Drosophila Sensory Neuroethology

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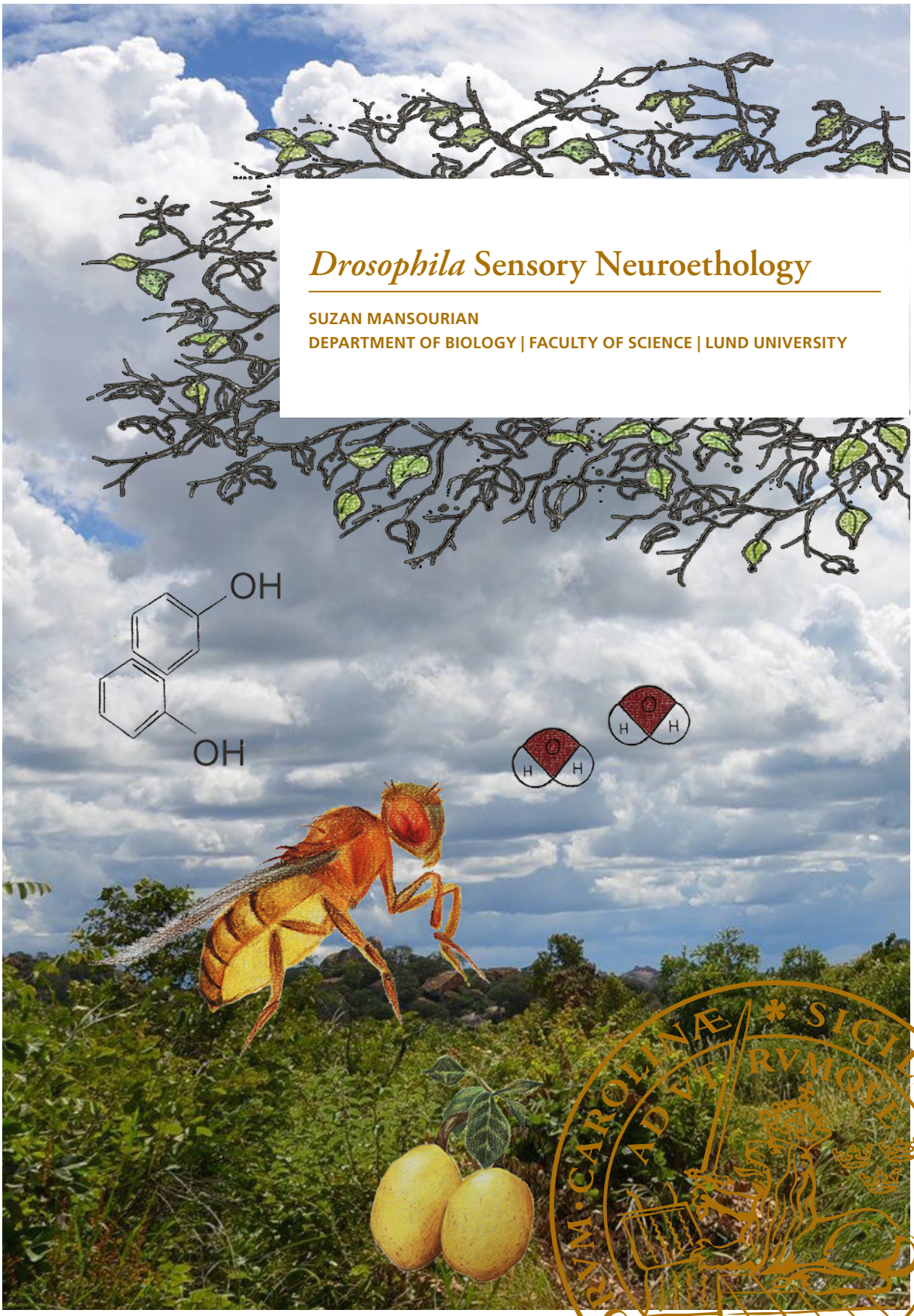

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Drosophila Sensory Neuroethology

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Abstract

Animals, like humans, need to perceive their surroundings via their senses in order to make sensible behavioral decisions, reproduce successfully, and survive. Animals are equipped with audition, vision, thermosensation, hygrosensation, mechanosensation, magnetoception, gustation, and olfaction, which detects physical and chemical changes in their habitats. Among these senses, olfaction is likely the most ancient sensory modality. Insects, the most abundant and successful group of the animal kingdom, predominantly use olfaction to find food, mates, breeding sites, and to avoid dangers. Moreover, hygrosensation is vital for insects to find a suitable habitat and to avoid risks of dehydration. Our understanding of the molecular, neuronal, and morphological organization of the insect olfactory system is today substantial, in large parts thanks to *Drosophila melanogaster* (vinegar fly) and the wealth of sophisticated genetic tools available in this classic model system. Our knowledge regarding the functional and molecular basis of insect hygrosensation, is, however, limited.

In this thesis, I show that the vinegar fly olfactory system do not detect odor molecules randomly, but capture and process specific odors associated with needs and dangers. I demonstrate how the olfactory system cope with toxic and harmful matters in the natural habitat and I identify an olfactory circuit that mediates repellency towards phenol, which is produced by pathogenic bacteria, predominantly present in carnivore feces. Furthermore, I show that flies have an innate and species-specific ability to find suitable humidity levels, related to their native habitat. Vinegar flies can sense humidity changes in their environment through a trio of ionotropic receptors expressed in the sacculus of the antennae. Although *D. melanogaster* is known as a generalist, I show that wild populations of *D. melanogaster* from a mopane forest within the potential ancestral habitat have a strong breeding preference towards marula fruit. This fruit is seasonally abundant, native to Southern Africa, and is presumably the ancestral host of the vinegar fly. I also argue that marula drove the *D. melanogaster* to become a human commensal.

In summary, the research presented in my thesis enhances our understanding of how the olfactory system operates, the behavior of wild flies, and introduces the genetic and neural basis underlying humidity sensation in insects. These findings might lead us to better strategies for controlling insect pests, as well as human disease vectors.

Key words: *Drosophila melanogaster,* vinegar fly, olfactory system, hygrosensation, receptor, ancestral habitat.

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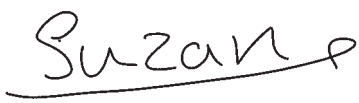
Signature  Date: 2018-04-19

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To my kindest mami and baba.
This journey began with your endless support.

“I encourage all of us, whatever our beliefs, to question the basic narratives of our world, to connect past developments with present concerns, and not to be afraid of controversial issues.”

Yuval Noah Harari

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Author contributions

I. **S.M** wrote the manuscript together with M.C.S.

II. Conceptualization, **S.M**. and M.C.S.; Methodology, **S.M**., M.D., and M.C.S.; Investigation, **S.M**., A.E., J.C., and M.C.S.; Resources; C.L. and M.C.S.; Writing – Original Draft, M.C.S.; Writing – Review and Editing, all authors; Funding Acquisition, M.C.S., M.D., and C.L.; Supervision, C.L., M.D., and M.C.S.

III. A.E., G.S.B.S., M.G., and M.C.S. conceived and designed the study. A.E. performed all humidity experiments and, with assistance from **S.M**., anatomical studies by confocal microscopy. A.E. performed two-photon Ca2+ imaging of humidity responses. E.E.Z. carried out all temperature-preference and two-photon anatomy experiments. D.D.F. performed two-photon Ca2+ imaging for temperature responses. A.E., M.G., and M.C.S. analyzed the data and wrote the manuscript with input from all authors.

IV. **S.M.** and M.C.S. conceived and designed the study. **S.M.**, E.J.,and M.C.S. performed the field work. **S.M.** performed all behavioral experiments and analyzed the data. **S.M.** and A.E. performed the Ca^{2+} imaging. V.R. and J.P. conducted the genetic analysis. M.C.S. and **S.M.** wrote the manuscript, with input from all authors.

Popular science summary

You have most likely seen the tiny pesky flies sitting on your fruit basket or flying around in your kitchen, especially during late summer. Have you ever wondered from where these flies come from and how they find their way to your fruits? These insects, known as vinegar flies or fruit flies, use all kinds of overripe and fermenting fruit – rich in yeast and sugar – for feeding and breeding. Vinegar flies, similar to other insects, rely on their strong sense of smell (olfaction) to find their way to fruits, to find suitable partners for mating and good breeding spots. Vinegar flies sense odor molecules in the environment by their organs of smell, the antenna and maxillary palps, which have a similar function to the human nose. The fly "nose", like the human nose, is equipped with dedicated receptors that capture odor molecules. These receptors do not detect odor molecule randomly, but capture odors associated with needs and dangers. For example, the fly has several smell receptors dedicated to odors coming from alcoholic fermentation and activation of these receptors guides the fly to suitable food sources rich in sugar and yeast (**Paper I**). Flies are also able to avoid toxic molecules and harmful matters by their sensitive "nose". Vinegar flies avoid carnivore feces as a breeding site because of the smell of phenol, which is produced by pathogenic bacteria presence in carnivore feces. Phenol accordingly alarms vinegar flies about the looming danger of pathogenic bacteria (**Paper II**).

The fly "nose" has, however, more tricks up its sleeve. Flies, like other insects, are in high risk of dehydration due to their small body size and small capacity to store water, therefore, flies have an innate ability to find suitable humidity levels. Flies can sense humidity changes in their environment trough humidity receptors, located in a invagination of the antenna. Humidity information is then processed together with thermal information in a dedicated region in the fly brain (**Paper III**).

The vinegar fly is today a cosmopolitan species, which means you can find them on all continents. However, the fly originated within southern central Africa (including Zambia and Zimbabwe). Although flies are known as generalists, wild populations of flies from Zimbabwe have a strong breeding preference towards marula fruit. This fruit is seasonally abundant, native to Southern Africa, and is presumably the ancestral host of the fruit fly (**Paper IV**).

This pesky fly has been a superhero in biological science for decades and has contributed to an astonishing amount of knowledge in genetics, neuroscience, embryology and modern biomedical research. The findings of my thesis further enhances the basic science behind olfaction and its mechanisms, the wild behavior of flies, and introduces the genetic and neural basis underlying humidity sensation in insects for the first time. These basic understandings may lead us to better strategies for controlling insect pests, as well as human disease vectors such as mosquitoes.

Populärvetenskaplig sammanfattning

Du har säkert sett de små irriterande flugorna sitta i din fruktskål och flyga runt i köket, särskilt på sensommaren. Har du någonsin undrat varifrån dessa flugor kommer och hur de hittar till din frukt? Dessa insekter, vanligen kallade bananflugor eller vinägerflugor, använder all slags övermogen eller ruttnande frukt – rik på jäst och socker – till föda och för att föda upp larver.

Bananflugor är, liksom andra insekter, beroende av sitt känsliga luktsinne för att hitta föda, lämpliga parningspartners och bra ställen att lägga ägg på så larverna får tillgång till mat. Bananflugor känner doftmolekyler i omgivningen med hjälp av sina luktorgan, nämligen antennerna och maxillarpalperna, som har samma funktion som våra näsor.

Flugans luktorgan är liksom människans näsa utrustad med dedikerade receptorer som fångar upp doftmolekylerna. Dessa receptorer detekterar inte doftmolekylerna slumpmässigt, utan fångar upp dofter associerade med behov och med faror. Exempelvis har flugan flera luktreceptorer dedikerade till dofter från alkoholjäsning och aktivering av dessa receptorer leder flugan till lämpliga födokällor rika på socker och jäst (**Artikel I**).

Bananflugorna kan också undvika giftiga molekyler och skadliga ämnen genom sina känsliga luktreceptorer. De undviker köttätande rovdjurs exkrement som äggläggningsställen eftersom det luktar fenol, som produceras av patogena bakterier i exkrementerna. Tack vare fenolen varnas flugorna för den hotande faran med patogena bakterier (**Artikel II**).

Bananflugan har fler ess i rockärmen. Liksom andra insekter löper den stor risk för uttorkning beroende på liten kroppsstorlek och låg kapacitet att lagra vatten. Därför har flugorna en medfödd förmåga att hitta lämplig nivå på den relativa luftfuktigheten. Detta är ytterligare en orsak till att kök är en favoritplats för flugorna. De föredrar en relativ luftfuktighet på omkring 70%, vilket är precis vad kök ofta håller. Bananflugorna kan registrera förändringar i den relativa luftfuktigheten med hjälp av fuktreceptorer, vilka är belägna i en räfflad del av antennen. Den registrerade informationen bearbetas sedan tillsammans med temperaturinformation i en dedikerad del av hjärnan (**Artikel III**).

Bananflugan är idag en kosmopolitisk art, vilket betyder att den återfinns på alla kontinenter. Ursprungligen kommer den emellertid från de södra delarna av

centrala Afrika (exempelvis Zambia och Zimbabwe). Även om bananflugan är känd som en generalist globalt har vilda populationer av arten i Zimbabwe en stark förkärlek för marulafrukter när det gäller att föda upp sina larver. Marula är ett vanligt inhemskt träd i hela södra Afrika och dess frukter finns i överflöd när det är säsong. Förmodligen är marula den ursprungliga värdväxten för bananflugan (**Artikel IV**).

Den lilla irriterande bananflugan har visat sig vara en superhjälte inom biologi under flera decennier och har bidragit till en förbluffande mängd ny kunskap inom genetik, neurovetenskaplig embryologi och modern biomedicinsk forskning.

Resultaten i min avhandling ökar också de grundläggande kunskaperna bakom luktsinnet och dess mekanismer, beteendet hos vilda populationer av bananflugan och påvisar för första gången den genetiska och neurala bakomliggande basen för fuktsinnet hos insekter. Dessa grundläggande kunskaper kan komma att leda till bättre bekämpningsstrategier mot skadeinsekter och mot smittbärande insekter som t.ex. myggor.

Persian popular summary

چکیده:

حشرات کوچک مزاحمی که بر روی سبد میوه‌های شما می‌نشینند یا درون آشپزخانه‌تان پرسه می‌زنند، بله! بی‌شک با آن‌ها به ویژه هنگام پایان تابستان مواجه شده‌اید. آیا تا کنون اندیشیده‌اید که این حشرات از کجا آمده‌اند و چگونه راه خود را برای دسترسی به میوه‌های شما پیدا کرده‌اند؟

این حشرات به مگس سرکه مشهورند و از انواع میوه‌های رسیده یا در حال فساد که سرشار از قند و مخمر هستند، تغذیه می‌کنند. مگس‌های سرکه همانند سایر حشرات جهت رسیدن به میوه‌ها و یافتن جفت برای تولید مثل و همچنین پیدا کردن محل مناسب تخم‌ریزی، به سیستم بویایی قدرتمند خود متّکی هستند.

مگس‌های سرکه ملکول‌های بوی موجود در محیط را از طریق اندام‌های بویایی خود که همان شاخک‌ها و پالپ‌ها هستند، تشخیص می‌دهند. به بیانی دیگر می‌توان این اندام‌ها را با بینی انسان مقایسه کرد.

"بینی" حشره مانند بینی انسان به گیرنده‌های اختصاصی مجهز شده که به یاری آن‌ها ملکول‌های بوی موجود در محیط را دریافت می‌کند. امّا نکته‌ی بارز درخصوص این گیرنده‌ها این است که آن‌ها این بوها را به صورت تصادفی جذب نمی‌کنند بلکه بوهای مرتبط با نیاز و بقاء، توسط حشره دریافت می‌شوند. برای نمونه بینی حشره به چندین گیرنده‌ی بویایی ویژه مجهز است که برای تشخیص رایحه‌ی پراکنده شده از فرایند تخمیر الکلی تخصص یافته‌اند و با فعال شدن این گیرنده‌ها حشره راه خود را به سوی منابع غذایی مناسب و سرشار از قند و مخمر پیدا می‌کند. (نگاشته‌ی ۱)

حشره‌ها همچنین قادر به دوری از ملکول‌های سمّی و مواد مضر به وسیله گیرنده‌های بویایی خود هستند.

مگس‌های سرکه از مدفوع جانوران گوشتخوار جهت تخم‌ریزی دوری می‌کنند، دلیل این امر وجود فنول بوده که حاصل فعالیت باکتری‌های پاتوژن موجود در مدفوع این جانوران است. فنول برای مگس

سرکه به معنی هشداری عمل می‌کند که نمایانگر وجود باکتری پاتوژن، درون مدفوع است. (نگاشته‌ی ۲)

"بینی" حشره همچنان شگفتی‌های دیگری دارد. مگس‌ها مانند سایر حشرات به دلیل اندازه‌ی کوچک و توان کم در ذخیره‌ی آب، همواره در معرض خطر از دست دادن آب بدن و مرگ قرار دارند. از این‌رو حشرات توانایی ذاتی در یافتن سطوح رطوبتی مناسب را دارند. مگس‌ها قادرند توسط گیرنده‌های رطوبتی که درون حفره‌ای در شاخک‌ها قرار گرفته، تغییرات رطوبت محیط را تشخیص دهند. سپس اطلاعات مربوط به رطوبت همراه با اطلاعات مرتبط با دمای محیط در بخشی از مغز مگس سرکه که برای این امر تخصص یافته، تحلیل می‌شوند. (نگاشته‌ی ۳)

امروزه مگس سرکه گونه‌ای همه جایی است. بدین معنی که در تمام قاره‌ها یافت می‌شود، هر چند که موطن اصلی آن‌ها جنوب آفریقای مرکزی (نواحی زامبیا و زیمباوه) است.

گرچه این مگس‌ها گیاهخوار عمومی هستند ولی جمعیت‌های وحشی آن‌ها در زیمباوه گرایش شدیدی برای تخمریزی روی میوه مارولا دارند. این میوه در فصل بهار به فراوانی یافت می‌شود و بومی جنوب آفریقا است. تفکر و تحلیل در این باب می‌تواند مبتنی بر این ادعای مهم باشد که سرآغاز مسیر تکاملی مگس سرکه جنوب آفریقا، و مارولا اولین میزبان این حشره بوده است. (نگاشته‌ی ۴)

این حشره‌ی مزاحم در طی دهه‌ها در بستر علوم زیستی همواره یک ابر قهرمان بوده به گونه‌ای که هماره جزیی از دستاوردهای شگرف در علم ژنتیک، عصب‌شناسی، رویان‌شناسی و تحقیقات نوین پزشکی بوده است.

یافته‌های پایان نامه‌ی من موجب ارتقاء دانش پیشین در مورد سیستم بویایی و نحوه عملکرد آن و همچنین برای نخستین بار منتج به معرفی ساز و کار ژنتیکی و عصبی گیرنده‌های حس رطوبت در حشرات شده است.

امید است این یافته‌های بنیادین ما را به سمت تدابیر بهتر جهت کنترل حشرات آفت و همچنین مدیریت ناقلین بیماری‌های انسانی مانند پشه‌ها رهنمون سازد.

The Scope of this Thesis

Animals, like humans, need to perceive their surroundings via their senses in order to make sensible behavioral decisions, reproduce successfully, and survive. Most animals do not only have more sensitive senses in comparison with humans but have also evolved more sensory systems enabling them to cope with special conditions in their natural habitat. Animals are equipped with audition, vision, thermosensation, humidity sensation (hygrosensation), mechanosensation, magnetoception, gustation, and olfaction which detects physical and chemical changes in their habitats. Among these senses, olfaction is likely the most ancient sensory modality. Insects, the most abundant and successful group of the animal kingdom, predominantly use olfaction to find food, mates, breeding sites, and to avoid dangers. Moreover, hygrosensation is vital for insects to find a suitable habitat and to avoid risks of dehydration.

Our understanding of the molecular, neuronal, and morphological organization of the insect olfactory system is today substantial, in large parts thanks to *Drosophila melanogaster* and the wealth of sophisticated genetic tools available in this classic model system. Our knowledge regarding the functional and molecular basis of insect hygrosensation, is, however, limited.

The aim of this thesis is to investigate how the olfactory system of Drosophila translates sensory inputs into behavioral responses (Paper I and II). Moreover, I have also studied the organization of the hygrosensory system and how Drosophila senses changes in humidity levels (Paper III).

Every biologist wonders about the origin of the creatures of earth and origin of life. This central question stimulated another aim in this thesis, the search of wild *Drosophila melanogaster* and its ancestral habitat (Paper IV).

This thesis begins with an overview of the fly and its astonishing contribution to the field of genetics and biology. Then the organization and function of the olfactory and hygrosensory systems are described. The thesis continues with the results and discussions from the four papers dealing with aforementioned aims. Finally, a conclusion and contributions to the field along with some ideas for future studies are described.

Background of the study

Drosophila melanogaster – Lord of the flies

Drosophila melanogaster or the vinegar fly (**Figure 1A**) has been one of the primary laboratory model organisms for over a century. The use of the fly as a genetic model organism goes back to the beginning of the 1900s with the work of Thomas Hunt Morgan (**Figure 1B**) at Columbia University, where he and his students were searching for a suitable animal model to study the laws of heredity. Morgan settled on the fly, and soon isolated a white-eyed mutant (wild-type flies have red eyes), and with the help of this sex-linked mutant he could show that the chromosomes carries the hereditary material (Morgan, 1910). Morgan was awarded the Nobel Prize in physiology or medicine for this discovery in 1933. Apart from this important discovery, Morgan together with Calvin Bridges, Alfred H Sturtevant, and Herman J Muller established most of the major principle of classic genetics such as the nature of genetic linkage and genetic maps, the genetic behavior of chromosome aberrations, the induction of gene and chromosome mutations by radiation, and the discovery of mitotic recombination (Bridges, 1921; Sturtevant, 1965). Calvin Bridges experimental evidence of genes and chromosomes linkage and the term "nondisjunction" was published as the first paper of the first issue of the journal Genetics (Bridges, 1916). Herman J Muller won the Nobel Prize in 1946 for using X-ray radiation to induce mutations in the fly (Muller, 1927). Since then, research using *D. melanogaster* has yielded astounding insights into processes of cell and molecular biology, neurobiology, embryonic development, learning and memory, behavior, and physiology. Fly research has received six Nobel Prizes to date, with the most recent one in 2017 for the discoveries concerning the molecular mechanism underlying circadian rhythms (Bargiello and Young, 1984; Bargiello et al., 1984; Zehring et al., 1984; Siwicki et al., 1988; Hardin et al., 1990).

Figure 1. *Drosophila melanogaster*, Lord of the flies.
A) A female *Drosophila melanogaster* perched on one of its favorite fruits, orange. Photo: Marcus Stensmyr B) Thomas Hunt Morgan (Columbia University, New York, USA), who introduced the fly as a genetic model organism. Photo: Wikimedia.

Today, the whole genome of *D. melanogaster* together with numerous tools to manipulate the genome is available, which allow for e.g. functional dissections of specific genes, as well as neuronal circuits (Adams et al., 2000). Therefore, mutant flies can be engineered through selective removal or replacement of gene sequences (Rong et al., 2002), the expression level of any gene can be reduced using RNA interference (Dietzl et al., 2007; Ni et al., 2009), any gene can be expressed in almost any tissue or cell using the yeast Gal4-UAS system (Brand and Perrimon, 1993; Pfeiffer et al., 2008). It is also possible to visualize the expression of genes of interest and monitor neural activity by introducing reporter genes, such as calcium indicators and green fluorescent proteins via the UAS system (Nakai et al., 2001; Marella et al., 2006). Moreover, with the UAS system one can also introduce genes that can modify neuronal activity, such as the tetanus-toxin light chain protein (TNT), which blocks synaptic transmission (Sweeney et al., 1995), or shibire that leads to depletion of vesicles in the synaptic termini (Kitamoto, 2001), or even light sensitive proteins, such as CsChrimson, which is a red-shifted channelrhodopsin enabling light activation of neurons (Klapoetke et al., 2014). With the aid of these tools, scientists have even succeeded in studying genes that are involved in neurodegenerative human diseases and their underlying mechanism such as Alzheimer and Parkinson (Lu and Vogel, 2009) in the fly.

D. melanogaster is an opportunistic human commensal species with a cosmopolitan distribution. This cosmopolitan species has originated within sub-Saharan Africa as evident from biogeography and genetic variation studies (Lachaise et al., 1988; Pool and Aquadro, 2006). A recent genomic study revealed that southern central African populations (e.g. Zambia and Zimbabwe) have the highest genetic diversity, suggesting that the ancestral range of *D. melanogaster* may be located within this area (**Figure 2A**) (Pool et al., 2006 and 2012). Most of Zambia and Zimbabwe are covered by seasonally dry miombo and mopane

woodlands, which have subtropical climate with a hot dry season from August through October and a hot wet season from November to March. Interestingly, African *D. melanogaster* in comparison with its close relatives has higher tolerance to dehydration and severe high temperatures (Stanley et al., 1980) that would fit its ancestral subtropical climate conditions.

D. melanogaster is considered a generalist, and can utilize a wide range of decaying vegetables, fruits and other plant matter as feeding and breeding substrate. Knowledge about the breeding substrates in the wild natural habitat of the fly is scant, at best, since essentially all reported populations even within Africa have been caught from sites within or near human settlements (Lachaise and Silvain, 2004). *D. melanogaster*, however, could maintain permanent populations in wild habitats after human activity has diminished and produce wild non-commensal generations. Lachaise (1988) reported 25 host plant species used as larval breeding resource by *D. melanogaster* in the Afrotropical region, e.g. *Lobelia* inflorescences in montane forests or *Pistia stratiotes* (water salad) from Kivu in Congo (Lachaise et al., 1988). 16 out of 25 identified host plants were native, which is high ratio for a domestic species like *D. melanogaster*.

Although vinegar flies are known as a generalist, they show strong egg-laying preference towards *Citrus* spp. – especially orange – over other type of fruits (Dweck et al, 2013). *Citrus* spp. originated in South-east Asia, hence, this fruit cannot be the hosts with which the fly has evolved. The ancestral host fruit of *D. melanogaster*, along with the history of this species evolution to a human commensal species is accordingly unknown.

The lord's close relatives

D. melanogaster belongs to the melanogaster species group of the *Sophophora* subgenus (or genus pending whom you ask). The *melanogaster* group is geographically widespread and contains over 170 species (Schawaroch, 2002). The *melanogaster* species subgroup compromises nine closely related species, occupying different ecological habitats and has probably evolved 13-15 million years ago (**Figure 2B**). Species within this subgroup are ranging from single host specialists to generalists (Lachaise et al., 2000). ***Drosophila sechellia*** is the most highly specialized member of this subgroup, and preferentially lay eggs on noni fruit (*Morinda citrifolia*), a fruit native to the Seychelles islands (**Figure 2C**). *Morinda* fruit, which ripens throughout the year, is toxic, or at least not palatable, to other members of the *melanogaster* subgroup because of its high continent of octanoic and hexanoic acid (Lachaise et al., 1988; Dekker et al., 2006).

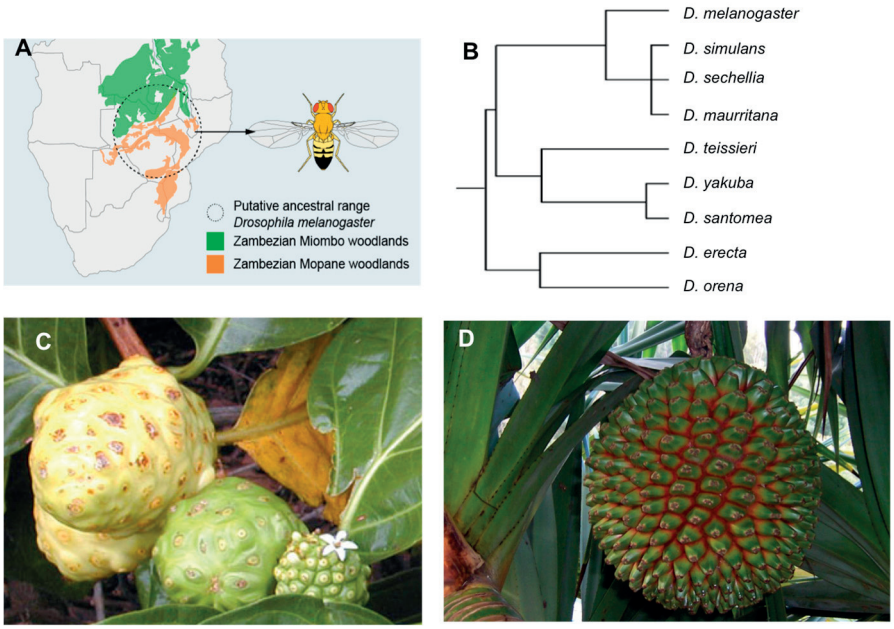

Figure 2. *Drosophila melanogaster* and its close relatives.
A) Putative ancestral range of *D. melanogaster* showed in the dashed circle. B) Phylogeny of the nine related species in the *melanogaster* species subgroup. C) Noni fruit (*Morinda citrifolia)*, host of *D. sechellia* Photo: E. Guinther. D) *Pandanus* cone, seasonal host of *D. erecta*. Photo: B. Navez.

Drosophila erecta and ***Drosophila orena*** are the two known seasonal specialist of this subgroup. *D. erecta*, which is endemic to gallery forests of West-central Africa, is a seasonal specialist on ripe fruits of *Pandanus* spp. (**Figure 2D**). *Pandanus* cones ripen once a year for two months, during which time *D. erecta* is exclusively found on this fruit. While *D. orena* for a long time was only known from a single location on a sole mountain in Cameroon (Tsacas and David, 1978), a recent discovery of another population on the West African island of Bioko revealed that the species has a close, but seasonal association with waterberrys *Syzygium staudtii* (Comeault et al., 2017).

On the other hand**, *Drosophila simulans*** and ***D. melanogaster*** are the only two cosmopolitan species of this species subgroup. As opportunistic human commensals, these two species have effectively spread out in the world by human transportation (David and Capy, 1988). *D. simulans,* however*,* is less associated with human activities and does not readily enter buildings, therefore, it is not found in all places in the world (Rouault and David, 1982). *D. melanogaster* is more tolerant to broader temperature variation and is accordingly more abundant in temperate regions than *D. simulans*. These two species are so similar morphologically that *D. simulans* was initially described as *D. melanogaster* in the beginning of the 20th century (Capy and Gibert, 2004).

Olfaction – Lord of the senses

Animals in their natural habitat are surrounded by volatile chemicals, some of these chemicals will convey important information to the perceiving animal regarding e.g. presence of food, sexual partners, and potential dangers. Most if not all animals are also equipped with peripheral detection systems for volatile chemicals, and dedicated brain centers, which translate the detected volatiles, or odors, into appropriate behaviors. The whole procedure of these transformations is called olfaction. Olfaction is probably the most ancient sensory modality in the animal kingdom (Strausfeld and Hildebrand, 1999). Over 2000 years ago, Aristotle noticed that animals rely more on their sense of smell for their survival than humans do, however, the principle and function of the olfactory system in general, is quite similar in all organisms ranging from a little fly to a large mammal.

The peripheral olfactory system of the fly

Flies are able to detect an array of volatile chemicals via two pairs of olfactory appendages on the head, the antennae and maxillary palps (**Figure 3A**). These organs are covered by sensory hairs, porous cuticular structures called sensilla (**Figure 3B**). Each sensillum houses dendrites of olfactory sensory neurons (OSNs), which express a distinct olfactory chemoreceptor (**Figure 3C**). These chemoreceptors come in three flavors, odorant receptors (OR), ionotropic receptors (IR) and gustatory receptors (GR), with each capturing specific environmental volatiles.

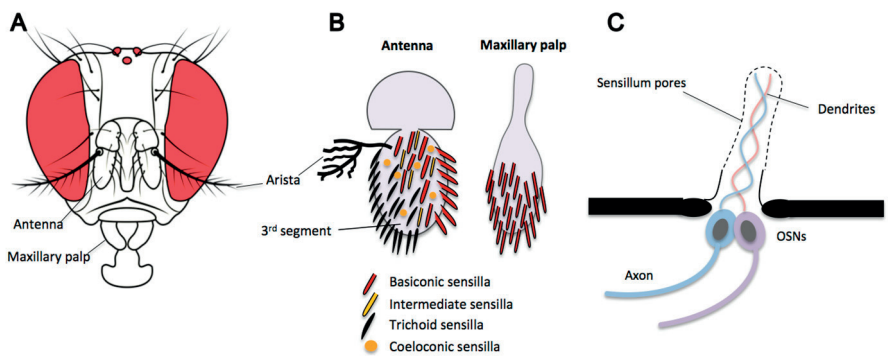

Figure 3. The peripheral olfactory system of the adult fly.
A) Head of the fly. B) Different types of sensilla (sensory hairs) are covering the two olfactory organs. C) Each sensilla house dendrites of 1-4 OSNs and axon of these OSNs projects to the antenna lobe.

The antennal olfactory sensilla of the fly are divided into four different morphological and functional types: basiconic, trichoid, intermediate, and coeloconic sensilla (**Figure 3B**). Each of these sensilla houses one to four OSNs. The maxillary palp has one type of basiconic sensilla (**Figure 3B)**, each housing two OSNs (Vosshall and Stocker, 2007). Antennal sensilla of different morphological types have special functions to some degree; OSNs housed in basiconic sensilla are tuned to fermentation, microbial, and plant volatiles, whereas coeloconic OSNs primarily detect amines and acids (Benton et al., 2009; Yao et al., 2005). Trichoid OSNs are mainly responding to pheromones, such as (Z)-11-cis-vaccenylacetate (cVA), which mediates courtship behaviors via activation of specific trichoid neurons (Clyne et al., 1997; Ha and Smith, 2006; Kurtovic et al., 2007). The function of intermediate sensilla remains somewhat unclear, but at least one type of OSN housed in this type of sensilla is involved in oviposition site selection (Dweck et al., 2013). Most of the maxillary palps' OSNs detect only one single chemical and mediate short and long range attraction to specific chemicals such as 4-ethylguaiacol, furaneol and methylether (Dweck et al., 2016). In total there are 1100-1250 OSNs on the antennae (Stocker, 2001) and 120 on the maxillary palps, which in total are expressing 62 ORs, 16 IRs and 12-14 GRs (Fishilevitch et al., 2005; Couto et al., 2005; Hallem et al., 2004; Benton et al., 2009; Menuz et al, 2014).

The larval chemosensory system

The structure of the adult and larval olfactory pathways is quite similar even though the number of OSNs is less in larvae in comparison with adults (21 OSNs in larvae (Cobb, 1999). The main larval chemosensory organs are located in the cephalic lobe, and include the dorsal organ, ventral organ and terminal organ (Singh and Singh, 1984). There are three other organs located on the thoracic and abdominal segments, which are probably involved in contact chemoreception (Scott et al., 2001). The dorsal organ expresses ORs and GRs and acts as both an olfactory and gustatory organ, while the ventral and terminal organs only have a taste function (Singh and Singh, 1984; Singh, 1997). OSNs on the dorsal organ respond to a wide variety of chemicals, such as alcohols, acetates, aldehydes, esters, and ketones (Cobb et al., 1992; Cobb, 1999; Heimbeck et al., 1999; Cobb and Domain, 2000; Boyle and Cobb, 2005). The olfactory response of larvae is age dependent; with stronger responses to chemical stimuli observed by increased age from first instar larvae to third instar, however, late third instar larvae shows very weak olfactory responses (Kaiser and Cobb, 2008). The olfactory information detected at the peripheral level, converge into the larval antennal lobe (LAL) (Tissot et al., 1997).

Odorant receptors

Odorant receptors (ORs) are the most prominent component of the olfactory pathway. Genes encoding olfactory receptors were first discovered in the rat by Buck and Axel in a 1991 milestone study (Buck and Axel, 1991), which led to the 2004 Nobel Prize in medicine or physiology. The olfactory receptor genes in vertebrates (and nematodes) belong to the family of seven transmembrane G-protein-coupled receptors (GPCRs). In 1999, with the identification of OR genes in *D. melanogaster* (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999), brought up an important distinction between the mammalian and insect olfactory systems, namely that the *Drosophila* OR genes showed no sequence similarity to the GPCRs of vertebrates and nematodes, but form a unique family. The insect ORs has moreover, a reversed transmembrane topology to that of GPCRs (Benton, 2006). The number of ORs varies between species, e.g. from 47 in the cotton leafworm (*Spodoptera littoralis*) (Poivet et al., 2013) to around 400 in the Indian jumping ants (*Harpegnathos saltator*) (Zhou et al., 2012), whereas mammalian genomes contain around 250 ORs in platypus to over 2000 in the African elephant (Niimura and Nei, 2007; Niimura et al., 2014).

The odorant receptor co-receptor, Orco, is highly conserved among insects, showing over 90% sequence homology between closely related species (Vosshall et al., 1999). Orco is co-expressed with the conventional receptor in both adults and larvae (Fig. 3b). Orco is involved in dendritic localization and critical for the function of the tuning ORs, but does itself not directly respond to odorants (Larsson et al., 2004). Orthologs of Orco from other animals than insects has not been reported yet.

Individual ORs has been examined using loss-of-function studies. Flies lacking specific ORs show a reduction in both behavioral and electrophysiological responses to distinct odors (Störtkuhl and Kettler, 2001; Dobritsa et al., 2003; Jones et al., 2007; Kreher et al., 2008; Kurtovic et al., 2007; Semmelhack and Wang, 2009). For example, mutant flies lacking a functional *Or67d* receptor revealed that the neurons in which this receptor is expressed regulate mating behavior. Both female and male *Or67d* mutant flies show defective mating behavior, including male-male courting (Kurtovic et al., 2007).

In *Drosophila*, like mammals, each OSN typically expresses a single OR gene apart from Orco, although, there are six classes of OSNs that express more than one OR. In four of these classes, the co-expressed ORs are closely linked and are probably the result of a recent gene duplication. An example of this case is found in the so-called ab3A OSNs, which co-express *Or22a* and *Or22b* (Hallem et al., 2004; Couto et al., 2005), but where only Or22a appears to confer odor sensitivity (Dobritsa and et al., 2003).

Ionotropic receptors

Another family of insect olfactory receptors are the IRs, which belongs to the highly divergent family of Ionotropic Glutamate Receptors (iGluR) (Benton et al., 2009). IRs do not only have a role in olfaction but also act as sensory receptors for humidity, temperature, and taste. *Drosophila* adults express at least 57 IR genes. At least 16 of these are expressed in the antenna (Benton et al., 2009; Croset et al., 2010). IRs expressed in antennal neurons are found in the arista (a feather-like protrusion from the third antennal segment), the sacculus (a three-chambered pit on the third segment), and in neurons housed in coeloconic sensilla. Up to three IRs are usually co-expressed in one sensory neuron together with Ir8a, Ir25a, or Ir76b, which are broadly co-expressed and likely act as co-receptors, required for proper IR function (Benton et al., 2009; Abuin et al., 2011; Hussain et al., 2016). The ligands of antennal IRs differ from ORs. For instance, Ir8a positive neurons in coeloconic sensilla respond mainly to carboxylic acids and aldehydes, while Ir25a or Ir76b expressing neurons predominantly detects amines (Silbering et al., 2011). For example, Ir41a is co-expressed with Ir76b. Activation of these two IRs enables the fly to detect polyamine volatiles from a long range, and induces egg-laying (Hussain et al., 2016).

In the first two chambers of the sacculus, combinations of Ir25a, Ir40a, Ir68a, and Ir93a are expressed, which are all required for humidity sensing in the fly (Enjin et al., 2016; Frank et al., 2017; Knecht et al., 2016 and 2017). In the third chamber of the sacculus, Ir64a is expressed with Ir8a as a co-receptor and mediate acid sensing (Ai et al., 2010). In the arista, Ir21a is expressed with Ir25a where they mediate cool sensing (Ni et al., 2016). Ir21a and Ir25a have a similar function in larvae (Ni et al., 2016). Ir25a and Ir93a are also expressed in the thermosensory cells of the arista (Benton et al., 2009).

IRs are also expressed on the proboscis, pharynx and legs of adults and on the taste organ of the larvae. Ir76b is not only expressed in OSNs but also in gustatory receptor neurons (GRNs). Activation of Ir76b together with Gr66a in the taste neurons, helps the fly to evaluate polyamine presence in decaying fruits (Hussain et al., 2016). IRs expressed in the male foreleg (Ir52c and Ir52d) become activated in contact with the female and mediate courtship in male flies (Koh et al., 2014). In summary, IRs perform a number of different functions, most of which likely unrelated to olfaction.

Gustatory receptors

GRs are expressed in sensory neurons on the mouthparts, maxillary palps, legs, wings, as well as the ovipositor (Stocker 1994; Vosshall and Stocker 2007; Ling et al., 2014; Depetris-Chauvin et al., 2015). A few are also expressed in OSNs on the

antennae (Jones et al., 2007; Kwon et al., 2007). The GR genes in insects have no sequence similarity to mammalian taste receptors, but are closely related to the insect ORs. Around 12-14 GRs are expressed in OSNs on the antenna (Menuz et al, 2014), including Gr21a and Gr63a, which together function as CO_2 detectors (Jones et al., 2007; Kwon et al., 2007). Another GR with high expression on the antenna is Gr10a, which is expressed in neurons in a large basiconic sensillum, but has unknown function (Hallem et al., 2004). The remaining GRs expressed on the antenna are presumably sugar detectors (Dahanukar et al., 2007).

GRs expressed on the labellum are mainly sweet and bitter detectors e.g. Gr5a, which together with a subset of related receptors detect sugar and trigger feeding, whereas, Gr66a and related genes, confer responses to bitter compounds and causes aversive behavior (Dahanukar et al., 2001; Chyb et al., 2003; Weiss et al., 2011). For example, flies detect and avoid caffeine via a combination of the bitter receptors Gr33a, Gr66a, and Gr93a (Moon et al., 2006; Moon et al., 2009; Lee et al., 2009). Male flies avoid courting other males by tasting (Z)-7-tricosene, a male sex pheromone and bitter stimuli via activation of Gr66a on the labellum (Lacaille et al., 2007).

Around 39 GRs are expressed in the larval taste organs, and most of them are supposed to be bitter receptors (Colomb et al., 2007; Kwon et al., 2011). Gr21 and Gr63 are also expressed in larvae and activation of these receptors cause aversive behavior in larvae as well (Jones et al., 2007; Kwon et al., 2007).

Gustatory sensory neurons (GSNs) from proboscis, mouth parts, and legs transmit gustatory information to the subesophageal ganglion (SOG), a dedicated taste center in the brain (Wang et al., 2004).

The central olfactory system of the fly

The OSNs, after detecting and discriminating odor molecules, send their axons to the brain. In the antennal lobes (AL), these axons coalesce and form glomeruli, which are spheroidal structures (**Figure 4**). The AL is the insect counterpart to the mammalian olfactory bulb. Like olfactory bulbs, ALs pre-process and integrate olfactory information (Strausfeld and Hildebrand, 1999; Ache and Young, 2005). Each AL of the fly consists of 49 glomeruli. Each single class of OSNs expressing the same OR genes projects to a distinct glomerulus in the ALs, where axonal branches synapse with dendrites of the corresponding class of projection neurons (PNs) (Vosshall et al., 2000; Goa et al., 2000; Bhalerao et al., 2003). PNs can be uniglomerular (branch in one glomerulus) or multiglomerular (branch in many glomeruli) (Galizia, 2014). The glomeruli of the AL are also innervated by local interneurons (LNs), which regulate the activities of PNs and assist communication

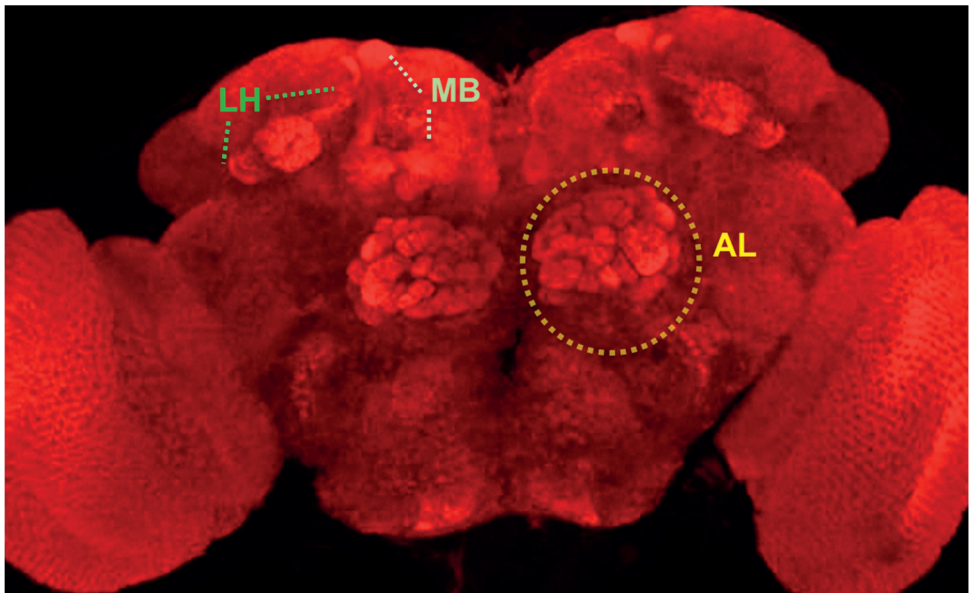

Figure 4. The *Drosophila* brain.
Axons of OSNs projects to the antennal lobe (AL), which is the primary olfactory center in the fly brain (dashed yellow circles). Projection neurons transfer the integrated olfactory information from the ALs to the mushroom body (MB) and terminate in the lateral horn (LH).

within and between glomeruli (Stocker et al., 1990; Wilson and Laurent, 2005; Seki et al., 2010; Wilson, 2013).

The axons from uniglomerular excitatory PNs relay olfactory information to the mushroom body (MB) (**Figure 4**), a center for olfactory learning and memory (Davis, 2005; Heisenberg, 2003), and to the lateral horn (LH), a less-understood higher-order center presumed to direct olfaction-mediated innate behaviors (Heimbeck et al., 2001). MBs are also involved in locomotor activity (Martin et al. 1998), male courtship behavior (Sakai and Kitamoto, 2006), and sleep regulation (Joiner et al., 2006; Pitman et al. 2006).

Antenna - a multifunctional sensory appendage

The insect antenna is a multifunctional sensory appendage that does not only detect volatile chemicals, but it is also involved in a number of other tasks, such as gustation, tactile sensation, thermosensation and hygrosensation (Schneider, 1964 ; Schafer and Sanchez, 1973; Norris and Chu 1974 ; Toh, 1977 ; Schaller, 1978; Altner and Prillinger, 1980 ; Chapman, 1982; Keil and Steinbrecht, 1984 ; Steinbrecht 1984; Lee and Strausfeld 1990). For instance, the nocturnal cockroach determines objects in the dark by the tactile sense on the antenna (Okada and Toh,

2000 and 2006; Okada et al., 2002). Cockroaches can also detect changes in the temperature by cold and hot receptor neurons on the antenna inhibited by heat and excited by cold (Yokohari, 1999; Mizunami et al., 2016). Moreover, some of the antennal sensilla of cockroaches carry moist and dry detecting neurons that respond to the changes in humidity as well as changes in air pressure (Tichy and Kallina, 2010). However, the molecular basis and the neural mechanism underlying hygrosensation is not well understood.

Hygrosensation - The sixth sense

Humidity is a major environmental factor affecting animal health, their reproductive success, and their geographic distribution (Shelford, 1918). Small-bodied insects have a small storage capacity for water and a large surface area for losing it (Kuhsel et al., 2016), they are therefore more sensitive to humidity variation in comparison with mammals and birds (Ludwig, 1945). To avoid rapid dehydration, the insects body is covered by a layer of wax and long-chain cuticular hydrocarbons (CHs). A recent study on *Drosophila* showed that the proportion of certain cuticular hydrocarbons (CHs) enables *Drosophila* to resist dehydration (Ferveur et al., 2018) and a gene named *dsat1*, which regulate CHs production in the fly, is involved in dehydration resistance (Ferveur et al., 2018).

More notably, unlike mammals and worms, insects have dedicated hygrosensory receptor neurons (HRNs). Insects use humidity cues to locate oviposition sites, identify hosts and nectar-bearing flowers, and to find a suitable habitat based on humidity range. For example the female mosquito *Anopheles gambiae* – the vector of malaria – use humidity cues to find stagnant water to lay her eggs (Okal et al, 2013). Moreover, the humid and warm breath of animals along with olfactory and vision cues guides *A. gambiae* and other heamatophagous species to favorable hosts for biting and feeding (van Breugel et al., 2015). The hummingbird moth *Hyles lineata* likely identifies flowers abundant in nectar based on humidity cues (von Arx et al., 2012).

Functional anatomy of hygrosensation in insects

Studies of honeybees, locusts, cockroaches and stick insects revealed that their antennae are equipped with a small number of hygrosensory sensilla (Altner and Loftus, 1985). Unlike olfactory sensilla, the hygrosensory sensilla are poreless, but some carry a plug on the apical surface, called a molten pore, the function of which is unknown. Hygrosensory sensilla are in most insects located in an invagination of the antenna where they are protected from wind, unlike the

olfactory sensilla, which are in a position with highest exposure to the surrounding air (Tichy and Loftus, 1996; Enjin, 2017). The hygrosensory sensilla house the HRNs, reported for the first time from the honeybee *Apis mellifera* (Lacher, 1964). The layout of hygrosensory sensilla is similar between most insect species; one moist neuron together with one dry neuron and one hygrocool neuron are located in a single hygro-sensillum forming a triad (Altner and Loftus, 1985).

Outcome and discussion of the studies

I. The chemical ecology of the fly

Chemical compounds are involved in most interactions between animals and their environment. Chemical ecology is the study of these interactions from a chemical, genetic, neural, behavioral, and ecological perspective. This fascinating field was commenced when the sex pheromone of the silk moth *Bombyx mori* was discovered (Karlson and Butenandt, 1959). Since then, we have gained a fairly detailed understanding about the ecological relevance of insect's chemical interactions with their natural habitat. In **Paper I** of this thesis, I review the chemical ecology of *D. melanogaster* and discuss the ecologically relevant functions of many of the fly's ORs, IRs, and GRs (Mansourian and Stensmyr, 2015). I argue that the vinegar fly's receptors do not sample volatile chemicals randomly but are tuned to subsets of volatiles associated with its ecological needs by bringing up several examples in host seeking, oviposition, and avoiding toxic matters.

Host seeking

Fermented fruit - rich in yeast and sugar – is the favorite food of *D. melanogaster* (Carson, 1971). In the process of fruit fermentation, yeast convert sugars into ethanol and CO_2. This process also generates a large number of other volatiles, which are irresistible to *D. melanogaster*. These volatiles activates several receptors e.g. Ir31a that detects 2-oxopropionic acid and pyruvic acid generated from sucrose (Silbering et al., 2011), Gr21a/Gr63a detect CO_2 released from the decarboxylation of pyruvate into acetaldehyde (Jones et al., 2007), Or92a detects diacetyl formed from acetaldehyde, and Or42b detects acetal generated from the reaction of acetaldehyde with ethanol (Mathew et al., 2013). Activation of these receptors guide flies towards suitable food sources. Yeast is the major source of nutrition for adults and larvae of most *Drosophila* (Begon, 1982), and affects larval growth and survival (Starmer and Aberdeen, 1990). Yeast itself primarily releases two groups of volatiles, acetate esters and phenolics, which activate several olfactory receptors in the fly (Hallem and Carlson, 2004 and 2006; Stökl et al., 2010) (**Figure 5A, B**).

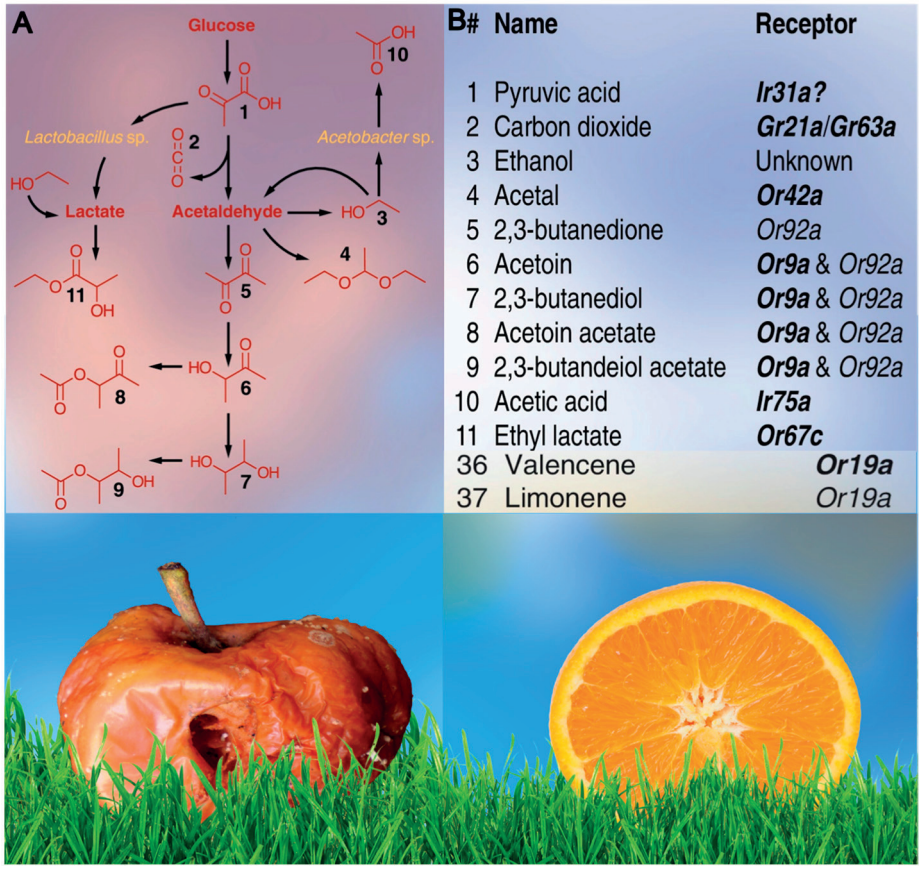

Figure 5. Alcoholic fermentation and orang smells
A) Volatiles emitted through the activity of yeast and fruit fermentation (e.g fermented apple) and B) the fly's receptors that detect fermented volatiles (from A) and volatiles from orange (limonene and Valencene).

Fruit volatiles are also important for flies for host seeking and oviposition site selection. Even though *D. melanogaster* shows a preference towards *Citrus* spp. (Dweck et al., 2013), *D. melanogaster* is an opportunist and is able to utilize a wide range of fruit. Accordingly, their peripheral olfactory detectors are also equipped with receptors that are broadly tuned to common fruit volatiles such as Or43b, Or47a and Or85a, which detect fruity acetate esters, e.g. ethyl acetate, isoamyl acetate, and amyl acetate (Hallem and Carlson 2004, 2006). Or22a is another important broadly tuned receptor detecting fruit esters, primarily ethyl hexanoate (de Bruyne et al., 2001; Stensmyr et al., 2006).

Volatile chemicals effecting oviposition

The fly larvae's mobility is limited, hence the selection of a suitable oviposition sites is a critical decision the female fly has to make. Olfaction guides the female in making the right decision in this matter. Examples of chemical compounds that trigger egg laying in the female are flavedo terpenes, such as valencene and limonene released by *Citrus* spp and other fruit with a thick epicarp, which activates Or19a (Dweck et al., 2013). The thick epicarp protects the fly's larvae from endoparasitoid wasps (Dweck et al., 2013) (**Figure 5B**).

The microbial composition of the substrates is of critical importance in the oviposition site selection process. The smell of acetic acid, produced by acetobacteria during the fermentation process, activates Ir75a and induces egg-laying behavior (Joseph et al., 2009). *Brettanomyces* yeast on the surface of ripe fruit produces volatiles that stimulate oviposition, as well as feeding via activation of Or71a (Dweck et al., 2015), while the presence of harmful microbes on fermented fruit abolishes oviposition (Stensmyr et al., 2012). These harmful microbes release, among other compounds, a volatile called geosmin. Geosmin is detected by a single receptor, Or56a, and activation of this pathway alerts flies to the presence of e.g. *Penicillium* molds and triggers aversive behavior (Stensmyr et al., 2012).

II. Foul feces fool flies

Feces is an abundant source of energy in nature, used by many organisms, not least by members of the order Diptera. Adult Mediterranean fruit flies (*Ceratitis capitata*) need to feed on animal fecal matter in order to develop their eggs (Hendrichs et al., 1993; Lauzon, 2003). Houseflies and blowflies feed and breed on animal feces (Amendt et al., 2004). In **Paper II** of this thesis, I study how *D. melanogaster* reacts to fecal matter.

I show that *D. melanogaster* displays aversive oviposition towards carnivore feces, while indifference or even attraction towards herbivore dung (**Figure 6A**). This aversive behavior is due to the presence of high amounts of a toxic volatile compound – phenol – specifically enriched in carnivore feces, and is detected by Or46a-expressing OSNs located on the maxillary palps (**Figure 6B**). Silencing this single neuronal population, through directed expression of TNT (Sweeney et al., 1995), abolishes aversion to phenol as well as carnivore feces (**Figure 6C, D**). I further demonstrate that phenol suppresses egg-laying in flies, even in an attractive odor background. This observation suggests that phenol itself affects both oviposition site selection and the oviposition rate in flies.

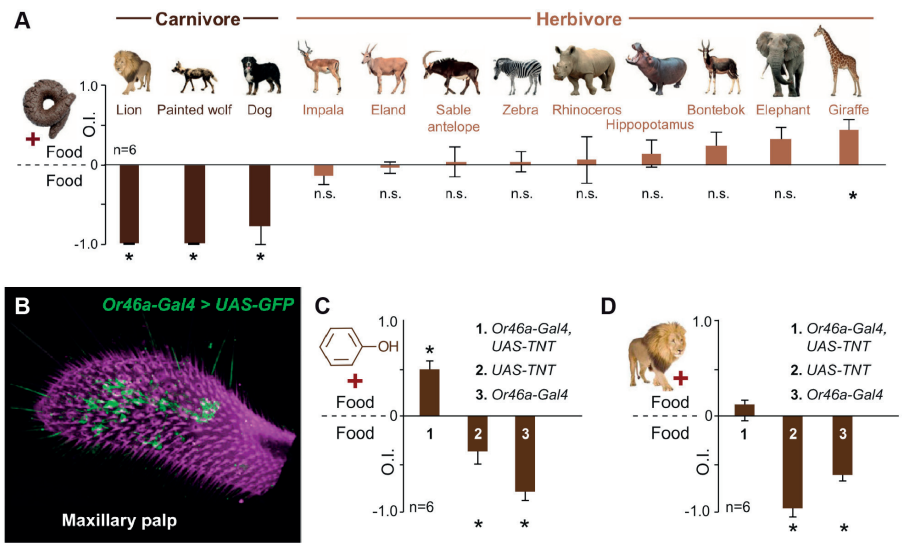

Figure 6. Flies avoid carnivore feces and the bacterial volatile phenol
A) Oviposition index (OI) of wild-type flies from a binary-choice test between standard cornmeal fly food and fly food mixed with mammalian feces. Error bars represent the SEM. Deviation of the response indices against zero was analyzed for significance with Student's t test ($p < 0.05$). B) Expression pattern of Or46a in the maxillary palp. C) and D) OI of flies expressing TNT from the putative promoter of Or46a in a binary-choice assay between standard cornmeal fly food and fly foodmixed with (C) phenol (10_2 dilution) or (D) lion feces.

Phenol signals pathogenic bacteria to the fly

Detecting and avoiding pathogens before infection is naturally beneficial, because once an animal is infected, it has to pay a high metabolic price to mount immune responses and to recover (McKean and Lazzaro, 2011; Kominsky et al., 2010). *D. melanogaster* can detect bacterial molecules called lipopolysaccharides (LPS), which alerts the fly's immune system to the presence of dangerous bacteria (Abbas et al., 2014). Gr66a detects these bacterial molecules, which mediates aversive feeding and oviposition responses (Soldano et al., 2016). In **Paper II** of this thesis, I show that carnivore feces, unlike herbivore feces is enriched by a family of bacteria – Enterobacteriaceae – that contains a wide range of pathogenic taxa causing severe diseases in humans (**Figure 7A**) (Rossetto et al., 2014). Some of these bacteria are able to produce phenol under special circumstances; when these bacteria grow in substrates containing L-tyrosine, an enzyme called tyrosine phenol-lyase convert L-tyrosine to phenol, pyruvate, and ammonia (Kumagi and Yamada, 1970). Accordingly, bacteria in carnivore feces are able to produce phenol from L-tyrosine, whereas bacteria in herbivore feces, scarce in L-tyrosine,

do not (**Figure 7B**). I further demonstrate that a dung beetle, *Scarabaeus (Kheper) lamarcki*, avoids carnivore feces and phenol presumably for a similar reason (**Figure 7C**).

Why would a fruit-dwelling fly then have a receptor for a fecal chemical? Fecal matter generates a wide range of volatiles, several of which are released by fruits as well. For example, limonene is emitted from thick epicarp fruit and also from many fecal samples. Flies in the native Southeast African habitat face feces more frequently than fruit, especially during the dry season when fruit is scarce. In this situation, distinguishing feces that contains harmful bacteria from fruit is necessary and beneficial for flies.

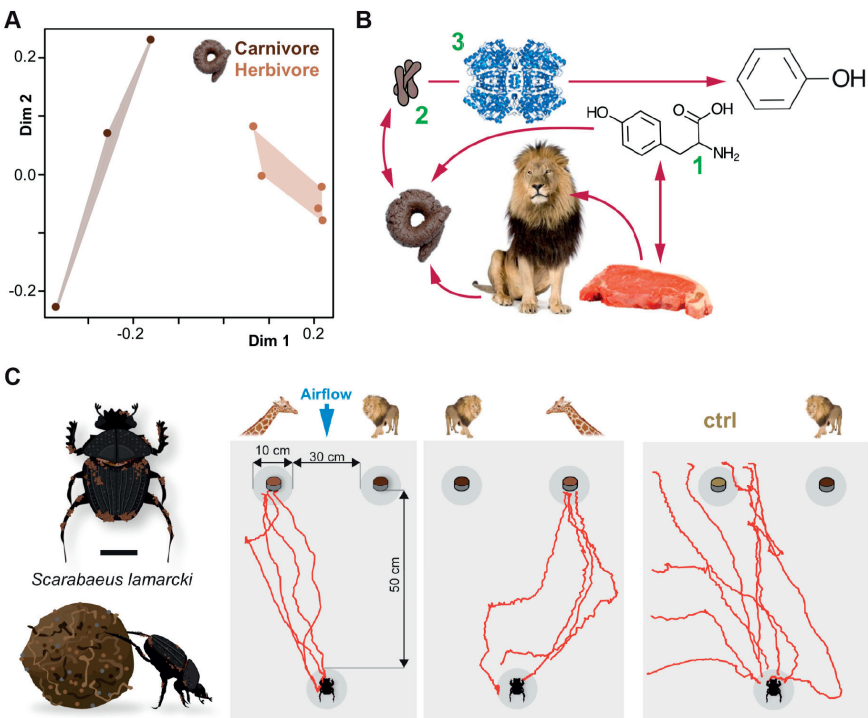

Figure 7. Dangerous dung
A) Multidimensional scaling plot from a Random Forest analysis of bacterial content in herbivore and carnivore feces B) Schematic model of phenol production by bacteria presence in the carnivore feces, presenting (1) L-tyrosine, (2) bacteria, and (3) L-tyrosine-phenol lyase enzyme. C) Dung beetles avoid carnivore feces. From left to right, tracks from beetles provided with a choice between lion and giraffe feces, confronted with lion feces and sand in an open olfactory binary-choice arena.

III. Humidity sensation in the fly

Insects must find a proper level of humidity for their survival, as they are in high risk of dehydration due to their low capacity to store water. Insects have evolved a sensory system to detect humidity changes in their environment, however, the molecular basis of humidity sensation has remained unknown. In **Paper III** of this thesis, I study humidity preference in the fly. In this paper, I present that drosophilid flies have an innate and species-specific preference towards various humidity levels in harmony with the climate of their native habitat. For instance, the cosmopolitan *D. melanogaster* prefers an intermediate humidity of 70% relative humidity (RH) at 25°C, whereas *Drosophila mojavensis*, a native to the dry Sonoran desert in southwestern United States and northern Mexico, prefers a 20% RH. *Drosophila teissieri,* native to warm and humid tropical rainforest in western Africa, prefers 85% RH **(Figure 8A-C).**

I have also searched for the hygrosensory sensilla and the molecular components of this sense on the antenna (Altner and Loftus, 1985). The hygrosensilla in *D. melanogaster* are located in the sacculus (**Figure 9A**), composed of three chambers (**Figure 9B**), each housing sensilla with distinct morphology. Those housed in chambers I and II are poreless, and show similar morphology to hygrosensilla of other insects. I show that Ir40a, and Ir93a along with the co-receptor Ir25a are expressed in neurons found in sensilla housed in chamber I and II (**Figure 9C**). Flies lacking any of these genes display deficient humidity guided behavior. Previous studies have proposed the requirement of thermosensory pathways for hygrosensation (Altner and Loftus, 1985), therefore, I test if these

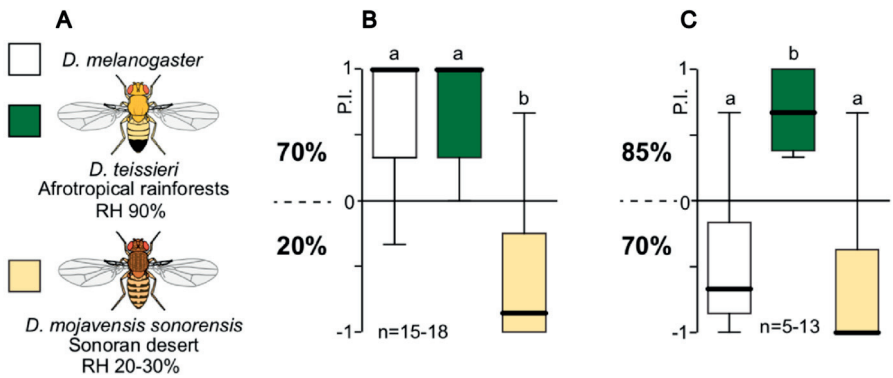

Figure 8. Innate and species-specific humidity preference
A) *D. teissieri* and *D. mojavensis*. B) Humidity-preference indices after 4 hr of *D. melanogaster*(white), *D. teissieri* (green), and *D. mojavensis* (yellow) tested in a binary humidity-preference arena with 20% RH versus 70% RH and, C) 70% RH versus 85% RH.

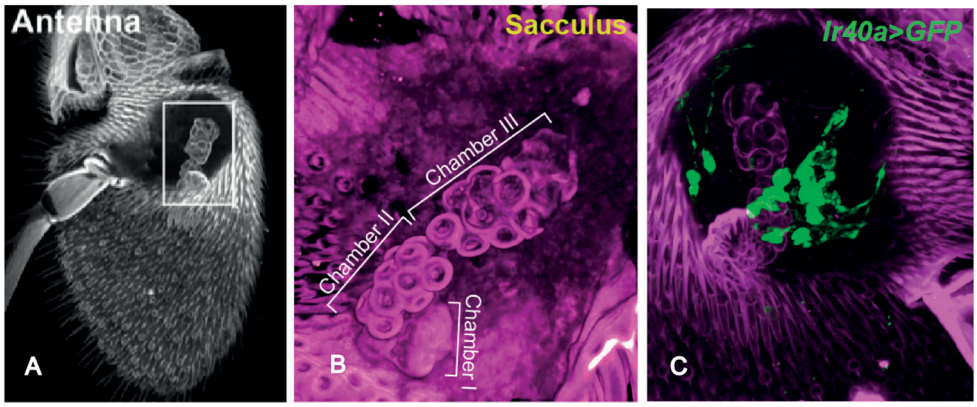

Figure 9 The humidity sensing apparatus
A) The antenna of the fly with the sacculus highlighted. B) Maximum-intensity projection of cuticular autofluorescence showing the outline of the sacculus. C) Maximum-intensity projection of antennae from a *Ir40a-Gal4>UAS-GFP* shows Ir40a is expressed in neurons targeting chamber I and II .

IRs are also involved in thermosensation. Genetic inactivation of Ir40a (through RNAi) caused a loss of the physiological and behavioral response only to the humidity stimulus, but not to temperature, whereas, both Ir25a or Ir93a mutant flies showed abnormal thermosensory along with hygrosensory responses. Ir25a and Ir93a are also expressed in the thermosensory cells of arista (Benton et al., 2009) and in the larval dorsal organ cool cells (DOCCs) (Knecht et al., 2016).

Early processing of hygrosensory information in the fly brain

In **Paper III**, I show that humidity information is processed in a region called the posterior antennal lobe (PAL) in the brain. The axon terminals of dry sensing neurons, expressing Ir40a, project to a glomerulus called “arm” while cool sensing neurons expressing the same receptor – Ir40a – project to “column” (**Figure 10A**). As Ir25a is required for both humidity and temperature sensing, neurons expressing this co-receptor projects to both “arm” and “column”, as well as “hot” and “cold” glomeruli in the PAL. Moreover, since Ir25a is also co-expressed with a subset of other odor sensing IRs, Ir25a positive neurons also project to olfactory glomeruli of the AL. Ir93a, necessary for humidity preference, projects to arm and column, as well as thermosensory glomeruli.

I also examine the response of “arm” and “column” to humidity and temperature changes by calcium sensors (**Figure 10B**). In imaging experiments the “arm” glomerulus showed increased calcium signal in response to stimulation with dry air, and a reduction in response to humid air, consistent with a function as the dry

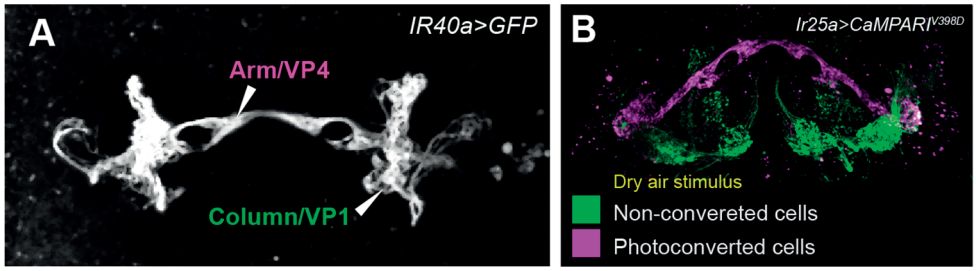

Figure 10. The arm glomerulus in the brain respose to humidty stimuli
A) Maximum-intensity projection from the PAL of a *Ir40a-Gal4>UAS-GFP* fly. B) The antennal lobe of a *Ir25a-Gal4>UAS-CaMPARI* fly following photoconversion during dry air stimulation.

neuron. In response to temperature stimulation, the "arm" glomerulus only responded to strong cold stimulation. The "column" on the other hand did not respond to humidity changes, but to changes in temperature, both hot and cold.

I did not succeed in identifying the moist neurons in this study, and accordingly I did not identify the corresponding glomerulus either. Subsequent studies have, however, found that these neurons express Ir68a, project to a glomerulus called "bean", which is adjacent to the "arm" and "column" (Frank et al., 2017; Knecht et al., 2017). The "hot" and "cold" glomeruli processing temperature information are found adjacent to these humidity-sensing glomeruli (Gallio et al., 2011; Knecht et al., 2017, Frank et al., 2017), and together form a hygro and thermo topographic map in the fly brain.

IV. Marula – The ancestral host of the lord of flies

As stated, *D. melanogaster* displays strong egg-laying preference towards *Citrus* spp. over other type of fruits (Dweck et al., 2013). This distinct host preference implies that *D. melanogaster* might form a close association with a specific fruit, or group of fruits, that probably share some characteristics with citrus in the native habitat. In preparation for **Paper IV** of this thesis, I searched for wild non-commensal populations of *D. melanogaster* within its native range, along with a search for its ancestral host. I searched mopane woodlands of the Matobos national park in Southwestern Zimbabwe (**Figure 11A**). This park is within the predicted ancestral range of *D. melanogaster* (Pool et al 2012), and covers 424 km^2, have no human habitation and is covered in mopane and kopje woodlands (**Figure 11B**). In the park I found a population of *D. melanogaster*, which turned out to be closely

associated with marula fruit (*Sclerocarya birrea*), a seasonally abundant resource during March to May. Marula, like citrus, has a thick rind, which covers a sweet-sour juicy pulp (**Figure 11C**). Marula is well-known for its high ethanol content (Morris et al., 2006), which would allow *D. melanogaster* to exploit its greater ethanol tolerance (David et al., 1986; Montooth et al., 2006).

We demonstrate that flies have a strong oviposition preference towards marula over orange both in the lab and in field traps (Figure.). We furthermore localized drosophilid larvae in all marula examined in the field traps, from which *D. melanogaster* adults emerged. Interestingly, we only found the wild population of *D. melanogaster* where the marula trees were present, but not in any other location of the Matobos with similar vegetation (**Figure 11D**), unlike *D. simulans*, which was present in high number at all trap locations (**Figure 11E**). This distribution pattern suggests that *D. melanogaster* has a specialized lifestyle, and is in fact a seasonal specialist, like its siblings *D. erecta* and *D. orena* in the *melanogaster* subgroup.

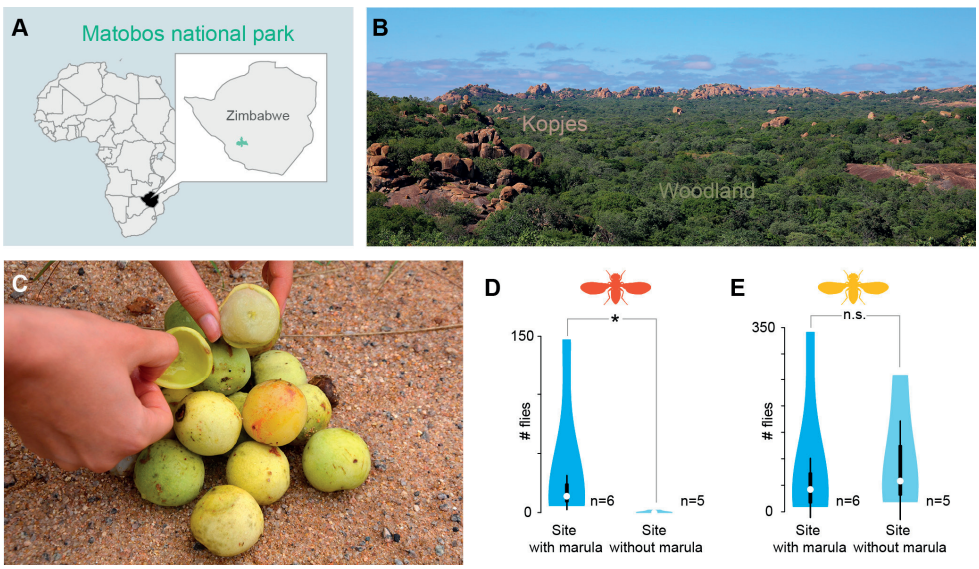

Figure 11 Picturetext headline
A) Location of the Matobos National Park, Zimbabwe. B) View of the park. Photo: M. Stensmyr. C) Marula fruit has a thick rind (in my right hand) and a soft flesh (in my left hand). Photo: E. Jirle. D) Number of *D. melanogaster* (red fly) traped at sites with and without marula. E) Number of *Drosophila simulans* (yellow fly) caught at the same sites as in D).

Or22a detects the marula volatile ethyl isovalerate

The two main chemicals of marula, ethyl isovalerate (an ester) and beta-caryophyllene (a sesquiterpene), together make up ~55% of the marula headspace and both trigger oviposition in flies. Similar to citrus, marula emits large amounts of terpenes. Citrus headspace is typically dominated by limonene, whereas, marula primarily emits beta-caryophyllene, which also activates the same olfactory pathway as limonene, Or19a (Dweck et al., 2013). Unlike citrus though, marula emits high amounts of esters, primarily ethyl isovalerate, which activates Or22a, as I show via calcium imaging (**Figure 12A**). Silencing the *Or22a* pathway via *Or22a-Gal4>UAS-TNT*, however, did not fully abolish the marula oviposition preference, suggesting that additional pathways are involved in this behavioral preference. The primary function of this pathway might be to locate the host over distance rather than mediating egg-laying preference. *Or22a* in general is broadly tuned to detect fruity esters in *D. melanogaster* and its eight close relatives (Stensmyr et al., 2003). On the other hand, Or22a has a selective and species specific receptive range based on the species preferred breeding substrates (Pelz et al., 2006; Linz et al., 2013). For example in *D. erecta* and *D. sechellia*, Or22a is most sensitive to the key ligands of their respective host fruits. In *D. erecta*, Or22a is most selective towards the *Pandanus* volatile, 3-methyl-2-butenyl acetate (Linz et al., 2013), while in *D. sechellia* this receptor detects the morinda volatile, methyl hexanoate (Dekker et al., 2006). Moreover, this selectivity and higher sensitivity is accomplished by a numerical increase in neurons expressing Or22a in both species in comparison to the other melanogaster species subgroup members (Dekker et al., 2006, Linz et al., 2013).

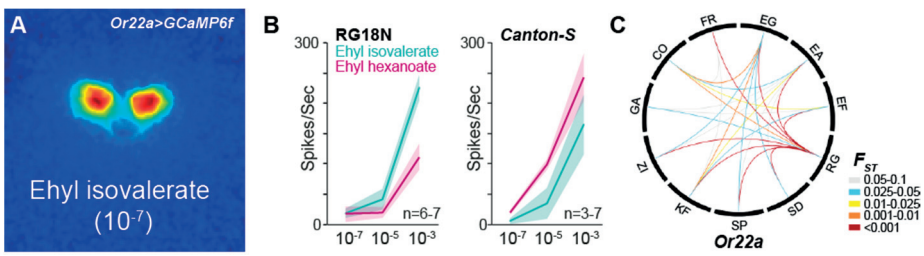

Figure 12. Picturetext headline
A) Pseudocolored image showing ethyl isovalerate-induced fluorescence changes in the antennal lobe (AL) of a *Or22a-Gal4>UAS-GCaMP6f* fly. B) Dose-response curve of ab3A neurons from the Rwanda (RG18N) and a lab strain of North American origin (Canton-S) toward ethyl isovalerate and ethyl hexanoate. Shaded area shows standard deviation. C) Genetic differentiation among populations at *Or22a* and *Or22b* is depicted via Circos plots [Krzywinski et al., 2009] based on F_{ST} quantiles. Only connections between populations with unusually high F_{ST} values (elevated genetic differentiation) is shown. The red color, for example, indicates that between this pair of populations, less than 0.1% of windows on the same chromosome arm have an F_{ST} value this high. Abbreviations display geographic origin of examined *D. melanogaster* populations e.g. RG stands for Rwanda, FR for France.

Interestingly, electrophysiological examination of OSNs expressing Or22a/Or22b from Sub-Saharan African flies (collected in Rwanda), revealed stronger responses to ethyl isovalerate than to ethyl hexanoate – the primary ligand of *Or22a* (Munch and Galizia, 2016). In contrast, the corresponding OSNs from a lab strain of North American origin, displayed stronger response to ethyl hexanoate than ethyl isovalerate (**Figure 12B**).

As a consequence of the functional difference of these *Or22a/Or22b* expressing OSNs between the two examined populations, I looked for signs of local genetic adaptation of *Or22a/Or22b* between *D. melanogaster* populations utilizing other hosts than marula. The results of comparing the local genetic differentiation within the Or family between genomes of ten African populations, plus one European revealed a strong genetic differentiation at the *Or22a*/*Or22b* locus between almost all population pairs (**Figure 12C**), whereas, most of the other ORs exhibited little or no sign of local adaptation. Taken together, unlike most members of the OR family in *D. melanogaster* that are conserved, *Or22a*/*Or22b* displays strong signs of local adaptation, in line with a function associated with host specific chemistry. Curiously, we found that the Sub-Saharan flies predominantly carries a specific allele at this locus, with a chimeric receptor formed from *Or22a* and *Or22b*. Whether or not this "*Or22ab"* gene indeed is responsible for the shifted ligand tuning of these neurons remains to be investigated. In brief, Southern African population of *D. melanogaster* not only detects ethyl isovalerate, they are even more sensitive to this marula ester than flies from outside Africa.

Concluding remarks

In this thesis, I have showed that the fly olfactory system does not detect odor molecules randomly, but capture and process specific odors associated with needs and dangers. For example, the fly has several olfactory pathways dedicated to odors coming from alcoholic fementation and activation of these receptors and the related pathways guides the fly to suitable food sources rich in sugar and yeast (**Paper I**). Although, we outline a range of behavioral and dedicated ecological functions for a number of olfactory pathways, a number of unknowns remain to be studied, such as identifying ligands for the few orphan receptors left e.g. Or2a and Or23a whose functions are likely important given their evident extreme specificity. In addition, the ecological significance of a number of receptors with known key ligands, like Gr21a/Gr63a that detect CO_2 remain puzzling. I have also investigated how the olfactory system cope with toxic and harmful matters in the natural habitat and I could identify an olfactory circuit that mediates repellency towards phenol, which is produced by pathogenic bacteria, predominantly present in carnivore feces (**Paper II**). Determining ligands for the few remaining orphan ORs should be a priority.

Furthermore, I reveal that flies have an innate and species-specific ability to find suitable humidity levels, related to their native habitat. Flies can sense humidity changes in their environment through a trio of IRs expressed in the sacculus of the antenna, which projects to the "arm" glomerulus in the brain (**Paper III**). But we still do not know what the real stimulus is that activates these hygroreceptive neurons. Moreover, the hygro-sensilla expressing IRs are poreless, which brings up the question "how these receptors detect changes in water vapor in the air".

Although vinegar flies are known as generalists, wild populations of *D. melanogaster* from a mopane forest within the potential ancestral habitat have a strong breeding preference towards marula fruit. This fruit is seasonally abundant, native to Southern Africa, and is presumably the ancestral host of the fly. I also speculate that marula was a vehicle for *D. melanogaster* to become a human commensal. Archaeological excavations from one of the caves in the Matobos uncovered 24 million marula stones (Walker, 1995). These fruit were collected by a San tribe settling in this region during the Late Pleistocene to the Early Holocene and stored for later consumption. The smell of stored marula from these caves would have invited flies to a massive source of food, making the favored host

available long after fruiting season. (**Paper IV**). The finding of a wild population of *D. melanogaster*, and the ancestral habitat, opens up for a range of interesting questions to be addressed. For example, how do these wild flies differ from their commensal relatives, i.e. which genetic factors underlie this shift in lifestyle? The finding that *D. melanogaster* has a close association with a single host fruit, will greatly facilitate studies in host specific chemosensory adaptations, which so far have had to be conducted in other insects in which the wealth of tools available in *D. melanogaster* are unavailable.

In summary, the research presented in my thesis enhances the basic science behind how the olfactory system operates and its mechanisms, the wild behavior of flies, and introduces the genetic and neural basis underlying humidity sensation in insects These basic understandings may lead us to better strategies for controlling insect pests, as well as human disease vectors such as mosquitoes.

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