The Fly Nose
-Function and Evolution

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Abstract

This thesis summarizes and discusses the results of four separate studies on fly olfaction. The aim of the thesis has been to investigate how odor information is decoded by the fly peripheral olfactory system, how this code has evolved and how it is used by the insects in their daily life.

Here I show that insect odorant receptors (ORs), as indicated by the response profile of olfactory receptor neurons (ORNs), are narrowly tuned to specific compounds. The ligands of the system are volatile chemicals characteristic for favored or rejected traits of preferred or avoided resources. These compounds each carry important information. By relying on key-ligands even a narrowly tuned system comprising a fairly small number of ORs can be used to locate and evaluate a large number of resources in complex odor environments.

This conclusion is supported by experimental data contained in the four publications comprising this thesis. In the first study we show that fruitfly ORNs respond most selectively to generic fruit and fungal volatiles, typical for favored characteristics of the flies' preferred resources. In the second study we show how the olfactory code has evolved among the eight close relatives of *D. melanogaster*. The evolutionary pattern we observe illustrates how drastic alterations in odor space and food choice can have direct effects on specific ORNs tuned to important key-ligands. The concept of key-ligand tuning is further nicely illustrated in the third and fourth study by the deceptive pollination system of the dead horse arum. This plant copies in remarkable detail a cadaver in order to attract carrion blowflies. Of particular importance is the chemical mimicry, through which the plant copies three specific cadaver volatiles. Our study shows that these three compounds are the sole mean through which blowfly identify carrion, even though carrion produces a large range of volatile chemicals. The odor mimicry is accompanied by further adaptations reinforcing the carrion mimicry, among which heat is most important. Our study provides rare evidence for a direct functional role of plant thermogeny as we show that the generated heat (up to 20°C above ambient temperature) is important for fine tuning the behavior of the flies.

Keywords: olfaction, *Drosophila*, olfactory receptor neuron, odor ligands, electrophysiology, morinda, *Aracae*, mimicry, pollination, plant thermogeny

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Appendix

Papers I-IV

This thesis is based on the following publications, which will be refereed to by their Roman numerals.

I   Stensmyr MC, Giordano E, Balloi A, Angioy A-M, Hansson BS. 2003. Novel natural ligands for *Drosophila* olfactory receptor neurones. *J. Exp. Biol.* 206, 715-724.

II   Stensmyr MC, Dekker T, Hansson BS. 2003. Evolution of the olfactory code in the *Drosophila melanogaster* subgroup. *Proc. R. Soc. Lond. B.* 270, 2333-40.

III   Stensmyr MC, Urru I, Collu I, Celander M, Hansson BS, Angioy A-M. 2002. Rotting smell of dead-horse arum florets. *Nature* 420, 625-626.

IV   Angioy A-M, Stensmyr MC, Urru I, Puliafito M, Collu I, Hansson BS. 2003. Function of the heater: the dead horse arum revisited. *Proc. R. Soc. Lond. B. (suppl.)* 271, S13-S15.

Objectives

The objective of this thesis was to investigate how insects, as represented by flies, decode their odor environment, how the code has evolved and how the code is used in the search for food.

Introduction

The sense of smell is probably the oldest sensory system in the animal kingdom (Strausfeld and Hildebrand 1999). Although perhaps not fully appreciated among humans, for many animals olfaction serves as the primary mean by which the environment is interpreted. Insects are a notable example of a group of animals for which olfaction is of critical importance. Odors are used to locate food, enemies and mates. Apart from being of importance to many animals, olfaction also serves as an important model system in neuroscience.

The aim of this thesis has been to study how flies perceive their odor environment. Specifically how the odor environment is decoded by the peripheral olfactory system, how the system has evolved and how insects use the decoded odor information in their natural environment.

In this thesis I show that insects have a narrowly tuned olfactory system, specifically configured for a small number of compounds. These compounds carry critical information by being representative of favored resources. By relying on these so called key-ligands, insects can even with a narrow olfactory system locate a large number of assets in complex odor environments.

Flies

Drosophilids

The fruitfly, *Drosophila melanogaster* is one of our oldest model animals. The scientific history of the fruitfly goes back to the beginning of the 20th century. Thomas Hunt Morgan at Columbia University wanted a simple animal model in which he could analyze the physical basis of the, back then, rediscovered Mendelian theory of heredity (Mendel 1866). After an extensive search, Morgan and his students came across the inconspicuous fruitfly *Drosophila melanogaster*. This little fly, which they found proliferating in the alleyways of upper Manhattan, turned out to be a most suitable candidate for the planned experiments. The flies were very easy to rear and also had a very quick generation time. In addition, and importantly, as Morgan was short on money, the flies were extremely cheap to rear. Morgan's first attempts to find mutations were unsuccessful, he however persisted and finally managed to locate a white-eyed mutant (wild-type flies have red eyes, as seen in Figure 1). With the help of this first mutant, Morgan managed

to demonstrate sex-linked inheritance. A couple of mutants later and Morgan and his students were able for the first time to show that the chromosomes were the carriers of the hereditary material (a classic study published by Calvin Bridges in 1916 in the first issue, first volume of the journal Genetics). For his work with the fruitflies, Morgan was awarded the Nobel Prize in 1933 (and a student of his, H. Muller received the prize in 1944). The rest is history, and the little fruitfly has been a favorite model for genetic research ever since. The genus Drosophila comprises a large number of species, with over 3000 so far described (Grimaldi 1990). *D. melanogaster*, belongs to the melanogaster group of the Sophophora subgenus. The melanogaster group is geographically widespread and contains over 170 species, (Schawaroch 2002). Eight of these are immediate close relatives to *D. melanogaster*, and together they form the *D. melanogaster* subgroup (Lemeunier et al 1986; Jeffs et al 1994; Lachaise et al 2000). The evolutionary cradle of the *D. melanogaster* subgroup is in the western Afrotropics, more specifically in the area that today is known as the Ivory Coast (Lachaise et al 1988). The group is thought to have evolved during the last 13-15 million years and the geologic activity along the Cameroon Volcanic Line is believed to have been instrumental for the speciation process within the group (Lachaise et al 2000).
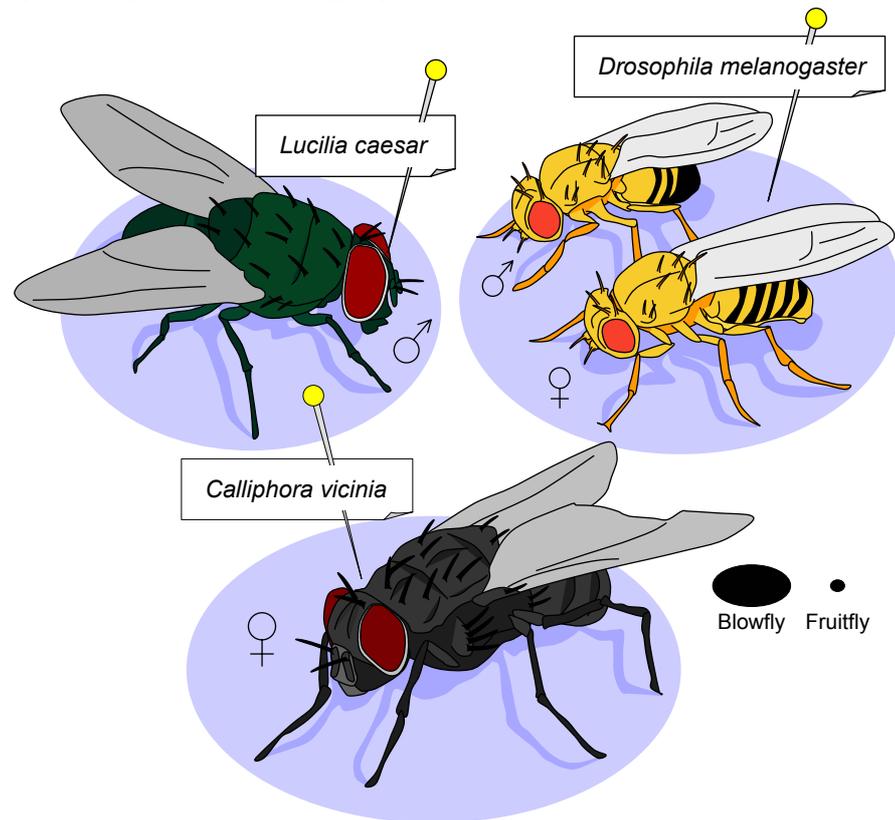

Figure 1. The main characters of this thesis. The fruitfly (*Drosophila melanogaster*), and the two blowflies: European blue bottle fly (*Calliphora vicina*) and green bottle fly (*Lucilia caesar*). The black spheres illustrate the size difference, as the flies are not drawn to scale.

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Calliphoridae blowflies

Two papers in this thesis have as leading characters not fruitflies, but Calliphoridae blowflies. These are, as far as genetics goes, a much less studied group than their smaller relatives. Nevertheless, although not model organisms, they have received quite some attention due to their economic importance (e.g. Fredeen 1985). Blowflies transmit diseases (Fischer et al 2004), torment livestock by causing myiasis (the infestation of live vertebrate animals by dipterous larvae) (Readshaw 1986) and is a general nuisance for e.g. cows, which result in decreased milk production (Miller et al 1973). The family Calliphoridae comprises a very large group of flies that contains more than 1000 species. Although considered pests, blowflies are extremely important in the community ecology and nutrient recycling as they remove and break down vertebrate carcasses (Byrd and Castner 2001).

The primary characters in papers III and IV are the green bottle fly *Lucilia caeser* and the blue bottle fly *Calliphora vicina*. The origin and evolutionary history of these two species is not fully investigated. However, both species are very common and have a widespread, almost circumpolar distribution (Byrd and Castner 2001). Blowflies are among the first insects to discover and colonize carcasses, the media in which the larvae develop. This aspect of blowfly biology has been extensively examined, as the timing of larval development is often used by forensic teams to establish time of death in criminal investigations. Both *Calliphora vicina* and *Lucilia caesar* are among the most common visitors on human cadavers (Byrd and Castner 2001). Although the larvae feed exclusively on carrion and excrement, the adult flies are opportunistic and have a very broad diet (Erzinclioglu 1996).

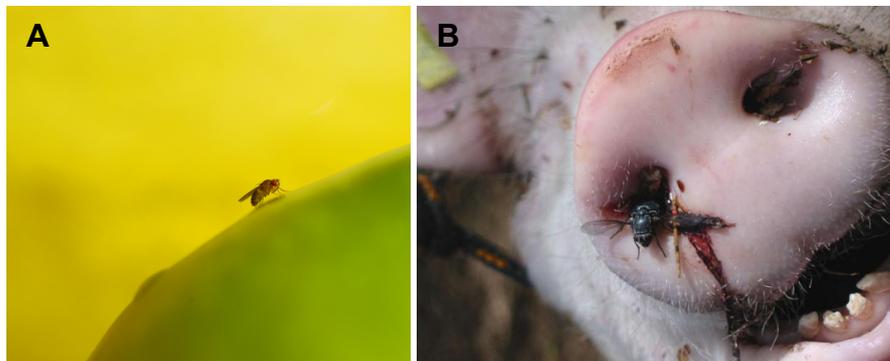**Figure 2**. Fly preferences. (**A**) A fruitfly investigating an apple. For the fruitfly, fruit is the main food source both as adult and during larval stage. (**B**) A blowfly (*Calliphora vicina*) crawling out of the snout of a dead pig. Blowflies are dependent on carrion during the larval stage. The adult flies however feed on a wide range of objects. Photo: Marcus Stensmyr (A), Claude Wyss (B)

The odor world of flies

Insects, including flies, use odors for a number of purposes. A well known type of odor compound in the insect system is pheromones. These compounds typically transmit information between individuals within a single species (Karlson and Lüscher 1959). The classic examples of these types of compound are the female released sex pheromones of nocturnal moths (Fabre 1879; Butenandt et al 1959). These compounds are traced in minute concentrations by the males, who upon detection immediately initiate a search for the source, which in most cases happens to be the female rear-end (Priesner et al 1986; Hartlieb and Anderson 1999). Insect pheromones are typically a blend of a small number of components that need to be present in specific ratios in order to elicit a behavioral effect in the targeted sex (Hansson 1995). The types of compounds which I will address in this thesis however are not related to sexual reproduction but include the odors that animals utilize in their search for food. Compared to the pheromone system, which for any given insect only encompass a handful of compounds, the number of volatiles of importance involved with food is vastly larger.
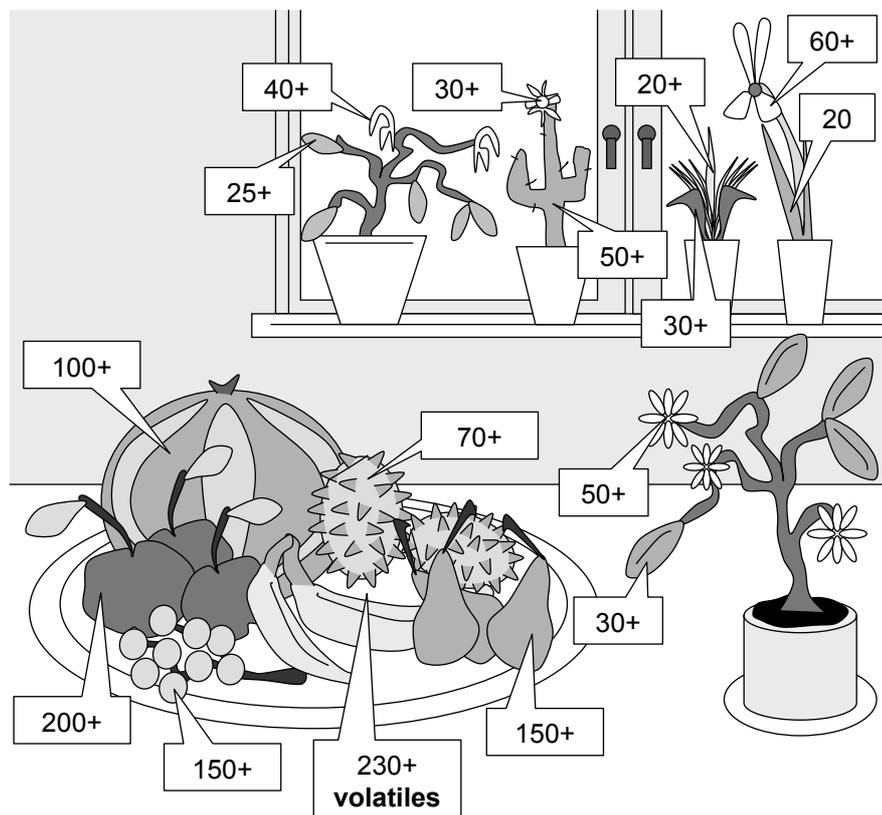

Figure 3. Even what we humans would perceive as a relatively odor poor environment, like a household living room, actually have a complex odor space, comprising a broad range of volatiles. The natural setting of the fruitfly, the Afrotropical rainforest have, naturally, a vastly more complex odor composition.

The staple food of fruitflies is, as their name imply, fruit, which produces a huge range of compounds. For example, in banana over 230 different volatiles have been identified (Macku and Jennings 1987) and in cherimoya (a tasty tropical fruit) and pineapple well over 200 (Idstein et al 1985; Flath et al 1990). The composition of the volatile content of fruit and the concentration of these volatiles also changes during the maturation, with different types of compounds prevailing during the different maturation phases (Macku and Jennings 1987). Young green fruit can be characterized by a high content of so called green-leaf volatiles, these are typically short chained alcohols and aldehydes, e.g. 1-hexanol and hexenal. These compounds have to the human nose a most characteristic smell, resembling freshly cut grass. As the fruit matures these compounds typically give way to an increased ester content. Dominant compounds in the ripe banana for example include isoamyl acetate (which is the compound used to make synthetic banana flavor) and assorted ethyl and methyl esters (Macku and Jennings 1987). After the ripe stage, the degradation starts and the high ester content is accompanied by more and more volatiles produced by degrading microorganisms. The volatiles produced by these bacteria and fungi, as a byproduct of their metabolism (Schnürer et al 1999), is a diverse and large collection of compounds belonging to many different chemical classes. Examples of typical microbial volatiles are acetoin and 2,3-butanediol (Phelan and Lin 1991; Nout and Bartelt 1998). For a fruitfly, with a very broad diet of rotting fruit, the number of potentially important compounds range into the high thousands (Figure 3).

The fly's olfactory system thus faces a daunting challenge. It must be able to detect the diverse and complex mixture of odors emanating from suitable resources in the even more complex and dense odor background of, in the fruitfly's case, the Afrotropical rainforest. Not only must the flies be able to detect which odors that originates from fruit, the flies must also be able to tell suitable fruit from non-suitable fruit and also identify the state of the fruit, as only rotting fruit are consumable for the flies.

Mechanisms of the olfactory system

The insect nose

In flies, as in most other insects, odors are primarily detected by the antennae, which are the insects' functional equivalent of the vertebrate nose. Insect antennae come in widely differing shapes and forms, although their basic organization is similar in most if not all insects (Keil 1999). The odor detecting part of the fly antenna is the club-shaped terminal segment, the funiculus (Siddiqi 1991; Clyne et al 1997; de Bruyne et al 2001; Paper 1). In addition, flies are also capable of detecting odors with the help of their maxillary palps (de Bruyne et al. 1999), which protrude from the mouth parts (Figure 4A).

The sensory organs responsible for odor detection are the olfactory sensilla, which in the fly densely cover the antennae (and the maxillary palps) (Venkatesh and Singh 1984; Singh and Nayak 1985; Stocker 1994). As with the antennae, olfactory sensilla come in many different forms and shapes, however they all

conform to the same principal organization (Keil 1999). A porous cuticle encloses a cavity filled with a viscous medium (the sensillum lymph) in which the dendrites of the olfactory receptor neurons (ORNs) reside (Figure 4B). The ORNs carry the receptors that bind the odor ligands. ORNs are bipolar and the axons connect directly to the brain, in a fashion similar to that of vertebrates.
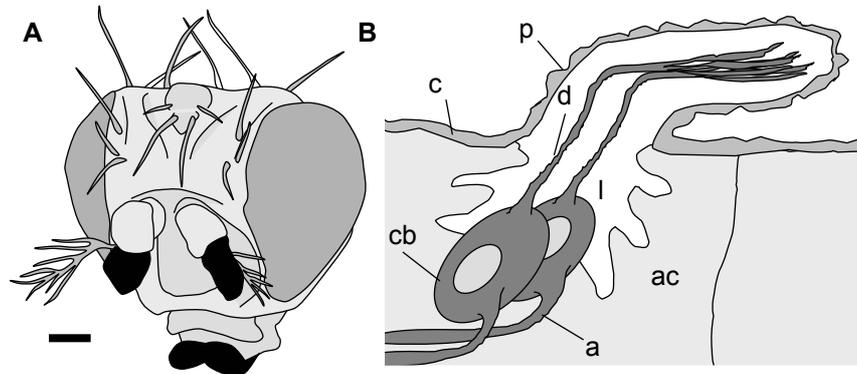

Figure 4. (A) Head of a fruitfly. Olfactory organs marked in black, antennae above and maxillary palps below. **(B)** Schematic drawing of an olfactory sensillum. The dendrites (d) of the olfactory receptor neurons extend into the sensillum lymph (l), which is protected by a porous (p) cuticle (c). Accessory cells (ac) surround cell bodies (cb) and axons (a).

The fruitfly antennae houses three morphologically distinct major types of olfactory sensilla, termed basiconicum, coeloconicum and trichodeum, which are in turn divided into further subgroups (Venkatesh and Singh 1984; Shanbhag et al. 1999; Stocker 1994). Details' regarding the classification, organization and distribution of these structures is outlined in Figure 5.

The odor receptors

The key components and the most critical element of the olfactory pathway are the odor receptors (OR). These were after a long search finally identified in the rat in a milestone study by Linda Buck and Richard Axel in 1991. As had been speculated (e.g. Lancet and Pace 1987), the ORs were indeed 7 transmembrane G-protein coupled receptors, related to e.g. the T-cell receptors (Figure 6A). After the initial finding, ORs were subsequently cloned from a number of other organisms (e.g. Ngai et al 1993; Troemel et al 1995; Mombaerts 1999) (Figure 6B). It soon crystallized that the family encoding ORs in the rat and in many other vertebrates comprised huge gene families. The mouse turned out to have an impressive ~1000-1300 OR genes, which makes it the single largest gene family in any mammalian genome, perhaps in any genome (Zhang and Firestein 2002; Zhang et al. 2003). Also humans turned out to have an impressive amount of OR genes, around 1000, although the majority are pseudogenes (only one third have an open reading frame) (Glusman et al 2001; Zozulya et al 2001; Niimura et al. 2003). Studies indicated that each ORN only express one OR gene (Ressler et al 1993; Vassar et al 1993; Chess et al 1994; Serizawa et al. 2003; but see Mombaerts 2004a for a review on the validity of the one neuron – one receptor

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"dogma"). The OR genes were first unambiguously shown to function as odor receptors by Zhao and coworkers in 1998 (Zhao et al 1998). Other studies have also shown undisputable support for the involvement of these genes in odorant recognition (e.g. Araneda et al 2000; Bozza et al. 2002). The identification of the OR genes had a profound effect on the olfactory research field and has spawned important discoveries and fundamental insights into the workings of the nervous system (Reed 2004).
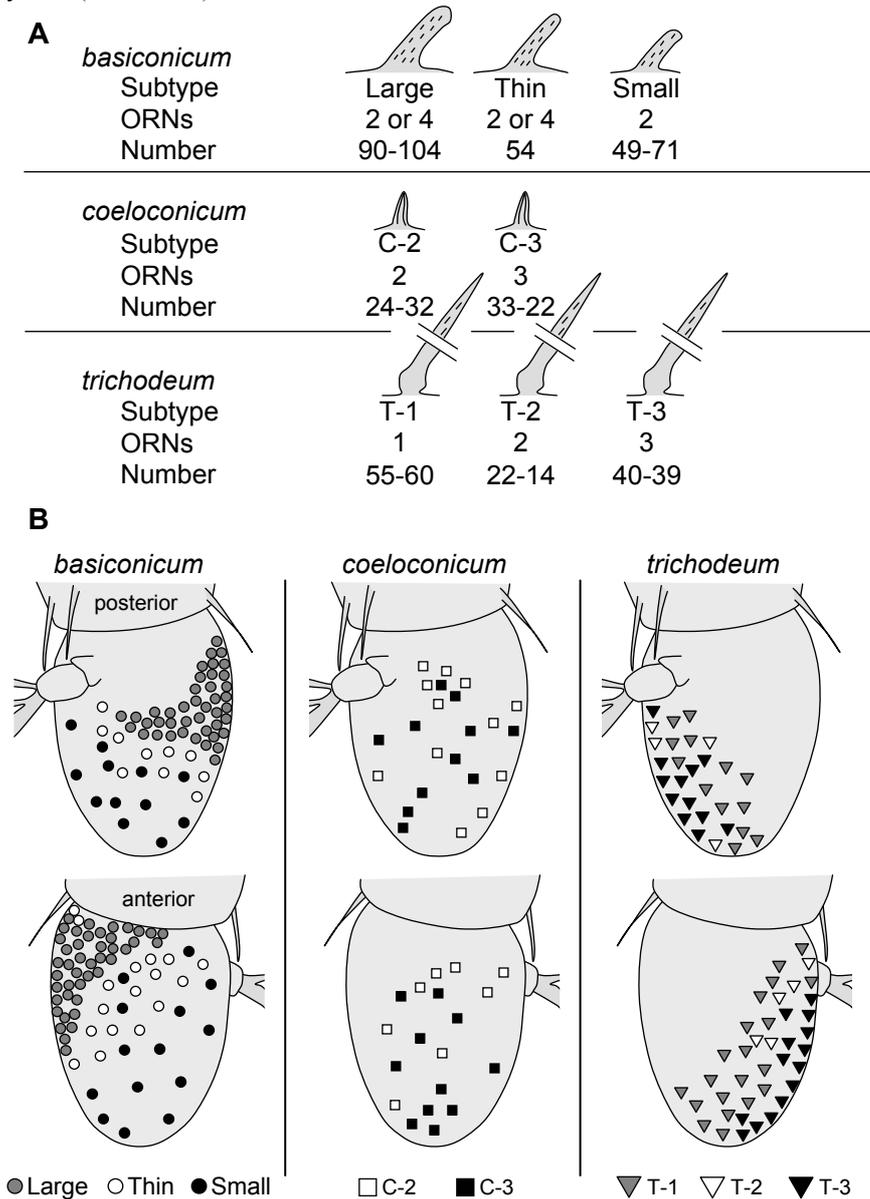
A

basiconicum			
Subtype	Large	Thin	Small
ORNs	2 or 4	2 or 4	2
Number	90-104	54	49-71

coeloconicum		
Subtype	C-2	C-3
ORNs	2	3
Number	24-32	33-22

trichodeum			
Subtype	T-1	T-2	T-3
ORNs	1	2	3
Number	55-60	22-14	40-39

B

● Large ○ Thin ● Small   □ C-2 ■ C-3   ▽ T-1 ▽ T-2 ▼ T-3

Figure 5. (**A**) The characterized morphological types of olfactory sensilla on the fruitfly antennae (from Shanbhag et al 1999). The classification is based on external and internal morphological characters. Each antenna is in total covered by ~430 sensilla

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innervated by ~1000 olfactory receptor neurons (ORNs). In the Number rows, the first number refers to sensilla counts from males, second from females. (**B**) Schematic drawing of the fruitfly antenna, showing the approximate position of the sensillum types (from Shanbhag et al 1999). The large basiconica (whose physiology is the topic of Paper II of this dissertation) have a distinct distribution along the proximo-medial zone.
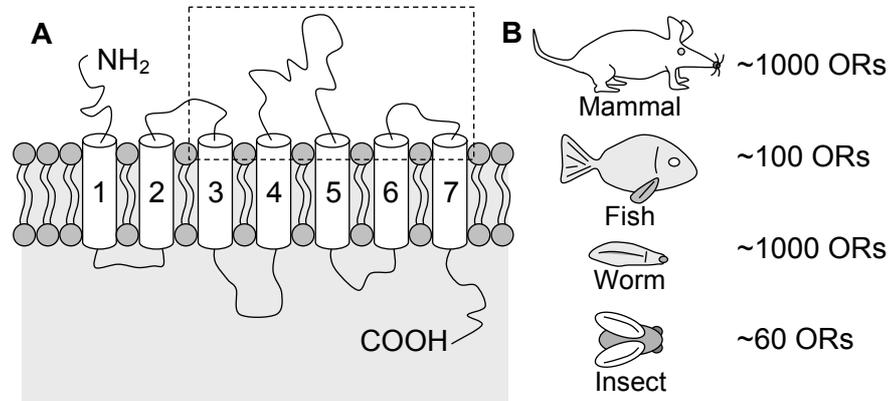

Figure 6. (**A**) An Odor Receptor (OR) protein. The seven transmembrane (TM) domains are numbered. Residues from TM regions 3-7 (boxed area) have been implicated to be involved in odor ligand binding. (**B**) The number of identified OR genes in various animal groups. Mammal, exemplified by the mouse (*Mus musculus*), fish, as in the zebrafish, worm, as in *C. elegans* and insect, as in *D. melanogaster*.

The insect ORs however eluded science for a long time. Attempts to find them based on a putative sequence homology with mammalian ORs turned out to be unsuccessful. Finally, Leslie Vosshall in the lab of Richard Axel and John Carlson and co-workers independently of each other managed to identify ORs from the fruitfly (Vosshall et al 1999; Clyne et al 1999 and Gao and Chess 2000). The approach both labs had taken was to screen the then partial genome of the fruitfly, scanning for putative transmembrane proteins. The fruitfly ORs turned out to share very little sequence homology to their vertebrate equivalents and interestingly also showed very little sequence homology within the family. The complete repertoire of ORs in the fruitfly comprised 57 genes. Of these, 32 were shown to be expressed exclusively in the antennae, 7 exclusively in the palps, one in both palps and antennae and 17 were not found expressed at all. (Vosshall et al 2000). These genes were subsequently shown to indeed function as odor detectors in two elegant studies. By over-expressing an OR (Or43b), Klemens Störtkuhl and colleagues were able to show that the electroantennogram response to a specific ligand (cyclohexanol) was increased over that found in wildtype flies (Störtkuhl and Kettler 2001). The second study, by John Carlson and co-workers, relied on a mutant strain (Δ*halo*) that lacks a particular OR (Or22a). By expressing assorted ORs in the "empty" neurons (which express Or22a in the wildtype) of the Δ*halo* mutant, Carlson and co-workers were able to show that the response characteristics of a single ORN is entirely due to the affinity of the expressed OR, i.e. by replacing one OR with another OR, the neurons shift their response characteristics accordingly (Dobritsa et al. 2003, see also Hallem et al 2004). As in the mammalian system, most insect ORNs seem to express only one functional OR. However, in the fruitfly the majority of the neurons also appear to express a

second OR, the enigmatic Or83b (Vosshall et al 2000). This particular OR is contrary to the other OR genes highly conserved among other insects so far investigated (Hill et al 2002; Krieger et al 2002), which in itself may imply an important role of this OR, separate from odorant recognition. Mattias Larsson and associates in the lab of Leslie Vosshall have recently managed to render a mutant phenotype lacking this protein. Flies lacking Or83b appear to be anosmic for most odorants, as the functional OR is not transported to the dendritic membrane, but remain in the ORN cell soma (Larsson and Vosshall unpublished). This observation suggests that Or83b may function as a chaperone, although more work is needed before other functions can be ruled out. Chaperone molecules associated with ORs have recently also been described for the mammalian olfactory system (Matsunami, unpublished).

The transduction pathway

The odor molecules enter the sensillum lymph through pores in the sensillum cuticle (Steinbrecht 1997). The sensillum lymph, that surrounds the dendrites of the ORNs, is an aqueous medium; a facet that causes a problem as most volatile odor ligands are hydrophobic (i.e. they dissolve poorly in water). Richard Vogt and Lynn Riddiford in 1981 discovered an abundant low weight family of proteins that were exclusively located in olfactory tissue. They termed these odorant binding proteins (OBPs), and speculated that these proteins were responsible for the transportation of odor molecules through the sensillum lymph to the receptor site (Vogt and Riddiford 1981). Subsequent analysis revealed OBPs as generally occurring throughout all investigated insects (Vogt et al 1999) and also being present in mammals (Tegoni et al 2000). In the fruitfly there are ~40 genes coding for OBPs (Shanbhag et al 2001). Modeling studies show that an odor molecule seems to fit snugly in a pocket of the protein. Although theory, modeling and some experimental data suggested a pivotal role of these proteins in the olfactory pathway (Kim et al 1998; Kaissling 2001; Kim and Smith 2001), direct functional evidence was long lacking. However, recent work in the fruitfly demonstrates the necessity of these proteins for functional olfaction. Through electrophysiological recordings from the *Lush* mutant, which lack the Lush OBP, Dean P. Smith and co workers managed to show that this particular OBP must be present in order to activate the receptor of the (putative) fruitfly pheromone cis-vaccenyl acetate (Smith et al 2004). Although evidently important in the olfactory pathway, the exact function of OBPs remains to be elucidated.

Once the odorant reaches the OR, it interacts with the protein. Residues of transmembrane (TM) regions 3-7 have been implicated in the ligand binding process (Pilpel and Lancet 1999; Singer et al 2000). Through *in silico* studies (computer algorithms based on the crystallized structure of the bovine rhodopsin receptor) residues of TM regions 3, 5 and 6 have been indicated to be directly involved in ligand binding (Floriano et al 2000). The interaction of the odor molecule with the OR presumably induces a conformational change, which activates the linked G protein that triggers a signal transduction cascade. The outcome of these processes results in an increased action potential firing rate of the activated neuron.

Higher brain centers

The axons from the peripheral ORNs target the antennal lobe. This structure, which is the functional equivalent of the olfactory bulb in vertebrates, is the first relay station of the olfactory signal. Within the antennal lobe, axons of neurons expressing the same OR coalesce and form a spheroid structure, a so called glomerulus. This important aspect of the system was first demonstrated in a noctuid moth by Bill S. Hansson and colleagues in 1992. Later work, relying on molecular techniques, have firmly demonstrated this principle in the fruitfly (Vosshall et al 2000), as well as in the mouse (Mombaerts et al 1996). In the fruitfly, the antennal lobe houses ~40 glomeruli, thus roughly correlating the number of expressed ORs (Laissue et al 1999). The glomeruli of the antennal lobe are in turn innervated by primarily two other forms of neurons, projection neurons and local interneurons. The latter group connects the glomeruli together, and may be of importance in fine tuning the code, as it is transmitted from the periphery (Hansson and Christensen 1999). The first group, the projection neurons assemble the signal from the glomeruli and transmit the information to higher brain centers, primarily to the mushroom bodies (MB) (Strausfeld 1976) and the lateral protocerebrum. The in most insect brains prominent MBs also receive input from other sensory modalities, and the MBs are thought to play an important role as an integrative center involved in learning and memory functions (Heisenberg 1998, Strausfeld et al 1998). The MBs are comprised of densely packed Kenyon cells (KC), and have as the name implies a mushroom shaped form. The "cap" (calyx) harbors the cell bodies of the KCs and the synapses between KCs and input neurons. The KCs extend their fibers down the "stem" (pedunculus) and into the "cup" (the α/δ and β/γ lobes). In the lobes the KCs connect with output neurons which in turn connect to e.g. motor centers (Strausfeld 1976).

Electrophysiological methodology

The first electrophysiological approach to insect olfaction was developed by Dietrich Schneider, one of the true pioneers of the field, in 1957. Schneider named the method electroantennogram (EAG), in analogy to the electroretinogram method used in the visual system. The EAG recordings represent the summed activity of the olfactory receptor neurons, and odor induced activity is visualized as a negative deflection in the baseline. The EAG method was further developed by Arn, Staedler and Rauscher in 1975, when they adapted the technique to facilitate stimulation via a gas-chromatograph (GC-EAD).

A technique that allows for a more detailed analysis of the peripheral olfactory system is the single-cell method (Hubel 1957). This technique was first adapted for the insect system by another pioneer in the field, Jürgen Boeckh in 1962. The procedure of this method is outlined in Figure 7. As with the EAG, this technique was also modified to work in combination with gas chromatographic detection (Wadhams 1982). All these methods have since been widely used and have proven to be indispensable tools in insect olfactory research.
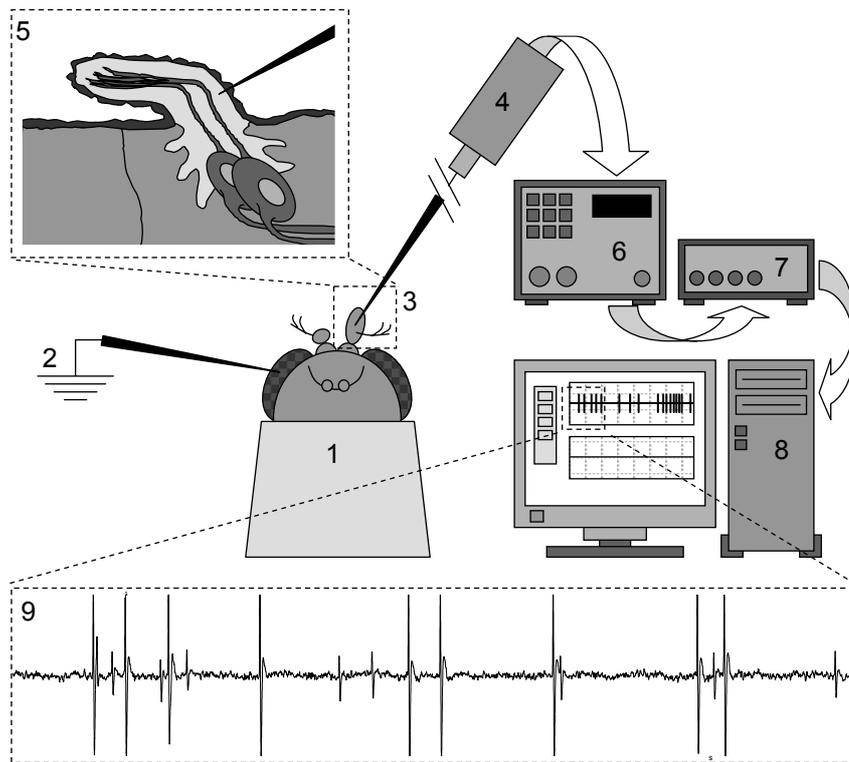
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Figure 7. Single-cell electrophysiology. An insect (1), grounded through the eye (2) is mounted with the antennae (3) protruding. A sharpened tungsten microelectrode (4) is brought into contact with a single olfactory sensillum (5), and the neural activity of the neurons housed in the sensillum is recorded extracellularly. The signal from the contacted neurons is amplified (6), passed on to a digital converter (7) and fed into a computer (8), where the signal is visualized and analyzed (9).

The peripheral olfactory code

Narrow vs broad tuning

How do insects decode their odor environment? A question that boils down to how the ORs operate and in what fashion they bind odor molecules. Traditionally two hypotheses of how ORs bind ligands have been proposed (Dethier 1972; Masson and Mustaparta 1990). The first hypothesis holds that ORs bind odorants most selectively, in principle this theory suggests that each OR only recognizes a single type of molecule or at most a handful. The information carried by the specific odor molecule would then be transmitted to the brain in the form of a *labeled line* system. The other proposed alternative would be that the ORs are rather promiscuous in their binding, resulting in that each OR binds a wide range of molecules. The identity of an encountered odorant would then be transmitted as the combined activation pattern of a number ORs, generating a so called *across fiber pattern* (Figure 8).
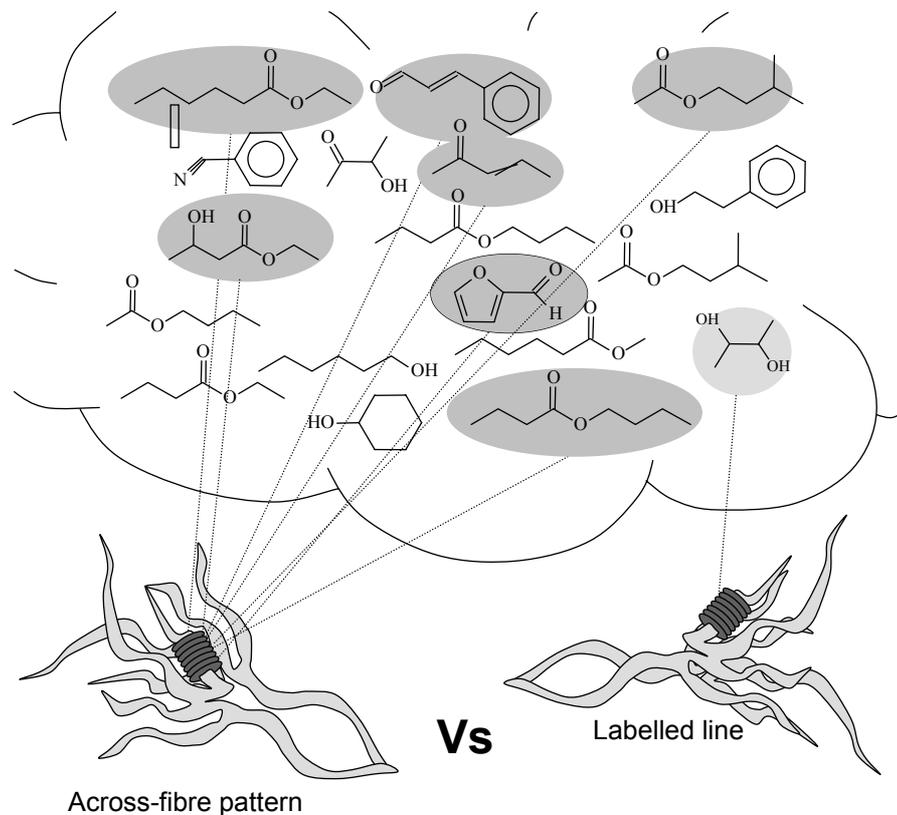
Figure 8. Odor coding principles. The across fiber pattern hypothesis holds that the odorant receptors are broadly tuned. The identity of an encountered odorant is accordingly decoded by the combined activity of a spectrum of ORs. A labeled line system on the other hand, in principle suggests that each OR is specifically configured for one ligand.

An example of a labeled line pathway would be the moth pheromone system, where highly dedicated and specialized neurons selectively bind a single type of molecule, a pheromone component. The way in which general odors, like those originating from food sources, are detected have traditionally served as an example of *across fiber* coding. This notion has been supported by a number of studies (Schneider et al 1964; Ma and Visser 1978; Den Otter et al. 1980; Selzer 1981; Dickens et al. 1984), however primarily by logical arguments. As the number of volatile compounds released by food resources, such as plants, is huge, a broadly tuned olfactory system is needed in order not to overlook (or rather oversmell) any potentially important odorants, coming from suitable resources. A narrowly tuned system would then according to this reasoning not be able to do the job. For a species like the fruitfly, where the number of potential odors of importance is in the range of several thousands, the arguments for *across fiber* coding are especially appealing. Even a specialized species would face problems with a narrowly tuned system. E.g. a banana specialist would preferably be able to detect a large proportion of the roughly 230 volatile components of the banana. In

addition, the olfactory system of the banana specialist should also be able to detect non-banana volatiles to ensure that the wrong host is not visited. Although these are attractive arguments for *across fiber* coding, the experiments that support this notion are all of an early date, and have been challenged by more recent work that suggests that general odor detecting neurons in insects can be as sensitive and selective as those detecting pheromonal components (Dickens 1990; Todd and Baker 1993; Anderson et al 1995; Blight et al 1995; Wibe et al 1997; Hansson et al 1999; Larsson et al 2001; Röstelien et al 2000; Stensmyr et al 2001), thus implying that general odors are perhaps detected along the same principles as in the pheromone system.

Still, the fact that the older studies do show that general odor tuned ORNs are broadly tuned raise some concern. A problem with many of these older studies however is the excessive concentrations of stimuli used, with odorants applied in concentrations far exceeding any amounts that the insects would ever encounter in their natural environment, under any conditions. Another problem is that these studies in many cases relied on compounds of no ecological relevance. Excessive stimulus amounts can even trigger the notoriously selective pheromone ORNs of noctuid moths into responding to non-pheromonal components (Hansson et al 1989; Carlsson and Hansson 2002; Peterlin et al 2002). Thus, the notion of broadly tuned odorant receptors may in fact stem from the use of extreme amounts of stimuli, and may perhaps not reflect how the system has evolved to operate.

The arguments in favor of broadly tuned general odor detecting ORNs are however still valid. Assuming that the ORs are selectively configured for a small number of molecules, how do insects then manage to locate food, if they are only able to detect a fraction of the released volatiles? This question is highly relevant if we consider the fruitfly system, with only ~50 ORs and a diet that release thousands of odorants. How do fruitflies decode their odor environment and how do they use the gained information to locate food? This is the question we have addressed in the first paper of this dissertation.

Odor coding in the fruitfly (Paper I)

In the first paper of this thesis we aimed to investigate the peripheral coding properties of the fruitfly olfactory system. We used a technique known as linked single-cell gas-chroamatography (GC-SC) (Wadhams 1982), a method that we have previously successfully used in the scarab system (Stensmyr et al. 2001). This approach which rely on simultaneous physiological detection (from contacted ORNs) with flame ionization detection (from the GC), facilitates the screening of a large number of odorants of known ecological relevance, as extracts of favored (as well as rejected) objects can be used. Hence, as GC-SC allows the testing of odor ligands that the olfactory system has evolved to detect, it makes a most powerful technique.
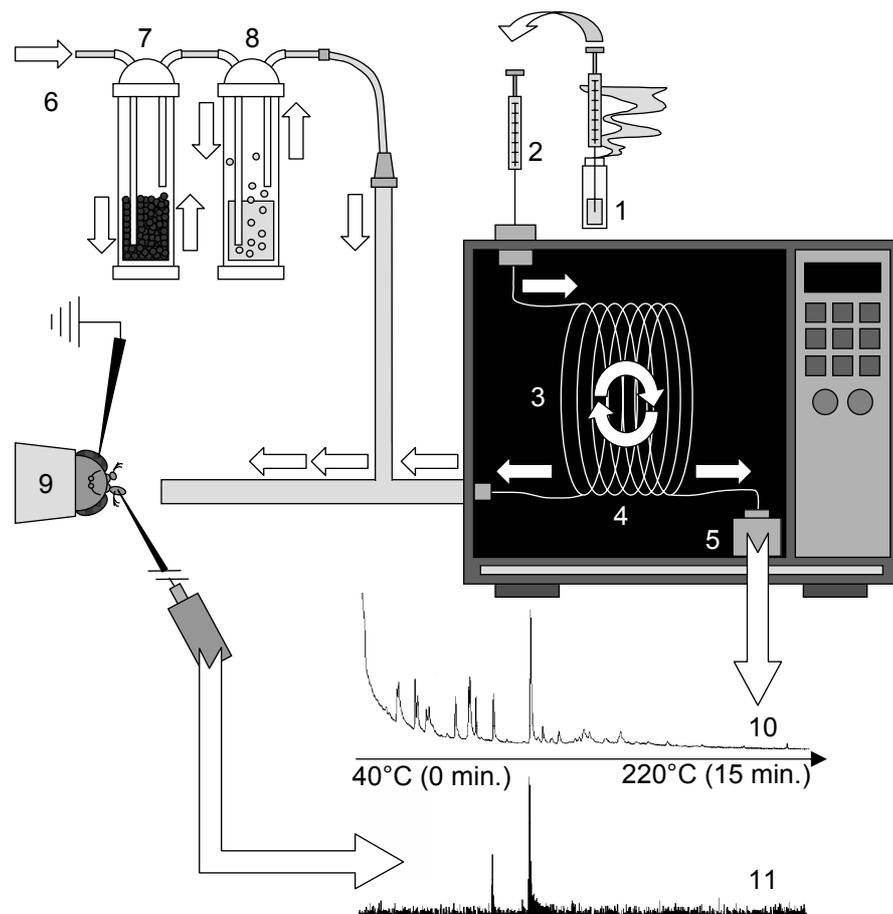
Figure 9. The GC-SC technique. Odor samples (collected as in Figure 10) (1) are injected (2) onto a GC-column (3), situated in an oven. As the temperature in the oven is increased, the components of the odor sample are separated by the active coating and travels down the length of the column. At the end of the column, a split (4) is installed, passing half the effluent to a flame ionization detector (FID) (5) and the other half out of the GC-oven into a glasstube. Through the tube, a constant stream (6) of charcoal filtered (7) and humified air (8) flows. The air stream passes the separated odor components over a mounted insect (9) from which single cell recordings are performed (following the procedure outlined in Figure 7), thus allowing for simultaneous recordings of FID (10) with physiological detection (11).

In addition, the GC-SC approach would also enable us to apart from figuring out aspects of the code, also to identify natural and novel odor ligands for the *Drosophila* system. Although the fruitfly has been used extensively in olfactory research, odor ligands of biological significance have long been lacking. Most studies have relied on stimulus sets chosen more or less arbitrary with no consideration for the ecology of the species. To draw conclusions based on experiments that test the properties of the system when it is challenged by stimuli that the system has never been designed to detect is naturally not fully satisfactory. Hence, novel ecologically relevant ligands for the *Drosophila* system were sorely needed.

First, we collected odors from different fruit, which were all readily eaten by the flies. The fruit came from a wide geographic area, which we speculated would ensure a broad range of volatiles. The fruits chosen were banana, litchi, papaya, mango, pineapple and passionfruit. In addition we also collected volatiles from yeast. We sampled odors from the fruit (and yeast) using head space technique (Figure 10).
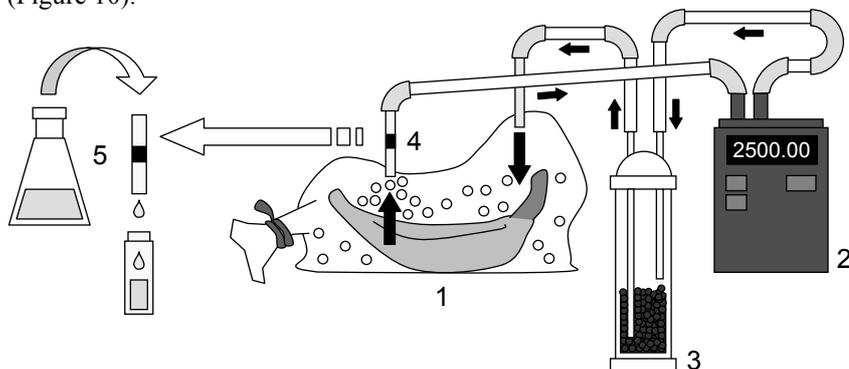

Figure 10. Head-space collection of odor volatiles. The volatile source (1) (in this case a banana) is encapsulated in a plastic bag; air is pumped (2) into the sampling bag after purification (by passage through active charcoal) (3). The pump then evacuates the air inside the bag through a charcoal filter (4), which traps the odor volatiles. The collected volatiles are then flushed from the filter with a solvent (hexane or methylene chloride) (5).

With the extracts at hand we proceeded with the GC-SC measurements. We performed a large number of recordings (~100) and the extensive data set allowed us to type response patterns and to classify functional groups of ORNs (Figure 11). In all, we found 12 distinct types of neurons. Based on the general position of the recording electrode we were able to match the physiology of 6 of these ORN classes with sensillum morphology.
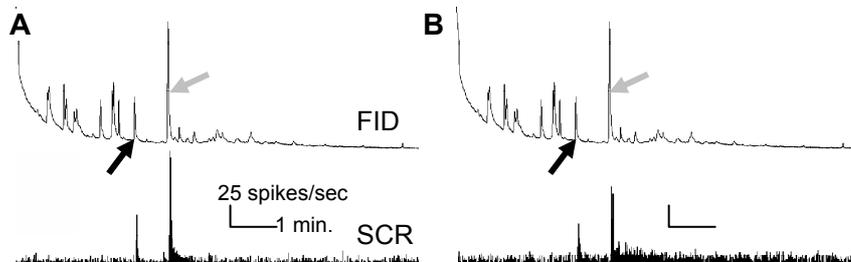

Figure 11. ORN classification. (**A**) A GC-SC recording, stimulating with a pineapple extract, showing discrete responses to two compounds. (**B**) Another neuron, stimulated with the same pineapple extract display identical response pattern. Similarities in response profile allowed us to type distinct physiological neuron classes. Grey arrow: ethyl hexanoate; Black arrow: methyl hexanoate.

The recordings revealed that the neurons responded most selectively to the screened compounds. In most cases a contacted neuron was only activated by just a few components and in some cases only by a single ligand; a remarkable selectivity considering the huge range of stimuli that we screened. Neurons

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responding to several compounds were primarily activated by components sharing both specific functional groups as well as sharing a high degree of similarity in overall structure, e.g. a neuron would respond to a group of highly similar ethyl and methyl esters, with highest affinity to compounds of a certain length. These findings strongly implicate that the fruitfly ORs have a very narrow ligand affinity and suggest strongly that the ORs are strictly configured for a specific ligand, a key-ligand. Thus, as far as selectivity goes, the fruitfly food odor detecting neurons show a great deal of similarity with the insect pheromone detecting neurons. The neurons did not only show high selectivity but also a high degree of sensitivity. In many cases these responded to compounds only occurring at the limit of the flame ionization detection capacity. Thus, also with respect to sensitivity the neurons appear to function much along the lines of the pheromone detection system.

To study the detection limit in more detail, we performed dose response trials with neat synthetics of the identified compounds from the fruit (Figure 12). The dose response relationships confirmed our initial observations of high sensitivity and selectivity. We tested the four most potent ligands for one of the neuron classes (left part of Figure 12) and the results show that these particular neurons are primarily configured for two most similar compounds. Deviations, even small ones, in the structure strongly decrease affinity. The detection level we noted is extremely low, a par with pheromone neurons and with the most sensitive plant odor neurons so far described (GLV neurons in a scarab, Hansson et al. 1999).
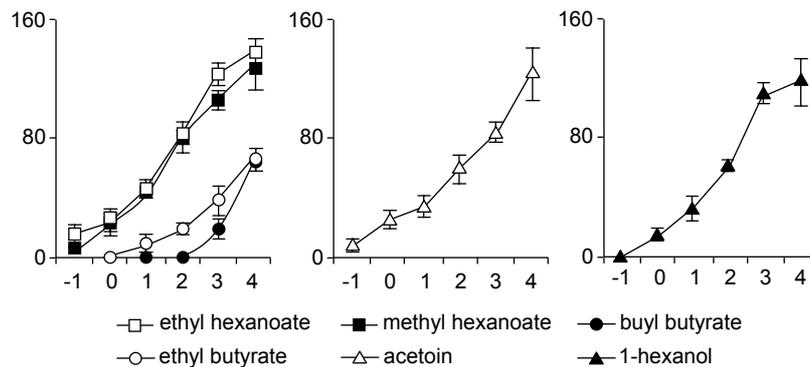

Figure 12. Dose-response functions. The S3A neuron stimulated with the four most potent ligands found for this neuron type (left graph). Ethyl and methyl hexanoate are much more potent than the other ligands and elicit responses at very low doses. Acetoin (middle) and 1-hexanol (right) likewise also produce responses at very low concentrations. Y-axis represent spikes/sec. X-axis dose in log ng.

The dose response curves also help to explain earlier results suggesting insensitivity and unselectivity on behalf of general odor detecting neurons. In the earlier experiments the concentrations used were in the right end of the graphs in Figure 12, at concentration levels where the neurons responds to more and more dissimilar components. In addition, as in many of these studies compounds of no ecological relevance were used (i.e. compounds that the system never has evolved to detect). The olfactory systems under scrutiny were never challenged down to

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the actual detection limit, as a consequence of the ligands simply not being efficient enough. Hence, the notion of insensitivity may perhaps really have been a consequence of a poorly chosen stimuli battery. Had correct stimuli been chosen, perhaps the notion of sloppy general odor neurons never would have arisen.

Assuming that most of the ~50 functional ORs expressed in the fruitfly are tuned along the same principle as the ones investigated here, how do fruitflies then manage to locate and detect food with what must be a most limited sensory system? We propose that they do so by relying on key components. The compounds that the ORs are primarily configured for are common and characteristic traits of favored resources. In the fruitfly, which primarily feeds on rotting fruit, we would expect a large proportion of the ORs tuned to general fruit volatiles (e.g. ethyl hexanoate), other ORs to compounds indicating microbial activity (e.g. acetoin). In addition the system should also be equipped with ORs configured for volatiles indicating unsuitable resources, in the case of the fruitfly e.g. volatiles characteristic of unripe fruit (green leaf volatiles, such as 1-hexanol). Combinatorial triggering of different ORs would in this fashion quite specifically relay information regarding the encountered odor source (Figure 13).
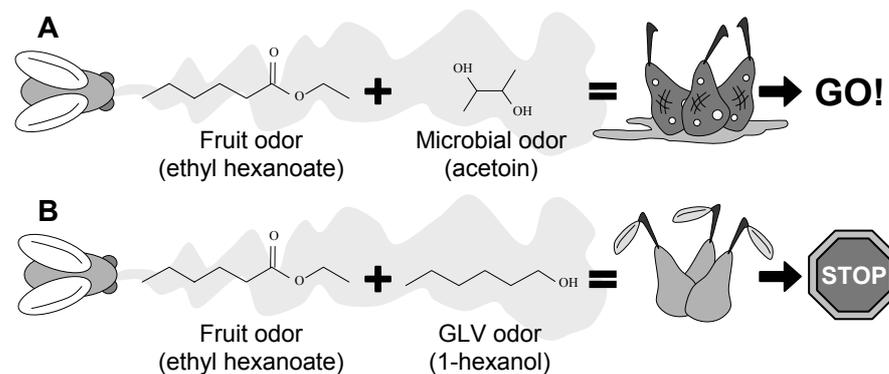

Figure 13. Combinatorial activation of a limited number of ORs can mediate specific information. (**A**) Fruit odor together with microbial odor indicates a preferred resource, i.e. rotting fruit. (**B**) Fruit odor linked with green leaf volatiles (GLVs) indicates a non suitable resource, i.e. hard unripe fruit. Combinations of, or even single key-ligands can in this fashion be used to locate a large number of resources.

Assuming that the flies rely on key-ligands, as described above, we would expect many of the physiologically active compounds found in this study to be attractive, as most of the identified ligands are indicative fruit volatiles. Furthermore, we would also expect many of the compounds to be attractive by themselves, and not only as part of blends if they indeed contain valuable information in themselves. We used a T-maze to investigate the behavioral effect of a subset of the ligands. The T-maze assay was originally developed by Tully and Quinn in 1985, and is an elegant method to study olfactory guided behavior in the fruitfly (Ayyub et al 1990; Alcorta 1991; Acebes and Ferrús 2001). The assay can measure both repellency as well as attraction and was originally developed for studies regarding olfactory memory and learning. The operation of and the design behind the T-maze assay is outlined in Figure 14.
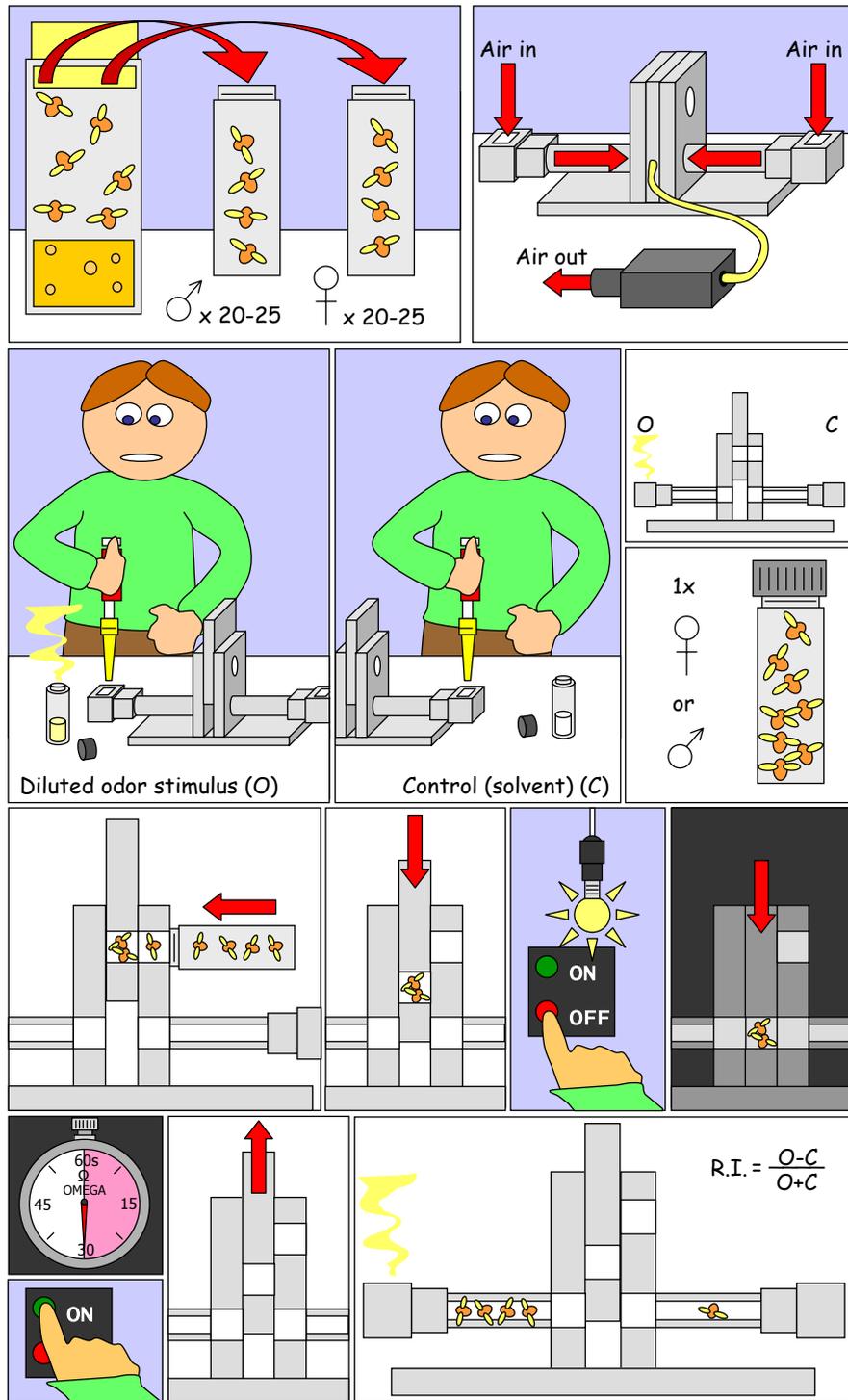
Figure 14. How to operate the T-maze assay. Cartoon read from left to right.

We tested the behavioral effect of seven compounds. As predicted, the fruit type volatiles were attractive, and the tested green leaf volatile (1-hexanol) repellent at all concentrations. Thus, the behavioral results support our notion of key-ligand tuning.
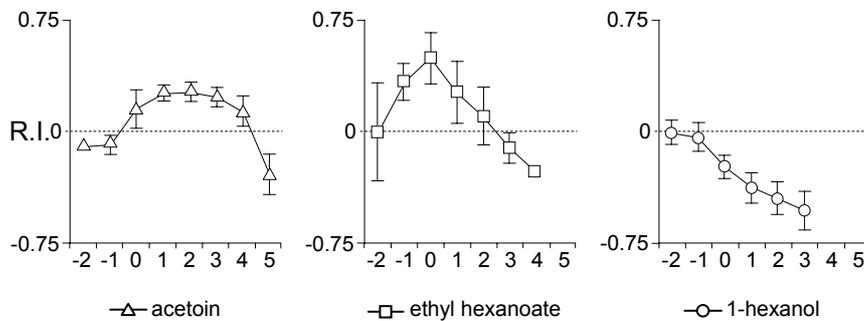

Figure 15. Results from the T-maze assay. The fungal volatile, acetoin and the fruit volatile ethyl hexanoate both elicit attractive behavior. At low concentrations the response index (R.I.) approaches 0, the value indicating indifference (the flies are not able to detect the compound). At moderate concentration levels the two compounds are attractive; at higher doses the compounds become repellent. On the other hand, 1-hexanol, a compound characteristic for green plant tissue, is repellent at all concentrations. R.I. calculated according to the formula in Figure 14. X-axis dose in log ng.

Odor coding in vertebrates

How do mammals decode odors? The general consensus has been that mammalian ORs have a broad binding affinity, with each OR capable of binding quite a range of molecules (Mombaerts 2004b). Several studies have shown a broad ligand affinity on behalf of mammalian ORs (Araneda et al 2000; Bozza et al 2002). Work by Michael Leon and coworkers, deploying 2-deoxyglucose imaging has showed that mammalian ORs bind selectively for specific characters of molecules, such as the presence of a certain functional group, whereas the overall structure of the molecule is ignored (Johnson and Leon 2000a, b). The method deployed in their studies is however not without its drawbacks. The technique requires lengthy stimulation periods (several hours) and may hence be criticized for not representing events as they occur under natural conditions, where odor molecules typically would only fleetingly interact with the animal.

The picture these studies presents is hence quite different from the one we observe in insects. Where not only the presence of a specific functional group is required for activation, but also a specific overall molecular shape and structure (e.g. Hansson et al 1999; Larsson et al. 2001; Stensmyr et al. 2001; Paper I). This difference may seem trivial, but have fundamental effects on how the peripheral olfactory system decodes odor information. We would by necessity have *across fiber* coding in the mammalian nose at all concentrations versus a primarily straight line of events in the insect antennae at low (natural) concentrations, which possibly switches to something akin to *across fiber* coding under very high (unnatural) concentrations.

Recent work, primarily done in the lab of Lawrence Katz suggests however that the mouse olfactory system may operate in analogous ways to that of insects. Combining, as we have done electrophysiology with gas chromatography, Katz and co-workers have shown a surprising degree of selectivity and sensitivity in the mouse olfactory system, a par with that described in insects (Lin and Katz 2004). Further work from the same lab, where they deployed a robotic stimulus delivery system, which enabled the screening of hundreds of synthetic compounds, also showed an olfactory tuning along the lines of the fruitfly and other insects. The neurons were tuned to compounds that shared an overall structural similarity, and were not activated by compounds only sharing a single feature but in other respect diverse (Davison et al 2004). Further work in the mouse and in other mammals is required before any firm conclusions can be drawn, work which will be most interesting to follow.

Evolving the olfactory code

Fitting the nose to the environment

In paper I, we show that the olfactory code is intricately linked with the food choice of the animal. As the food choice naturally is restricted by the available resources within the animal's given environment, the olfactory code is thus in extension a consequence of the animal's habitat. The question is what happens with the olfactory code over evolutionary time if the animal is transferred to a novel setting? We would expect to see a shift and a reconfiguration in the code towards the conditions of the new environment. Colonization of new habitats furthermore in many cases also result in speciation, i.e. that the transferred population evolve into a novel form distinct from the ancestral form (Hawthorne and Via 2001; Nosil et al 2002). Island colonization is a perfect example of how transfers can lead to speciation and the most bizarre adaptations (Wallace 1880), e.g. flightless birds such as the dodo (and its close association with the *Calvaria major* tree) (Temple 1977) and the Aldabra rail (Huxley 1979). The intriguing question of how the olfactory system has been affected by environmental transitions and through speciation events has however received virtually no attention. Electrophysiological data from the peripheral olfactory system of a number of species is present, however direct comparisons between these data sets are not feasible. The species investigated are in many cases to distantly related for any meaningful comparisons, in addition differences in methodology, such as varying stimulus sets, make it difficult to draw any firm conclusions. To study the evolution of olfactory coding we would need to make comparisons from orthologous structures in closely related species, preferably with a known phylogeny.

Evolution of the olfactory code in *Drosophila* (Paper II)

In the second paper of this thesis (II) we have addressed the question of how the olfactory system has been affected by the speciation process. As a model to study

this, we used the *Drosophila melanogaster* subgroup (Figure 16), which is an excellent target for these questions as the group comprises closely related species occupying widely different niches. In addition the species also display most varying food preferences, with species ranging from single host specialists to true generalists (Lachaise 1988; Lachaise et al 2000).
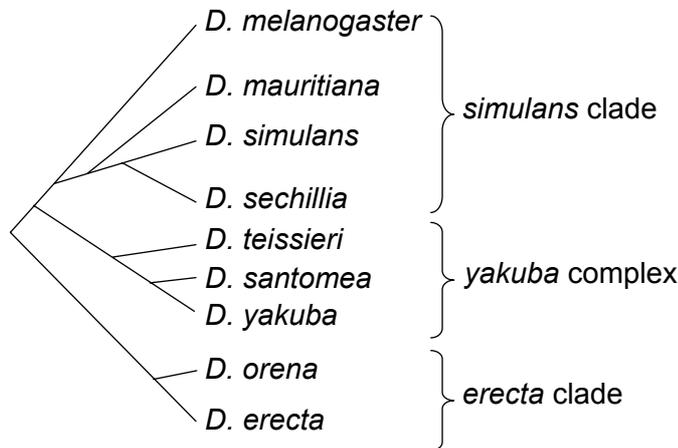

Figure 16. Phylogeny of the nine sister species of the *Drosophila melanogaster* subgroup. Tree based on Lemunier et al 1986, Jeffs et al 1994 and Lachaise et al 2000.

In this study we again used the single-cell electrophysiology approach (Figure 7). Although, in this study we used a set-up enabling a much more detailed approach than in Paper I. We used a system with a very high magnification factor (<1500X) equipped with piezo-linked motor-driven micromanipulators that allowed us to position the recording electrode with extreme accuracy. We concentrated our investigation to the response characteristics of a subset of neurons housed in a specific morphological type of sensilla, the large basiconica (Figure 5).

The recordings revealed that the ligand affinity, pairing rule and action potential amplitude of eight distinct ORN types (housed in three separate sensillum types, denoted ab1, ab2 and ab3) present in *D. melanogaster* had been conserved to a large extent in its eight sibling species. We only observed distinct changes in three of the species, *D. simulans*, *D. sechellia* and *D. mauritania*. In these species the differences were restricted to a shift in tuning profile of a single neuron type (the ab3A in *D. simulans*, *D. sechellia* and *D. mauritiana*) and the loss of a sensillum type (the ab2 in *D. sechellia*). Except for these changes, these three species shared the same characteristics as the other siblings.

We were quite surprised to note this high level of similarity. As today, two of the species are cosmopolitan (*D. melanogaster* and *D. simulans*), three occur only in the Afrotropics (*D. yakuba*, *D. teissieri* and *D. erecta*) whereas another four are endemics, of which three are confined to islands (*D. sechellia*, *D. mauritiana* and *D. santomea*) and one restricted to a sole mountain top (*D. orena*) (Lachaise et al
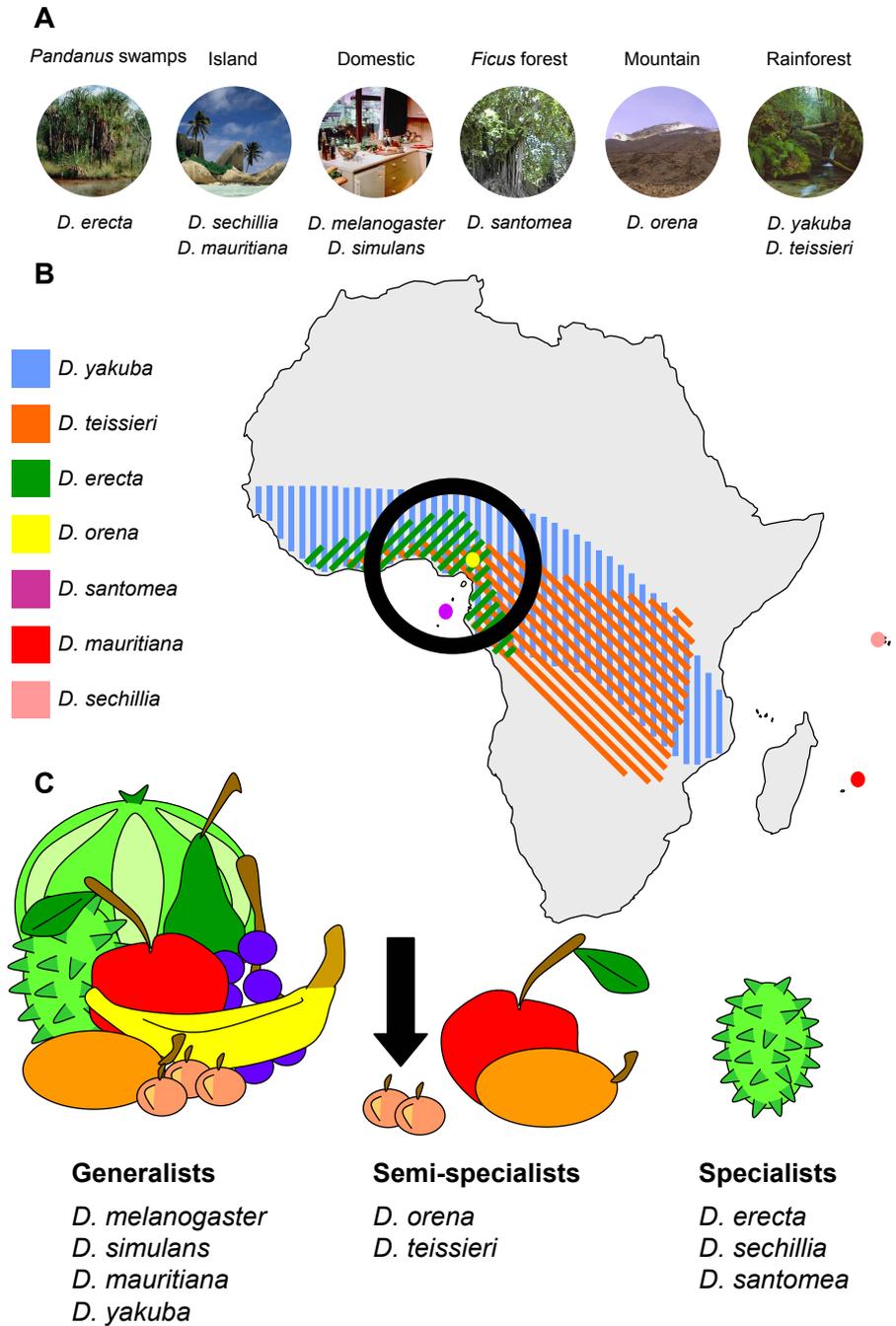
A

Pandanus swamps — *D. erecta*

Island — *D. sechillia*, *D. mauritiana*

Domestic — *D. melanogaster*, *D. simulans*

Ficus forest — *D. santomea*

Mountain — *D. orena*

Rainforest — *D. yakuba*, *D. teissieri*

B

- *D. yakuba*
- *D. teissieri*
- *D. erecta*
- *D. orena*
- *D. santomea*
- *D. mauritiana*
- *D. sechillia*

C

Generalists	Semi-specialists	Specialists
D. melanogaster	*D. orena*	*D. erecta*
D. simulans	*D. teissieri*	*D. sechillia*
D. mauritiana		*D. santomea*
D. yakuba		

Figure 17. (**A**) Habitats of the nine sister species of the *D. melanogaster* subgroup. (**B**) Distribution range. *D. melanogaster* and *D. simulans* have as human commensals an almost global range. The black circle marks the believed evolutionary cradle of the subgroup. (**C**) Food choice in the group range from broad generalists to single resource specialists.

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1988; Lachaise et al 2000) we expected to see more signs of adaptations to local settings. These highly differing environments, characterized by very different sensory information, i.e. odor space, should have exerted equally varying selective pressures, shaping the olfactory sense of the flies to the different unique settings. And, as mentioned, the species also display most varying food preferences (Figure 17). Still, to a large extent the characteristics of the olfactory system of the fruitfly, as seen through the large basiconica, have gone largely unchanged over evolutionary time.

The high degree of similarity is likely a consequence of the fruitfly siblings' ancestral polyphagous nature, which has generated an olfactory system efficient in detecting a large range of resources and capable to cope with quite varying circumstances. The almost circumpolar distribution range of *D. melanogaster* is in itself a proof of the effectiveness of the fruitflies' sensory system, and show that the fruitfly "nose" can operate competitively in almost any environment. Although similar in most of the species we did observe strong shifts in two of the species, both of which are insular endemics. Thus, an interesting question arise whether these shifts are the outcome of genetic drift or driven by natural selection, as direct adaptations to changes in the effective odor space?
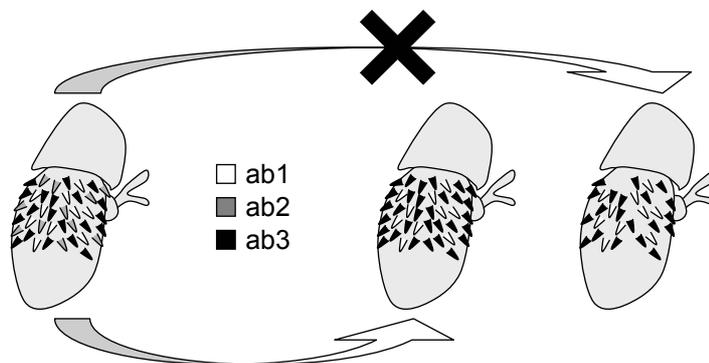

Figure 18. The ab2 type sensillum was not detected in *D. sechellia.* Sensillum counts and frequencies of the ab1 and ab2 indicate that the ab2 have not simply been lost (above scenario), but have been replaced by a higher proportion of ab3 sensilla (below scenario).

In *D. sechellia* we found in place of the missing ab2 sensilla, an increased proportion of ab3 sensilla (Figure 18). Could this possibly represent a host-specific adaptation towards the *Morinda citrifolia* fruit, the single host of *D. sechellia* (Tsacas and Bächli 1981; Louis and David 1986 Cariou et al 2001)? The specialization of *D. sechellia* towards morinda fruit is intriguing, as fresh and ripe morinda fruit is presumably toxic to all *Drosophila* species, except for *D. sechellia* (Legal et al 1992) (Figure 19). The toxicity of the fruit comes from the high acid content, to which *D. sechellia* is more tolerant, as a consequence of a number of specific adaptations (R'kha et al 1991; Jones 1998). Ripe morinda fruit, apart from having a high acid content also release large amounts of esters, including ethyl and methyl hexanoate, which give the fruit a characteristic aroma. The odorants detected by the missing ab2 neurons are on the other hand not found in morinda fruit (Farine et al 1996). We propose that having a larger proportion of neurons

devoted to the detection of ethyl and methyl hexanoate should enhance the ability of the flies to locate their host. The toxic and foul smelling fatty acids have been shown to be important for inducing oviposition in *D. sechellia* (Amlou et al 1998). We suggest that the esters are important cues used by the flies to detect morinda fruit over long-distance. As the patches of morinda trees are dispersed on the islands, the flies need to have a sensitive olfactory system, capable of picking up the faint odor traces of the morinda while navigating from one patch to another.
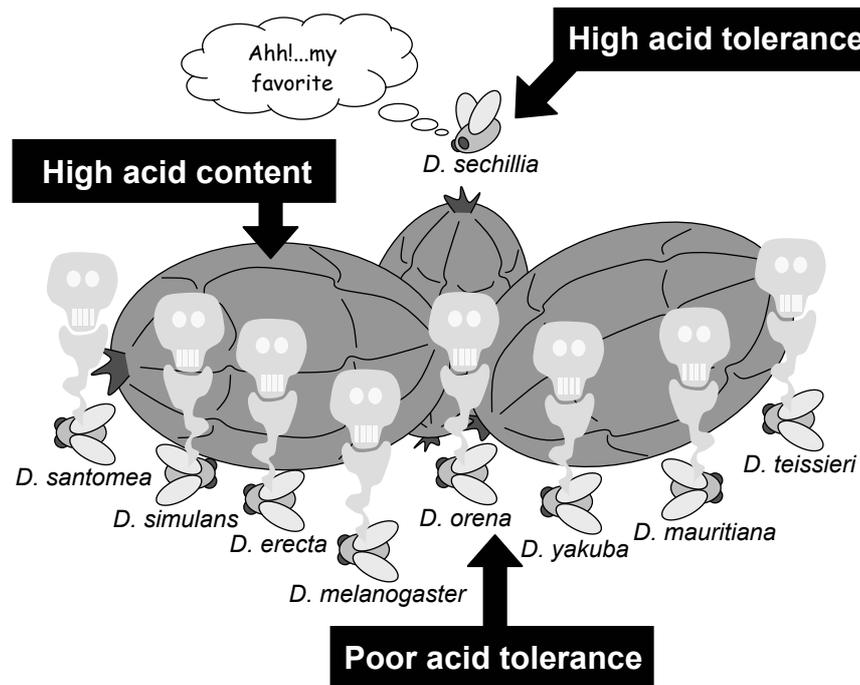

Figure 19. Morinda fruit, which is the single host of *D. sechellia,* has a very high acid content. *D. sechellia* is resistant to the toxic effect of this fruit, whereas to the other eight sister species of the *D. melanogaster* subgroup exposure to the fruit is lethal.

The other notable deviation in the study was the lost capacity in *D. mauritiana* to efficiently detect ethyl and methyl hexanoate in favor of ethyl butyrate (Figure 20). As we showed in paper I, these types of esters are highly attractive to *D. melanogaster*. As *D. mauritiana* reputedly shares food preference with *D. melanogaster* (David et al 1989), it is notable that in *D. mauritiana* the ability to detect these very common and indicative fruit components has been lost (Paper I). The present day population of *D. mauritiana* feeds primarily on plants introduced by humans (David et al 1989). The host / hosts of the founding population, prior to the arrival of humans can of course only be speculated upon; however the shift to ethyl butyrate may very well represent an adaptation for an as yet unknown primitive resource, and the shift in coding the result of an adaptation along the lines of *D. sechellia*. As the native Mauritian flora is poor in fruit producing trees, the ancestral stock of *D. mauritiania* had at arrival a limited selection of resources to choose from, which furthermore, given the poor island setting, were most likely

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already in use by other native species. The limited number of resources, and the fierce competition for these, would create strong selective pressure on behalf of the new arrivals to adapt their olfactory sense to the key-ligands of these resources in order to be competitive. A possible candidate native host is the *Spondias borbonica* tree, one of the few, for a fruitfly, suitable fruit-bearing trees on the island. Relatives of this Mauritius endemic have been shown to have ethyl butyrate, the component detected by the ab2a neurons in *D. mauritiana*, as a major or prominent volatile constituent (Jirowetz et al 1999; Augosto et al 2000).
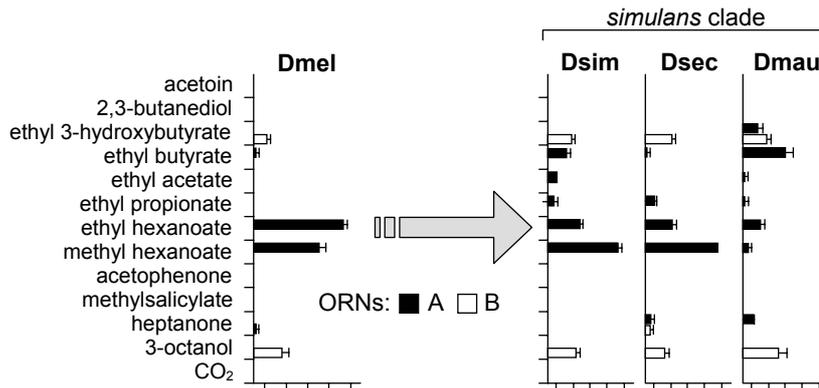

Figure 20. The tuning of the ab3A neurons have shifted in the *simulans* clade from the norm of the group. In *D. simulans* (Dsim) and in *D. sechellia* (Dsec) the key ligand have gone from ethyl to methyl hexanoate. In *D. mauritania* (Dmau) a more potent shift has occurred and the ab3A neurons instead respond primarily to ethyl butyrate. The tuning of the ab3B neurons has not been altered.

The coding shifts in the island endemics, most notably in *D. sechellia* nicely illustrates the concept of key-ligand tuning as shown in paper I. The shift shows that single ligands representative of preferred resources are indeed of importance, and that single ligands, even a ubiquitous fruit component like methyl hexanoate, can be single handedly used to locate a specific resource under certain conditions. For *D. sechellia*, the presence of methyl hexanoate in the air signals the close whereabouts of morinda fruit, whereas for most of the other siblings the presence of this compound, in the context of their more diverse habitats, would signal the proximity of fruit in general.

The olfactory code exploited

Of flowers and bees

A perfect example of how key ligands are of importance to insects comes from deceptive pollination systems. Most pollination systems have evolved as mutualistic relationships in which both parties are rewarded. The pollinator obtains a reward, such as pollen, nectar, waxes or scents from the flower whereas the plant achieves reproduction through the transfer of its pollen by the pollinator

(Simpson and Neff 1983). Chemical mimicry has evolved in pollinator/flower systems where no reward is received by the pollinator. The plant, which is the sole benefactor, hence lures the pollinator to its flower by producing specific scents, which may copy pheromones, food sources, broodsites or prey odors (Dafni et al. 1984). Perhaps the most well known case of chemical mimicry comes from the *Ophrys* orchids. These orchids mimic virgin females of their pollinators, thus attracting the males that are subsequently fooled into pollination. The *Ophrys* orchids are primarily pollinated by bees, and each *Ophrys* species has evolved its own unique relationship with one pollinator species (Linneaus 1745; Kullenberg 1961; Borg-Karlson 1990). The orchids both mimic the shape, color and texture of the specific bee they want to attract, as well as the pheromone composition of the virgin female bees. The complex pheromone blends of the female virgin bees are copied in detail, down to even the natural variation in pheromone composition. The visual and chemical mimicry of the orchids trigger sexual behavior in the males, who finally attempt to copulate with the flowers. The pollinia get attached to the heads of the males during their eager copulation attempts (Schiestl et al 1999, 2000; Ayasse et al 2003). Another equally fascinating system which also relies on sexual deception is the pollination system of the Australian orchid *Chiloglottis trapeziformis*. These plants, copy the shape and the single female pheromone compound (which turned out to be a novel and most unusual compound) of the wasp pollinator. As in the *Ophrys* system, the males are attracted to the flower and while attempting to copulate incidentally pick up the pollinia (Schiestl et al 2004). These two systems both exploit pheromone ligands of insets in order to achieve pollination. Following, I will describe a system that instead exploits food odor key-ligands for pollination.

The dead horse arum

The dead horse arum (Figure 21A) is native to the western Mediterranean region, with a distribution restricted to Sardinia, Corsica and the Balearics (Figure 21B). It can primarily be found on small rocky islands (Figure 21C), which often house extensive gull colonies (although a small population of the plants can also be found in the Corsican highlands) (Friedlender 2000; Rosello and Saez 1997). The most prominent aspect of the plant, apart from its bizarre appearance, is perhaps its obnoxious smell, which is most reminiscent of a carcass (Figure 21D) (Kite 2000). The foul smell is evidently highly attractive to insects, which are drawn to the plants in large numbers (Figure 21F). For pollination to occur, the arum needs to fool the attracted insects into entering a trap chamber (Figure 21G), which houses the female and male florets (Figure 21H). Once inside the chamber, spines and filaments blocks the insects' exit, effectively trapping them. Insects carrying pollen from another plant fertilize the receptive female florets as they buzz around the chamber. The chamber remains closed overnight and the insects trapped until the next morning. Then the spines and filaments blocking the exit starts to wilt and the male florets (situated at the entrance of the chamber) start to produce pollen and the female flowers cease to be receptive (in order to avoid self-pollination) (Figure 21I). When leaving the chamber, the insects have to get past the male florets and are anew coated with pollen (Paper III).
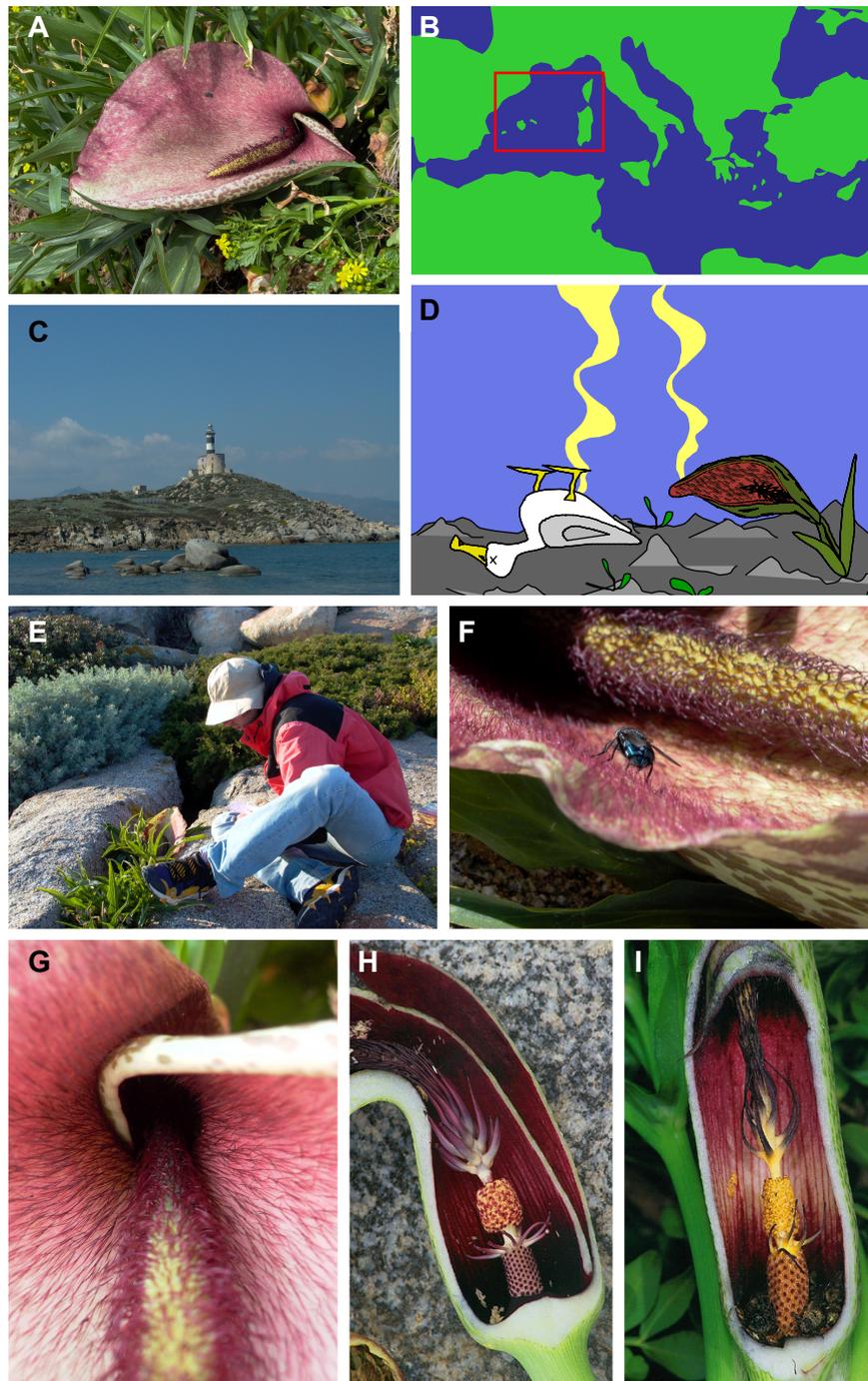
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Figure 21. (**A**) The inflorescence of a dead horse arum (*Helicodiceros muscivorus*). (**B**) Distribution range of the arum is restricted to Sardinia, Corsica, Minorca and Mallorca. (**C**) Typical habitat of the arum; small rocky islands. Here the island Cavoli where the field work was carried out. (**D**) The arum produces a truly foul smell, resembling carrion, such as

that coming from a dead gull, the primary carrion resource in the field site. (**E**) The author of this thesis deeply engaged in front line research. (**F**) Insects (here a blowfly, *Calliphora vicina*) are drawn in large numbers to the plants. (**G**) For pollination, attracted insects must be fooled to enter the trap chamber. Note the similarity with a mammalian rectum, complete with a "tail" and hair like structures "growing" out of and encircling the opening. (**H**) The trap chamber during first day of flowering with receptive female florets below, and male florets above, which at this stage are not producing pollen. Spines separate the female from the male florets and serve to enclose attracted insects around the female florets at the bottom of the chamber. (**I**) The trap chamber during the second day of flowering. The female florets are no longer receptive, whereas the male florets have started to produce pollen (the yellow powder visible in the image). The spines have wilted allowing the trapped insects to escape. While escaping the insects get anew coated with pollen, which they transfer to the next plant, repeating the procedure. Photo: Marcus Stensmyr (A, C, F and G), Salvatore Spanno (H and I), Amanda Sternhufvud (E).

An important aspect of this system is the fact that the attracted insects are not rewarded for their efforts, thus the system likely relies on mimicry. As the common name of the plant implies, the plant has been assumed to copy carrion, and the target for the mimicry accordingly carrion insects.

The olfactory deception (Paper III)

In our first study of the dead horse arum (Paper III) we investigated the assumed chemical mimicry. Our first attempt was to identify the targets of the mimicry. If the plant is truly copying carcass key-characteristics, then we would expect carriophilic insects to be the prime visitors. Thus, we caught insects visiting arum plants and carrion resources (dead gulls) in the study site. The trap catches showed that the primary victims of the fraud were almost exclusively female blowflies, primarily from the two species *Calliphora vicinia* and *Lucillia caesar*; moreover these flies were also the principal carrion visitors in the study site.
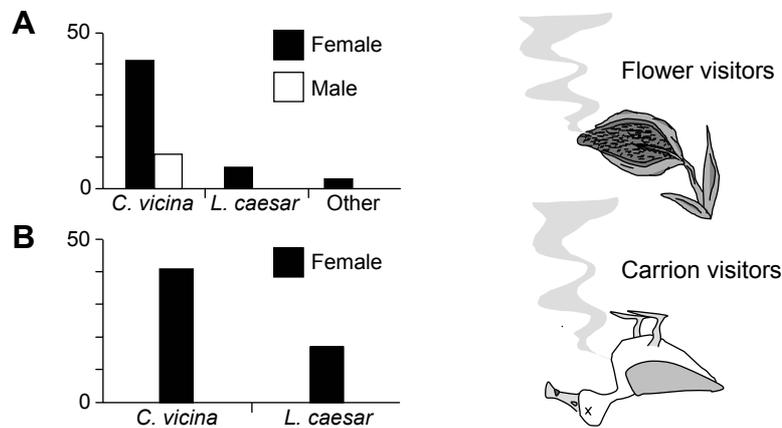

Figure 22. Total catches of insects visiting the arum (**A**) and carrion (dead gulls, *Larus michaellis*) (**B**). Both media attracted the same visitors; females of the two blowfly species *Calliphora vicinia* and *Lucillia caesar*. Y-axis indicates number of caught individuals. Insects were trapped from 12 different plants and from three different carcasses.

Having confirmed the targets as carrion insects, we next sought to identify the odor ligands involved in recruiting the blowfly visitors. To identify the odor compounds we again used the approach of linked electrophysiology with gas chromatography. Although, this time we linked the FID recordings with electroantennogram detection (GC-EAD) from blowfly antennae.

We collected odors from freely growing arum plants during their first day of flowering, following the procedure outlined in Figure 10. In addition we also collected odors from authentic carcasses (rotting pig meat), as we assumed that if the arum is indeed mimicking carrion we would expect to see similarities in the odor composition of the arum with that of true carrion. The GC-EAD recordings stimulating with arum odor showed that the fly antennae selectively detected four components (Figure 23A). Recordings with carrion odor also triggered four responses (Figure 23B), which after GC-MS analysis turned out to be the exact same components as was present and active in the arum. Furthermore, the response pattern elicited by the arum and carrion odor was identical (Figure 23C).
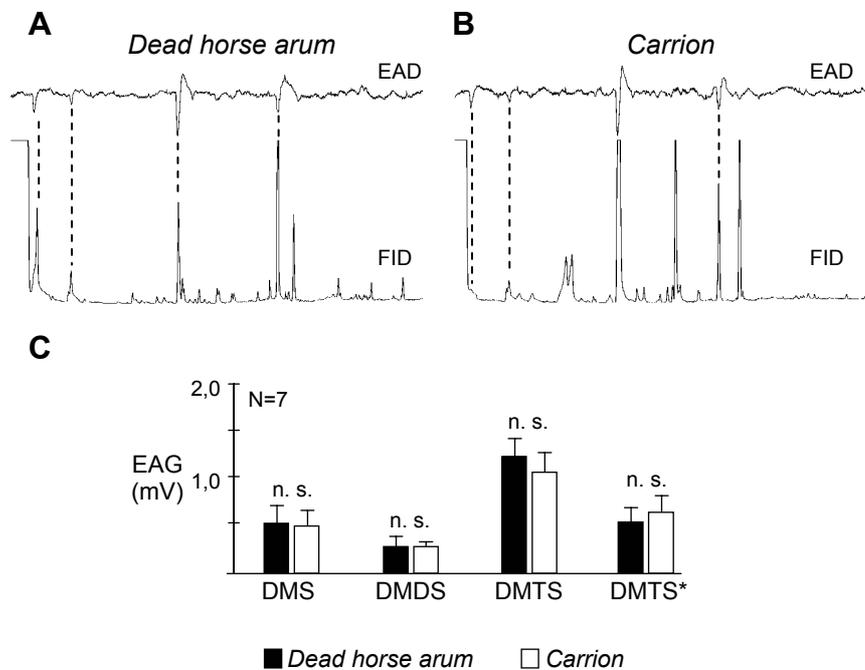

Figure 23. (**A**) Stimulation with arum odor produces four distinct responses (indicated by dashed lines) from blowfly antennae. (**B**) Stimulation with a carrion extract reveals that the same four components are also present in rotting meat. (**C**). Mean (± s.e.) EAD responses elicited by the active oligosulphides. The response patterns to both odor media are identical.

Thus, the GC-EAD recordings show that to blowfly antennae the odor coming from the arum is indistinguishable from that of true carrion. Hence, by relying on olfactory cues alone a fly would not be able to tell the difference between the plant and a carcass.

The foul smell, due to the presence of the three oligosulphide components, is only produced during the first day of flowering, while not detected in odor samples taken from day two. Although odorless during the second day, the plants look the same as on the first day. We recorded the number of flies visiting arum plants during the two-day flowering period, and found that the plants were primarily attractive to blowflies during the first day, while the odorless state during the second day attracted significantly fewer flies (Figure 24A). To test the behavioral importance of the identified oligosulphides, we added these to dental cotton rolls, which we then placed inside the trap chamber of the plants during their second, odorless day of flowering. Then we recorded the number of fly visits starting from day one (plants with natural scent) through day two (artificial scent). Thus, if the oligosulphides were an important part of the mimicry, we would expect to restore the arum's attractiveness on day two to similar levels as recorded on day one. Indeed, we found that adding the odors fully restored the arum's attractiveness during day two of flowering (Figure 24B). Odors thus play a crucial role in attracting flies to the flower.
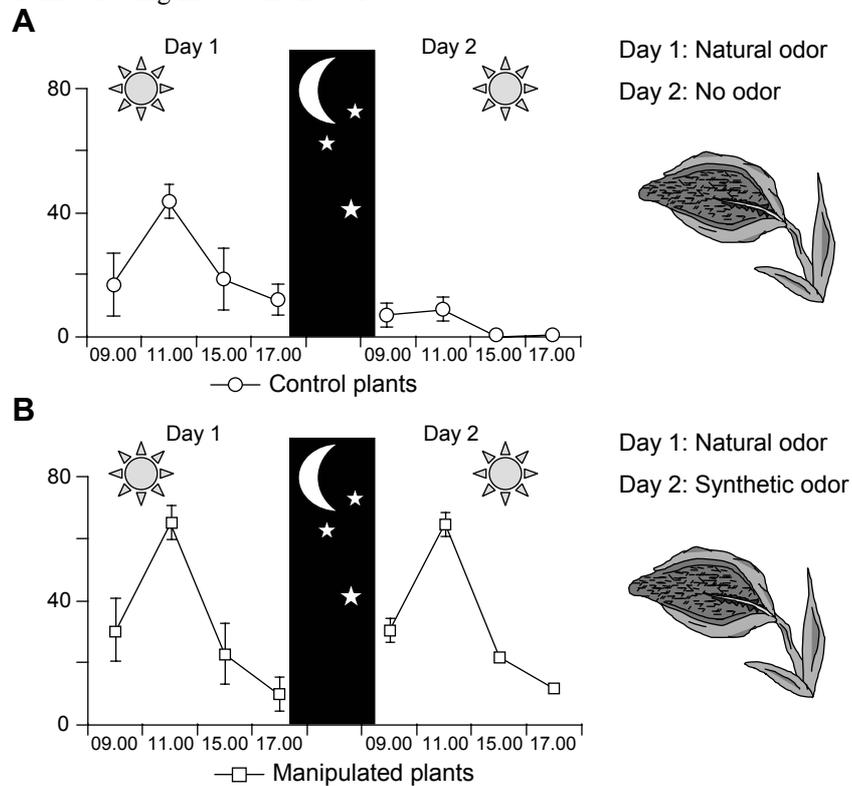

Figure 24. The behavioral effect of the oligosulphides. (**A**) Blowflies primarily visit the arum plants during the odorous first day of flowering. (**B**) By adding a synthetic mixture of the oligosulphides to the arum plants during the odorless second day, the attractiveness of the plants are restored to the same level as on the first day of flowering. Y-axis represents mean number of fly visits ($\pm$ s.e.). X-axis time of day.

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These sets of experiments are the first solid evidence for one of the fundamental principles of how mimicry systems are supposed to work. Theory holds that for a mimicry system to persist, the operator (in our case blowflies) must not be able to tell the copy (arum plants) apart from the model (carrion). Our physiological and behavioral data nicely illustrates this principle.

The pollination system of the dead horse arum shows that specific odorants, characteristic for specific traits of favored objects are indeed used by insects. The oligosulphides represent a superb example of key-ligand tuning. Although carrion produces a vast range of volatiles, carrion insects actually only rely on the oligosulphides to locate this, for them, most precious resource. The oligosulphides are typically found in the odor of decaying meat, as they are created in the protein decomposition process (Brown 1982) and are rarely found in other media, with the exception of certain bat-pollinated plants of Central America (von Helversen et al 2000). Thus, the oligosulphides can reliably be used by the blowflies to locate carcasses. The arum system is in many ways analogous to the sexual deception of the *Ophrys* orchids. Any fly failing to be attracted to these odorants will most likely also fail to localize a large fraction of the available true carrion resources. In both cases the plants mimic odors that the insects cannot afford to ignore when encountered.

Multisensory mimicry (Paper IV)

As outlined in Paper III, odors play a crucial role in this mimicry system and serve to attract flies over distance to the plants. However, once the plant is encountered, the plant must be interesting enough for the flies to stay and explore. Most importantly the plant must ensure that the attracted flies are also fooled to enter the trap chamber. How do the plants manage this feat? We suggest that the plants copy multiple aspects of a carcass, thus creating a multisensory targeting mimicry that is irresistible to the flies (for an excellent in-depth review on integrated sensory signals produced by plants, see Raguso 2004).

An intriguing aspect of the arum is its capacity to produce heat. This thermogenic ability is shared with many other species in the Araceae family (e. g. Seymour and Schultze-Motel 1999; Gibernau and Barabé 2000; Barabé et al 2002). The dead horse arum is among the most thermogenic plants known, with a capacity to produce temperatures 20°C over ambient temperature (Seymour et al 2003a). Despite this being rather well studied phenomena, a general consensus as to why plants would produce heat has not been reached. Most likely thermogeny has evolved for several different reasons, and not in response to meet a single common need

Thermogeny have been proposed to have evolved originally as a form of energy reward for beetle pollinators (Seymour & Schultze-Motel 1997). The beetle pollinated *Philodendron solimoesense* of French Guiana produces heat that indeed warm their beetle pollinators, that are attracted to the plant in large numbers and stay inside the plant over night, saving considerable amounts of energy in the process (Seymour et al 2003b). Thermogeny solely functioning as beetle "nightclubs" is not likely, as presently not all thermogenic plants are beetle

pollinated. Accordingly, other ecological roles for thermogeny have been proposed, for example to increase volatilization of specific chemicals directed towards pollinators (e.g. Seymour and Schultze-Motel 1999) and as a carrion mimicry reinforcing stimuli (e.g. Uemura et al 1993).

In paper IV, we investigated the functional role of thermogeny in the dead horse arum. First we recorded the temperature production capacity in our study population. We found, as Seymour and co-workers (2003a), an amazing heat production, with individual plants reaching 20°C above ambient. On average the plants held a temperature of 12.4°C higher than ambient (Figure 25A). The heat was solely produced along the appendix. Assuming that thermogeny in the arum assists the carrion mimicry; we would expect to see similar heat development in carrion. Thus, we measured the body temperature from a number of gull cadavers over the decay period. The dead gulls likewise had a rapid temperature development and reached temperature levels in parity with that of the arum (Figure 25B).
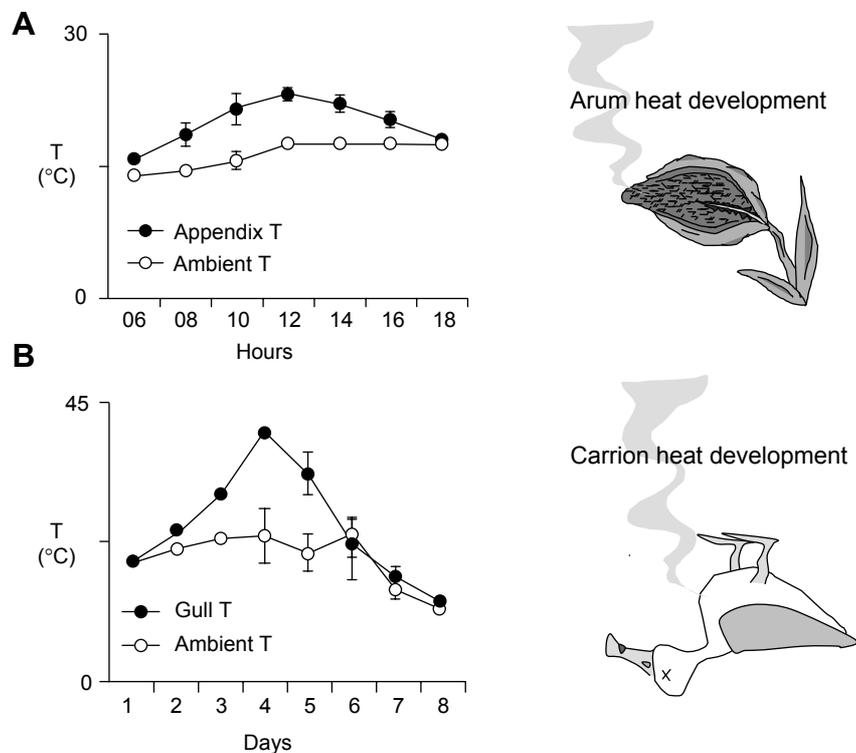

Figure 25. (**A**) Average temperature levels reached along the appendix (the sole site displaying thermogeny) during the first day of flowering in the arum. Plants on their second day of flowering do not produce heat. (**B**) The internal temperature development during the decay process of a dead gull.

We had observed that most of the visiting flies that proceeded to enter the trap chamber appeared to do so from the appendix and in fewer cases directly from the spathe. To verify this observation, we scored the behavior of attracted flies during

the first day of flowering, and found that the majority (~70%) of the flies that entered did so from the appendix. We also noted that by finding the appendix the flies were more than three times as likely to enter the trap chamber compared to those that did not (Figure 26A). Thus, that the attracted flies find the appendix at the outset should be of major importance, as the pollination system of the arum requires that the attracted flies are also fooled to enter the trap chamber. Could thermogeny have an important role in this process?

To address this question, we manipulated the heat production of the arum. We designed an experiment that would show if the presence of thermogeny increases the attractiveness of the appendix. As in Paper III, we exploited the conditions set by the two-day flowering period, which allowed us to manipulate the heat production on the second day of flowering. We monitored fly behavior on the first day and recorded the ratio of the attracted flies that also visited the appendix. On the second day we added odor to one group of plants and in another group we added odor plus heat. The heat was supplied through a thin resistance wire that we coiled around the appendix. The foul odor (the oligosulphide mix) was applied in the trap chamber, as in Paper III.
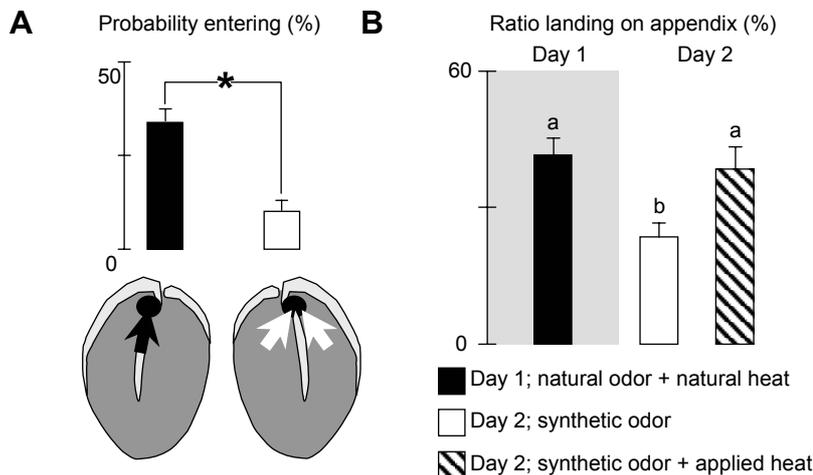

Figure 26. (**A**) The probability that the flies will enter the trap chamber if they find the appendix (black) vs. if they go directly from the spathe (white). Flies are three times as likely to enter the trap if they find the appendix. (**B**) Ratio of attracted flies that visit the appendix during the first and second day, with heat and odor manipulated on the second day. Both heat and odor is required to restore the behavior on the second day to the same level as on the first day.

We did not expect heat alone to enhance the overall long range attractiveness of the plants, as the oligosulphides single-handedly fully restores the long range attractiveness (Paper III). We however hypothesized a difference in behavior, dependent on the heat, once the flies had landed. During the first day of flowering we found that ~42% of the flies that were initially attracted to the plant also found the appendix (Figure 26B). During the second day, in the group where we only added odor, the proportion of the flies that visited the appendix dropped significantly to ~24%. In the group where we added both odor and heat, ~40% of

the attracted flies also found the appendix, i.e. the same proportion as on the first day. Thus, adding both heat and odor, fully restore the behavior of the flies and render the appendix as attractive on the second day as on the first day.

This study provides the first evidence for a direct functional role of plant thermogeny as modifier of pollinator behavior. The generated heat works together with the odor cues and fine tunes the overall deceit. Heat has previously been shown to be an important oviposition cue for blowflies (Cragg 1956; Erzinclioglu 1996). The arum pollination system relies on a multisensory mimicry of the cadaver model, where heat and odors are parts in a more complex mimicry including also color, shape and texture. These stimuli together create for the flies an irresistible super-stimulus, likely representing a gigantic mammalian rectal opening.

Conclusions and future directions

What I have found…

In this thesis, I show that insect odorant receptors, as seen through the response profile of the olfactory receptor neurons, are narrowly tuned to specific compounds. The ligands of the system are volatile chemicals characteristic for favored (or rejected) traits of preferred (or avoided) resources. These compounds each carry important information. By relying on these key-ligands even a narrowly tuned system comprising a fairly small number of ORs can be used to locate and evaluate a large number of resources in complex odor environments.

If I had four more years…

…I would probably devote my time to address the following questions.

The ligands identified in Paper I should play an important role in future research regarding the fruitfly olfactory system. However, as we only covered less than a third of the functional type of ORNs that should be present (assuming that each OR represents one functional neuron type) more work is needed in order to identify natural ligands for the remaining neuron classes. In addition, screening of volatile collections from repellent objects should also be done; as such objects were not included in Paper I. Potent ligands mediating avoidance would be most useful as many behavioral paradigms in the fruitfly system rely on scoring repellency.

The paper dealing with the evolution of olfactory coding opens up for many interesting research angles. The replacement of one sensillum type with another as we observed in *D. sechellia* likely involves some substantial reconfigurations of the olfactory nervous system. Future work should attempt to address the question of how this sensillum swap has been hardwired, e.g. whether the swap has altered the architecture of the antennal lobe. Most interesting would also be to see how this shift has been genetically accomplished.

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Whether or not the tuning shift of the ab3A neurons (from methyl hexanoate to ethyl butyrate) in *D. mauritania* represents a host-specific adaptation should be further investigated. The *D. sechellia* – morinda system has been widely studied and have given valuable insights into the processes involved in host specialization, a similar system, involving a species from the same clade, which operates in a similar habitat would be a highly valuable asset for comparative analysis. The shift from ethyl hexanoate to methyl hexanoate in *D. simulans* and *D. sechellia* in both cases likely involves minor changes in orthologous ORs. The presence of a minor, yet distinct difference in ligand affinity, caused by what most likely are highly similar ORs, opens up an excellent opportunity to identify the critical amino acids involved in the actual ligand binding. Interesting would also be to investigate how olfactory coding has evolved in other groups of fruitflies. A suitable target group would be the cactophilic fruitflies of the Sonoran desert, which have a most interesting evolutionary history and also display intriguing host specializations.

Several aspects of the arum's pollination system remain to be explored. Specifically, the importance of color and tactile cues should be investigated as well as several purely plant ecology related questions such as e.g. efficiency and success rate of the system. Furthermore, it would be interesting to investigate a plant relying on mimicry of a non-carrion resource, to see if such a system follows the same principles as the arum. In addition, a comparison between the highly evolved arum with a more primitive relative would be very interesting as it would produce valuable insights into how intricate pollination systems have evolved.

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