mixtures

In nature flies do not encounter odors singly, but as blends. We therefore examined the response of the fly to blends of synthetic *Morinda* volatiles. After GC injection with synthetic standards we calculated the proportion of methylhexanoate, 2-heptanone and hexanoic acid in *Morinda* fruit as 8.1%, 0.5%, 91.4% respectively. These ratios were subsequently used in our blend assays (Figure 3 paper II).

D. sechellia was highly attracted to *Morinda* odor mimic when tested versus water, whereas *D. melanogaster* was repelled by it, especially at high concentrations. The preference of *D. sechellia* for *Morinda* volatiles appeared also when testing the ternary mixture versus one single compound as well as to all binary mixtures. Conversely, *D. melanogaster* was highly repelled by the combination of MH and 2HP.

The increased imput of OSNs in *D. sechellia* antennal lobe has clearly consequences for the code generated in the antennal lobe. Glomeruli are not

stand-alone units, but convey information to each other via a dense network of lateral inhibitory connections (Shang et al., 2007; Root et al., 2007; Olsen & Wilson, 2008; Root et al., 2008) and excitatory connections (Olsen et al., 2007). How the excitatory and inhibitory connectivity shape the overall output pattern in an AL dominated by an MGC is unknown. Moreover, repulsion at high concentrations is common for odors (Ayyub et al., 1990), and is thought to be caused by unspecific responses from olfactory receptor neurons dominating the olfactory code generated in the antennal lobes (Sachse & Galizia, 2003; Wang et al., 2003; Wilson et al., 2004). Although the enlarged glomeruli reflect the ecological and behavioral significance of their key ligands, on the basis of another study (Acebes & Ferrus, 2001) we would have expected an increased repulsion not attraction to these odorants. Interestingly, the concentrations of the individual compounds in the noni-mimic were attractive to *D. melanogaster* when tested singly, but repellent when combined (i.e., a 10-1 Morinda mimic contains roughly 10-1, 10-2 and 10-3 of hexanoic acid, methyl hexanoate and 2-heptanone, which are attractive singly (figure 2 paper II). We conjecture that in *D. sechellia* odors are not read out by the circuitry as unitary elements, but rather as a combinatorial activity pattern across glomeruli (Silbering et al., 2007; Riffell et al., 2009 a). Here we demonstrate that the MGC in *D. sechellia*, in addition to sensitivity, can have a disproportional effect on the salience of the olfactory code and may as such modulate preference.

Ecological value of olfactory specialization

The olfactory system of *D. sechellia* is rearranged compared to its sibling *D. melanogaster* and it's clearly adapted toward the use of *Morinda*.

We suggest that the increased number of ab3 sensilla and the increased sensitivity to MH helps *D. sechellia* to locate from distance *Morinda* fruit, which is necessary because *Morinda citrifolia* shrubs are patchily distributed over the Seychelles. Whereas the acids are probably detected only when the fly is very close to the host, and mediate behavior such as oviposition. Once ripe Morinda fruit fall off and deteriorates in a few days. The toxicity of *Morinda* fruit is related to its acids content (Jones, 1998; Amlou et al., 1998), and it is inversely proportional to the fruit stage of ripeness. We don't know if *D. sechellia* oviposits on a fresh fallen fruit or on the fruit still in the tree. However, *D. sechellia* have a short time to oviposit on *Morinda* as the fruit rapidly deteriorates and sometimes *D. simulans* was found breeding on rotten *Morinda* (David et al., 1989). We think that the increased sensitivity to MH along with the behavioral attraction at high concentrations of Morinda

compounds singly and even more as blend it's advantageous for the fly not only to search and find new fresh fruits in the right stage of ripeness but also to compete for the food source and oviposition site.

Electrophysiologically and behaviorally active natural ligands for Ionotropic receptors
(Paper III)

In the third paper of this thesis we investigated natural ligands for ionotropic receptors in *Drosophila*. We aimed to study the significance of the coeloconic OSNs and their ligands in odor coding and in fly attraction.

Drosophila antenna, house three major morphological types of sensilla: *basiconica*, *trichoidea* and *coeloconica*. *Sensilla coeloconica* can be found in many insect orders (Steinbrecht 1997) and in terms of evolution are hundreds of million years old (Keill, 1999; Rebora et al., 2008). In *Drosophila* four different types of coeloconic sensilla are known and are classified as ac1, ac2, ac3, and ac4. The OSNs inhabiting these sensilla express receptors from a different family than other olfactory receptor neurons. Whereas most OSNs express either an odorant receptor (Or) or a gustatory receptor (Gr, Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999), most coeloconic OSNs express a new family of chemosensory receptors, called ionotropic receptors (iGluRs or Irs, Benton et al., 2009). Most of the response profiles in Ors are known e.g. fruit odors and pheromones (Hallem & Carlson 2006; reviewed in Hansson et al., 2009). For the Irs family, of which members are expressed in coeloconic sensilla, few ligands have been identified (Yao et al., 2005).
Here we screened for putative natural odour ligands for olfactory sensory neurons (OSNs) housed in coeloconic sensilla. We used headspace collections from ecologically relevant sources such as various fruits at different stages of ripeness, yeast and vinegar. By using a combined gaschromatographic–electroantennographic detection (GC-EAD) and single receptor neuron recording (GC-SS) we set out to identify natural compounds that elicit a response in coeloconic neurons of *Drosophila* (Figure 1 paper III). Through GC-mass spectrometry we identified 2-methyl propanoic acid (2MPA); 2-methyl butyric acid (2MBA); ethyl-3-(methylthio)propanoic acid (ETP). We checked the sensitivity of these

OSNs with a dose–response function. Subsequent screening with the natural ligands as synthetic compounds showed that the neurons were capable of detecting the natural odorant at relatively low concentrations. Finally we performed behavioral tests with *Drosophila* wild type (wt) as well as mutants, in which only coeloconic sensory neurons were functional (*Or83b*^{-/-}), to study the biological significance of the coeloconics OSNs and their natural ligands in odor coding (Figure 3 paper III).

Contrary to the Ors, the Irs seem to be expressed in a combinatorial manner in coeloconics OSNs (Benton et al., 2009). We have found that two of the natural ligands, 2-methylpropanoic acid and ethyl-3-(methylthio)propionate elicit a response in the ac3A neuron and ac4B neuron, respectively. Ac3A neurons express Ir75a, and Ir75b. The receptor Ir75a is also expressed in the ac2A neuron, but this neuron responds only unspecifically to 2MPA, and more specifically to 1,4 diaminobutane (DAB, Yao et al., 2005). Therefore, we infer that 2 methylpropionic acid is key ligand for Ir75b, that propionic acid is a key ligands (Yao et al., 2005) for Ir75a, and that the Ir receptor that respond to 1,4 diaminobutane has not been identified yet. We have found the also the receptor expressed in ac4B neuron it may has not been identified yet. Ac4B neurons express Ir75d and respond to ETP. This receptor is also expressed in one of the ac2 OSNs and in one of the ac1 OSNs, but neither ac2 nor ac1 neurons respond to ETP. The expression of 46 members of the Irs repertoire is still unknown (Benton et al., 2009) therefore identification of ligands for coeloconics will help understanding if and in which OSNs unknown Irs are expressed, and indicate the functional significance of the Irs combinatorial expression.

Both *D. melanogaster* wt and *Or83b*^{-/-} were attracted to almost all acids at high concentration. Attraction to odorants at high concentration is unusual, normally, at 100µg/µl most compounds are repellent (Ayyub *et al.* 1990). It's possible that acids are ecologically relevant for the fly only at close range, this is in agreement with their relatively low sensitivity to these compounds. Acids can be detected elsewhere. Benton and colleagues (2009) showed that antennal Irs are also detected in the proboscis. In addition, loss of function of OBP57e in fly tarsi increased the attraction to high concentrations of hexanoic acid (Matsuo et al., 2007), implying volatile detection of acids is partially mediated through the legs as well. Therefore we propose that the behavioral response is due to a combination of olfaction, and most likely taste. Ammonia and ethyl–3–(methylthio)propionate were repellent at high concentration for *D. melanogaster*, this is ecologically relevant in fact AMM is

an indicator of not edible breeding source and ETP seems to be a microbial breakdown product indicative of overrotten fruit (Moreira et al., 2002).

Putrescine or 1,4 diaminobutane, an odor reminiscent of putrefying flesh, does not elicit a a clear attraction or repellency behavior in both *D. melanogaster* wt and *Or83b*^{-/-} flies. Ac2A neuron is sensitive to DAB, it is possible that amines are ecologically relevant for *D. melanogaster* only in combination with other compounds.

Alternatively, it is possible that the DAB receptor is still of ecological use in related Drosophilids, but no any longer in *D. melanogaster*.

Finally, PAA, a floral odor eliciting a response in ac4A neurons (Yao et al., 2005), is behaviorally repellent at high concentration and attractive at low concentration. This compound is detected also by other OSNs (ab1A /Or67a, Hallem and Carlson, 2006) In that light it is interesting to note that in wt *D. melanogaster* PAA at 0.01% is highly attractive, whereas for flies with only coeloconics functional the same odorant is repellent.

In this study we have found 3 natural compounds, 2 short chain organic acids and 1 ester, which are ligands for three different ionotropic receptors expressed in coeloconics OSNs. We have shown that OSNs in coeloconics have low sensitivity, to these compounds and that this is reflected in the fly behavioral response to these compounds i.e. attraction at high concentration to acids.

On the basis of the functional and behavioral analysis we have done it seems that OSNs in *coeloconica sensilla* detect mainly odorants at close range.

Olfactory specialization of *Drosophila sechellia* at larval stage (Paper IV)

In the forth paper of this thesis we investigated if the *D. sechellia* adult fly specialization was also paralleled by alteration in the olfactory code at larval stage.

Differences in animal lifestyles are expected to correspond to differences in chemosensory performance. In holometabolous insects, i.e. all *Drosophila* species, adults and larvae display very distinct lifestyles. Adults have to fly and orient over considerable distances to find food, mates, and oviposition sites, larvae live directly on their food source, therefore a long range odor detection is not of vital importance. The sensory system of the larva differs

from that of the adult in terms of cell numbers ,21 olfactory sensory neurons (OSNs) compared with 1300 of the adult (Python & Stocker 2002a, Fishilevich et al., 2005, Kreher et al., 2005, Ramaekers et al., 2005).

However, the two stages share a similar design of sensory projections and central pathway (reviewed in Vosshall and Stocker, 2007; Gerber and Stocker, 2007). Nevertheless, depending on the adult or larval primary needs, the same inputs may or may not lead to different activity patterns in the chemosensory pathways.

Changes in the environment or in the animal's habit could also lead to changes in the olfactory system. In previous studies we showed that adaptation of the specialist fly *D. sechellia* to its sole host, the toxic noni fruit, was paralleled in the adult by alterations in the antennae and in the first olfactory center in the brain, the antennal lobes. The changes in *D. sechellia* olfactory system are reflected in the behavior e.g. increased attraction to morinda fruit and its synthetic mimics (AL, Dekker et al., 2006; Ibba et al., submitted).

Here we asked if the *D. sechellia* adult fly specialization was also paralleled by alteration in the olfactory code at larval stage. Therefore we observed in detail the olfactory responses of the two larval species to noni, banana and synthetic noni mimics. The direct observation of larval behavioral responses showed that also in the larval stage the two sibling species show a clear different preference (Figure 1 paper IV). The larval stage of *Drosophila sechellia* prefers the odors of its sole food substrate, *morinda* fruit whereas *D. melanogaster* prefers banana.

This offers great potential for research on how 'preference' is defined in an olfactory circuitry, as the simplicity of the larval system may allow more readily to find the key factor(s) involved in this. On the other hand the system is potentially also more complicated, as larvae show positive chemotaxis at almost all odors (Fishilevich et al., 2005). In agreement with larvae general attraction to odors, when *Drosophila* larvae have been tested for the single fruit (data not shown) or for the synthetic noni mimic (Figure 2 Paper IV) both larvae species showed high attraction to both fruits and the noni mixture.

The noni mixture repulsive to adults can be attractive for larvae. This finding may reflect different lifestyle requirements of adults and larvae. Larvae will rarely displace far away from fruit, and according to the immediate danger of dessication this implies that they cannot afford to neglect the odor of any potential food source. This may be why as a rule *D. melanogaster* larvae show positive chemotactic responses to almost all odors (Fishilevich et al., 2005). Apparently, differential response cannot be easily

tested against clean air or water control and needs more elaborate choices to show the differences between species.

Be it as it may, larvae do apparently show preferences for odor sources showing that they do discriminate among blends. Such capabilities are also underlined by olfactory learning experiments (Scherer et al., 2003). Results of a bootstrap analysis of the larvae Ors sequences showed the close link between the Ors orthologs of *D. melanogaster* and *D. sechellia*. Further studies should verify if the different behavior of the two larvae is due to small changes in the amino acid sequence or if there is a different ligand affinity of the Ors of the two *Drosophila* sibling larvae. Electrophysiological or optical imaging tools would be very helpful to understand the larval neuronal correlates of the behavior observed here.

Conclusions

In this thesis I showed:

1) How the olfactory system of the specialized sibling species of *Drosophila melanogaster*, *Drosophila sechellia* has evolved to adapt to its sole "life" source *Morinda citrifolia* fruit.

D. sechellia peripheral olfactory system is rearranged to detect chemical volatiles of its sole host. These changes in the periphery are followed by changes in the first center in the brain. The rearrangement in its olfactory system is clearly reflected in its behavior.

2) How odor information from ecological relevant sources is coded by the fly's peripheral system.

We identified natural compounds that elicit a response in OSNs housed in *sensilla coeloconica* of *Drosophila*. These OSNs were capable of detecting the natural odorants at relatively low concentrations and finally these odorants were of biological significance for the fly.

3) The adult *D. sechellia* fly specialization was also paralleled by alteration in the olfactory code at larval stage.

The behavioral responses of *D. sechellia* and *D. melanogaster* larvae showed that also in the larval stage the two sibling species of *Drosophila* have a clear different preference.

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