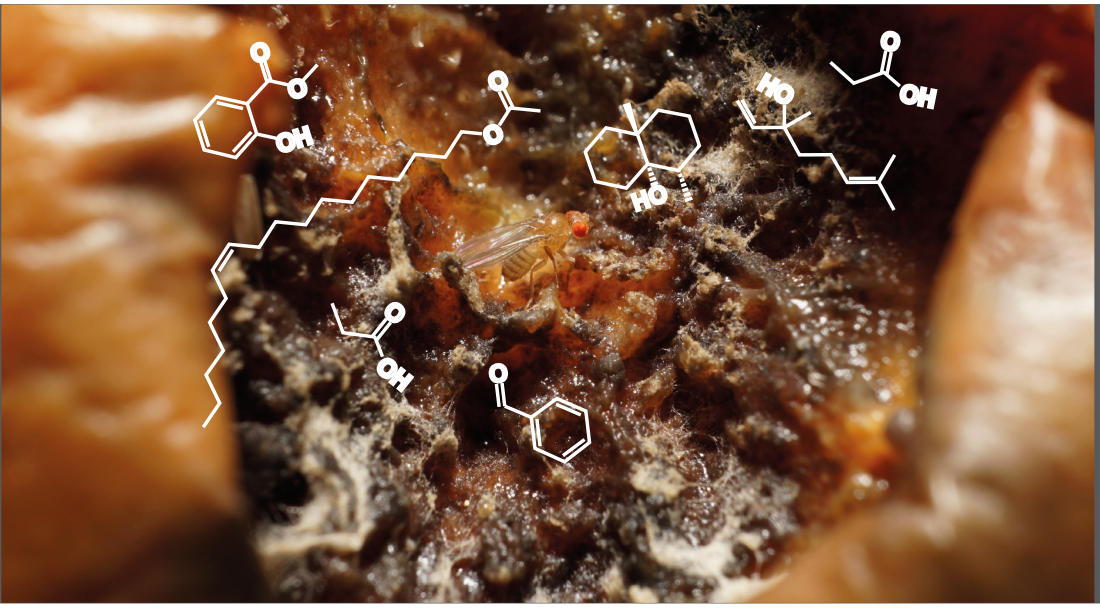
Exploring unknown avenues of intra- and interspecies communication in *Drosophila*



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Exploring unknown avenues of intra- and interspecies communication in *Drosophila*

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Introduction

"The fragrance of white tea is the feeling of existing in the mists that float over waters; the scent of peony is the scent of the absence of negativity: a lack of confusion, doubt, and darkness; to smell a rose is to teach your soul to skip; a nut and a wood together is a walk over fallen Autumn leaves; the touch of jasmine is a night's dream under the nomad's moon."
— C. JoyBell C

General

Everybody who has ever suffered from a head cold probably learned to appreciate– even if only for a brief time period- the immense role of the sense of smell (olfaction) in our day-to-day lives. Besides these rare occasions when we temporarily lose the functionality of our olfactory system, humans in modern, industrialized societies tend to underestimate the influence of this sensory modality on their well-being, decision making processes and social communication (Stevenson, 2009). However, the nose of humans is capable of striking performances, detecting even trace amounts of specific volatile substances (odorants), such as ethyl mercaptan, which is therefore added to gas as a signal/marker substance informing us about gas leaks (Whisman *et al.*, 1978; Mitchell, Kahn and Knasko, 1995). Humans living in indigenous, hunter-gatherer societies still rely on the remarkable sensory modality of olfaction for their day-to-day survival often mirrored in their extensive olfactory vocabulary describing a plethora of different smell qualities (e.g. *a stinging smell,*(de Valk *et al.*, 2017; Majid *et al.*, 2018)). In comparison the English language typically only use words that link an odor to a source (e.g. *smells like pear,* (de Valk *et al.*, 2017; Majid *et al.*, 2018)).

Nevertheless, even languages with an extensive olfactory vocabulary are no match in numbers for the almost intangible amount of odorants surrounding animals on planet earth. Detection and processing of these seemingly limitless varieties of volatile compounds was presumably the driving force behind the evolution of our and other animals' often complex olfactory system. A prominent example for a sense of smell, outstanding in its level of performance and requirements on its system, in the class of Mammalia, is the dog breed of bloodhounds which can distinguish between individual human scents and track those odor trails reliably for up to 48 h (Polgar et al. 2016, Harvey LM and Harvey JW 2003). While dogs are widely known to have a highly sensitive olfactory sense, the striking feature of the olfactory system of the African elephants is less obvious but yet unprecedented. Dogs possess on average a repertoire of 811 intact olfactory receptor (Or) genes which code for the first initial

detecting units of odorants. In contrast, African elephants possess 1948 genes of the gene family of olfactory receptors (Niimaru et al. 2014). Whether these high numbers of Ors also allow the elephants to have an extraordinary sense of smell is yet unknown as higher numbers of Ors may not necessarily go in hand with a keener olfactory sense.

From the vastness of volatile chemicals one group of odorants and their detecting as well as their processing circuity has been of particularly high interest to scientists due to their extraordinary role in an animal's life, which is an involvement in the control of sexual behavior (Darwin 1874, Fabre 1912 and Gomez-Diaz & Benton 2013). These odorants as well as non-volatile compounds with the same role in inducing stereotypic reproduction behaviors or endocrinological changes are therefore grouped together and referred to as sex pheromones (Wyatt, 2003; Touhara and Vosshall, 2009; Gomez-Diaz and Benton, 2013). Interestingly, elephants, albeit now Asian and not the previously mentioned African elephants, come up yet again as a prime example as they are one of the few mammal species in which a candidate for a sex pheromone has been identified to date (Rasmussen *et al.*, 1996; Wyatt, 2003; Gomez-Diaz and Benton, 2013). The exact structure of this candidate female sex pheromone has been elucidated ((Z)-7-dodecen-1-yl acetate, bombykol) and linked to a hardwired, non-habituating behavior in which the male elephants curl back their upper lip followed by an inhalation of air through the mouth cavity alone (flehmen response,(Rasmussen *et al.*, 1996; Gomez-Diaz and Benton, 2013)). Surprisingly, the volatile compound bombykol was already known to have the function of a sex pheromone, however not from other mammals or even vertebrates but rather in the insect *Bombyx mori,* also known as the silk moth (Butenandt *et al.*, 1959). In fact bombykol has been the first ever purified, identified and characterized sex pheromone; this succeeded in the middle of the twentieth century (Butenandt *et al.*, 1959). Since then bombykol has been found as a female-specific sex pheromone in various moth species and the aforementioned Asian elephant. Researchers explain the extraordinary importance of this one odorant with a possible example for convergent evolution of volatile hydrocarbon derivatives (Rasmussen et al. 1997, Gomez-Diaz & Benton 2013).

The olfactory system of insects is often extremely sensitive to sex pheromones due to the outstanding importance of these compounds in the mediation of reproductive behavior. Male moths can detect the female pheromone even over very long distances and use their species-specific blend of sex pheromones to pinpoint the location of their mating partner (Fabre, 1916). Even more remarkably, in *B. mori* the activation of already only 1 % of the pheromone receptors on the moths' main olfactory organ, the antenna, is sufficient to elicit wing fanning in a significant amount of tested male moths as part of their courtship behavior (Kaissling and Priesner, 1970).

While human and other mammals are capable of perceiving odors that are emitted by food sources of insects or that are produced by insects as repellents, the detection of insect pheromone (blends) is a unique intraspecies communication channel.

Besides various moth species that are popular model organisms for olfactory research in invertebrates, especially because of their extremely sensitive and sophisticated pheromone detection systems, the vinegar fly *Drosophila melanogaster* has been an equally attractive system to study the function, organization and the development of olfactory systems. Not only does *D. melanogaster* share core features, albeit simplified, of its olfactory system with vertebrates (Stocker, 2001; Vosshall and Stocker, 2007; Touhara and Vosshall, 2009), but also studies on the vinegar fly hold the opportunity to combine genetic manipulations with the analysis of olfactory behavior or olfactory physiological function *in vivo* (Clyne *et al.*, 1997). Furthermore, in the last decade the genus *Drosophila* with its highly diverse species richness has emerged as a model for research on ecology and speciation. Increasing our understanding of the sense of olfaction in insects does not only help us gain fundamental knowledge about neurobiological principles and evolutionary processes but also has practical applications since insects represent some of the world's most relevant pest species and disease vectors.

The following sections of the introduction intend to give a more detailed overview of (I) the architecture of the adult olfactory system in *D. melanogaster*, (II) olfactory signal detection in insects, (III) *D. melanogaster* as a model organism in chemoreception research, (IV) the genus *Drosophila* as a model for evolutionary neuroethology and chemical ecology, (V) odor-guided behavior in *D. melanogaster* flies and finally (VI) *Drosophila*-microbe interactions. The introduction concludes with (VII) the objectives of this thesis and its contribution to the research field.

I. Morphology of the olfactory system in *D. melanogaster*

Adults of *D. melanogaster* perceive chemical signals (chemosignals) in their environment predominantly with two pairs of bilaterally distributed olfactory organs on their head, the antennae, or more precisely the third antennal segments (funiculi), and the maxillary palps (Shanbhag, Müller and Steinbrecht, 1999; Martin *et al.*, 2013). On each of the third antennal segments a three-chambered invagination can be found, the sacculus, as well as a feathered arista towards the lateral edge of the funiculus. The club-shaped maxillary palps are located directly at the base of the fly's proboscis and are tucked away between the head and labellum if the proboscis is not extended (**Figure 1**, (Shanbhag, Müller and Steinbrecht, 1999)). Odorant

detecting sensory neurons (olfactory sensory neurons, OSNs) are found at the base of hair-like structures with porous shafts that cover the surface of the main olfactory organs and that are referred to as olfactory sensilla. On each of the funiculi around 400 sensilla are situated, and the maxillary palps each house approximately 60 sensilla (Vosshall and Stocker, 2007; Martin *et al.*, 2013). Olfactory sensilla on the olfactory organs can be classified by size, morphology and their response profile to different chemical classes, resulting into four different sensilla types: small and big basiconic sensilla that are club-shaped, intermediate sized sensilla, coeloconic sensilla that look like small knobs with a single, pin-like protruding structure and the thin, elongated trichoid sensilla (**Figure 1**, (Stocker, 1994; Shanbhag, Singh and Singh, 1995; Shanbhag, Müller and Steinbrecht, 1999; Yao, Ignell and Carlson, 2005; Su, Menuz and Carlson, 2009; Martin *et al.*, 2013; Lin and Potter, 2015)). Olfactory sensory neurons of basiconic, intermediate and coeloconic sensilla detect general odorants that consist of esters, alcohols, aldehydes, amines and acids, respectively (Yao, Ignell and Carlson, 2005; Hallem and Carlson, 2006; Benton *et al.*, 2009; Ai *et al.*, 2010; Dweck *et al.*, 2013; Ronderos *et al.*, 2014; Münch and Galizia, 2016; Sharma *et al.*, 2016), whereas OSNs of trichoid sensilla are exclusively tuned to the detection of pheromones, which mostly represent long-chained fatty acids (Clyne *et al.*, 1997; Kurtovic, Widmer and Dickson, 2007; van der Goes van Naters and Carlson, 2007; Datta *et al.*, 2008; Dweck, Ebrahim, Thoma, *et al.*, 2015). Basiconic sensilla are present on both, maxillary palps and funiculi, while the other three sensilla types can only be found on the funiculi (Shanbhag, Müller and Steinbrecht, 1999). Different sensillum subtypes are classified based on their sensillum category (b: basiconic, t: trichoid and c: coeloconic), location on antennae or maxillary palps (a: antennal, p: palp) and a specific identification number in *D. melanogaster* (e.g. antennal basiconic sensilla ab1-ab10).

Into the shaft of the olfactory sensilla extend the dendrites of the OSNs and embedded into the dendritic membrane the initial detection units for chemosignals, the olfactory receptors, are located. Generally, OSNs express only one type of olfactory receptor but in some cases these sensory neurons contain two types of olfactory receptors, for example the antennal basiconic sensillum ab5 expresses the receptors Or47a and Or56a. Per sensillum one-to-four OSNs are housed and are surrounded by the thecogen, trichogen and tormogen cell, also referred to as auxiliary cells (Shanbhag, Müller and Steinbrecht, 2000). The auxiliary cells form tight interconnections with each other and the enclosed OSNs via specialized cell junctions such as desmosomes or septate junctions (Keil, 1999; Shanbhag, Müller and Steinbrecht, 2000). Trichogen and tormogen cells are involved in the formation of the outer sensillum-lymph cavity and the production of the sensillum lymph, including the production and secretion of odorant or

pheromone binding proteins (OBPs, PBPs). Since the dendrites of OSNs are surrounded by aqueous sensillum lymph, OBPs and PBPs bind, solubilize and mediate the transport of odorants, including pheromones, that both have hydrophobic properties, through the lymph to Ors in the dendritic membrane (Vogt and Riddiford, 1981; Keil, 1999; Leal, 2013). The thecogen cell wraps the OSNs from the outer over the inner dendritic segments to the axonal regions in a glia-like manner (Shanbhag, Müller and Steinbrecht, 2000).

Axons from OSNs of the antennae and maxillary palps project into the antennal lobe (AL) that is the primary processing center in the insect olfactory system and represents an analog of the olfactory bulb (OB) found in vertebrates (Strausfeld and Hildebrand, 1999; Touhara and Vosshall, 2009). The projections of OSNs expressing the same Or(s) converge onto one of 52 spherical subunits with topographically fixed location, called glomeruli, in the AL (Gao, Yuan and Chess, 2000; Vosshall, Wong and Axel, 2000; Grabe *et al.*, 2015). In total the AL consists of 54 glomeruli, including around a dozen subunits that correspond to OSNs, which co-express Ors, and two non-chemosensory subunits with thermosensory function (Gallio *et al.*, 2011; Grabe *et al.*, 2015, 2016).

After initial integration of olfactory information in the AL, projection neurons (PNs) convey the information subsequently to two higher brain centers: the mushroom body calyx (MBc) and the lateral horn (LH). The MBc resembles the mammalian piriform cortex and is involved in olfactory associative as well as context-depending learning and memory, allowing the flies to constantly adapt to changes in the environment during their lifespan (Davis, 1993; Heisenberg, 2003; Fiala, 2007; Waddell, 2013). Whereas, the LH is a higher brain center that has been proposed to predominantly mediate odor-guided innate behavior (Heimbeck *et al.*, 2001), such as repellence towards geosmin, which is a compound produced by microbes harmful to *D. melanogaster* (Stensmyr *et al.*, 2012). The insect LH has similar properties analog to the vertebrate cortical amygdala (CoA, (Miyamichi *et al.*, 2011; Sosulski *et al.*, 2011)) and sends projections to a variety of other brain areas including multiple different regions in the protocerebrum (Tanaka *et al.*, 2004; Frechter *et al.*, 2019). Finally, since the output neurons of the LH do seemingly not directly mediate information to the ventral cord, it is likely that information is further processed before ultimately reaching the motor output (Namiki *et al.*, 2018; Dolan *et al.*, 2019).

II. Transformation of the olfactory chemical signal into an electrical information

II.I Olfactory receptors in insects

In the early 1990s, Ors were first discovered in rats (Buck and Axel, 1991) followed by the discovery of Ors in *Caenorhabditis elegans* in 1995 (Troemel *et al.*, 1995), while a subsequent identification of Ors in insects was hampered due to the unique sequence features of insect Ors that are unrelated to the G-protein coupled Ors found in vertebrates and nematodes. Eventually in the late 1990s, insect Ors were described and isolated from *D. melanogaster* through the efforts of multiple laboratories (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999). Insect Ors were characterized as seven-transmembrane receptors that are ligand-gated non-selective cation channels with an intracellular N-terminus and an extracellular C-terminus (Benton, Sachse and Vosshall, 2006; Lundin *et al.*, 2007; Smart *et al.*, 2008). In the dendritic membrane of OSNs the Ors occur as heteromeric structures with two subunits: the Or itself, which is the variable part of the functional ligand detecting unit, with striking intra- and interspecies sequence diversity between Ors, and which mediates ligand specificity, and a co-receptor (Orco, formerly known as Or83b) that is highly conserved between different insect species. Without its Orco subunit Ors will not assemble correctly, their trafficking to the dendritic membrane is impaired as well as their functionality (Larsson *et al.*, 2004; Benton, Sachse and Vosshall, 2006), interfering with the insect's olfactory behavior (Larsson *et al.*, 2004; Degennaro *et al.*, 2013; Trible *et al.*, 2017; Yan *et al.*, 2017). Besides the Orco subunit, the Hedgehog signaling pathway has been identified to play a role in the regulation of Or trafficking towards their final destination in the ciliary membrane of OSNs in *D. melanogaster* (Sanchez *et al.*, 2016). Until recently the structural basis of the OrX-Orco heterodimers has been unknown, but Butterwick and colleagues finally succeeded in the ground-breaking elucidation of the Orco crystallographic structure (Butterwick *et al.*, 2018); whereby, Orco can form tetrameric channels with four loosely assembled subunits that surround, symmetrically an ion-conducting central pore and which interact with other subunits over a small intracellular anchor domain (Butterwick *et al.*, 2018). For optimized detection of long-chain fatty acid derived pheromones like 11-cis-vaccenyl acetate (cVA) the OrX-Orco receptor units require further the presence of an additional transmembrane protein called Sensory Neuron Membrane Protein 1 (SNMP1), which belongs to a family of CD36-homologue proteins (Xu *et al.*, 2005; Benton, Vannice and Vosshall, 2007). In *D. melanogaster* without SNMP1 the spontaneous activity of OSNs expressing Or67d is altered and the response to cVA is either depleted or response kinetics are changed (Benton, Vannice and Vosshall, 2007; Li *et al.*, 2014; Gomez-Diaz *et al.*, 2016).

Since the discovery of Ors, two more receptor families have been identified to play a role in the detection of odorants and were therefore classified as olfactory receptors: the receptor family of ionotropic glutamate receptors (Irs) that detect airborne organic acids, aldehydes and amines (Stocker, 2001; Yao, Ignell and Carlson, 2005; Benton *et al.*, 2009; Silbering *et al.*, 2011; Menuz *et al.*, 2014) and two members of gustatory receptors (GRs, Gr21a and Gr63a) that are mediating the detection of CO_2 (Jones *et al.*, 2007; Kwon *et al.*, 2007). Like Ors, Irs and the CO_2 sensing Gr, Gr21a, depend on the presence of co-receptors for trafficking and wild-type functionality (Jones *et al.*, 2007; Kwon *et al.*, 2007; Benton *et al.*, 2009; Abuin *et al.*, 2018). The expression of Gr21a and Gr63a is restricted to basiconic sensilla on the antenna (ab1 sensilla, (Kwon *et al.*, 2007; Yao and Carlson, 2010), while IRs are expressed in coeloconic sensilla predominantly on the antennae but can also be found in other body regions (Benton *et al.*, 2009; Croset *et al.*, 2010; Abuin *et al.*, 2018; Sánchez-Alcañiz *et al.*, 2018).

Although the olfactory system of insects, humans and other vertebrates allows for the perception of a magnitude of different odorants there is a great discrepancy between the virtually countless arrays of volatile compounds that can be detected and the quantity of Or genes, which exist in rather discrete amounts. In mammals the numbers of functional Ors are usually ranging from a few hundred (e.g. 387 Ors in humans and 811 Ors in dogs (Niimura and Nei, 2007)) to a few thousands (1207 Ors in rats and ~2000 Ors in elephants (Niimura and Nei, 2007; Niimura, Matsui and Touhara, 2014)), which can in contrast detect hundreds of thousands of different odorants. Insects have even fewer numbers of functional olfactory receptors (~62 in adult *D. melanogaster*, 79 in mosquitos and ~170 in honeybees (Vosshall, Wong and Axel, 2000; Hill *et al.*, 2002; Couto, Alenius and Dickson, 2005; Robertson and Wanner, 2006; Jones *et al.*, 2007; Kwon *et al.*, 2007; Yao and Carlson, 2010)) but yet their olfactory system enables them to perceive and discriminate an immense range of different odorants, comparable to performance levels of vertebrate olfactory systems.

Consequently, both, vertebrates and insects, use a combinatorial code to enable a discrimination of the vast number of diverse odorants encountered in their day-to-day life (Malnic *et al.*, 1999; Fiala *et al.*, 2002; Wang *et al.*, 2003; Galizia, 2014; Haverkamp, Hansson and Knaden, 2018). Only recently is has been shown that besides the activation of olfactory receptors through agonistic ligands also the inhibition of OSNs via antagonistic odorants has odor coding quality and behavioral relevance for insects (Cao *et al.*, 2016; MacWilliam *et al.*, 2018). Together odorant specific excitation and inhibition of olfactory receptors lead to a unique pattern of OSN activity and this combinatorial code is read-out by the higher brain centers and assigned a certain odorant. Furthermore, additional players in perireceptor events such as

OPBs, PBPs and SNMPs that are differentially expressed in different sensilla types have the potential to add another level of complexity and aid an even greater odor-coding capacity (Larter, Sun and Carlson, 2016).

II.II Signal transduction of olfactory stimuli

In insects, events that take place after binding of the odorant to the OrX-Orco units have not yet been definitively elucidated and several models are controversially debated with the possibility that diverse insect genera employ different signal transduction mechanisms upon ligand binding. It is only certain that signal transduction processes in insects vary from the G-protein dependent, metabotropic signal transduction cascades found in vertebrates, as insect Ors do not possess any conventional G-protein binding sites (Benton, Sachse and Vosshall, 2006; Lundin *et al.*, 2007; Kaupp, 2010). Initially there were two main hypotheses for the translation of the chemical information of an odorant into an electrical signal in insects: A) Insect OrX-Orco complexes are odorant-gated ion channels with cation permeability features where the signal transduction does not involve any secondary messengers (**Figure 1 D**, (Sato *et al.*, 2008; Yao and Carlson, 2010)) or B) after ligand binding to the OrX-Orco complex, a cation influx triggers metabotropic processes, involving G-protein activity, that lead to the formation of an action potential if the stimulus exceeds the threshold (**Figure 1 E**, (Kain *et al.*, 2008; Wicher *et al.*, 2008; Deng *et al.*, 2011; Sargsyan *et al.*, 2011)). These two hypotheses were first seen to exclude involvement of the opposing signal transduction mechanism. Since then it has been suggested that upon ligand-binding either mixed ionotropic-metabotropic processes take place or that Ors are second messenger modulated ionotropic receptors. Both explanations describe G-proteins as secondary messengers of the metabotropic processes (Nakagawa and Vosshall, 2009; Wicher, 2010). However, very recent work shows that at least in Or47b expressing OSNs of *D. melanogaster* signal amplification upon odorant stimulation can be achieved without the involvement of G-proteins, solely via Ca^{2+}-influx mediated activation of downstream sodium-channels (**Figure 1 F**, (Ng *et al.*, 2019)). If ionotropic signal amplification is a common mechanism shared between different OSN types, this would explain how insect signal transduction and signal amplification can be accomplished without shared second messenger binding sites and without sequence similarity between the highly diverse Ors found in insects (Ng *et al.*, 2019). Furthermore, since Orco subunits can form homotetramers with cation channel properties it has been suggested that beside Orco's function in OrX trafficking and functionality, in moth, Orco homomers could have the function of pacemaker channels that control the membrane potential of OSNs, bringing the membrane potential closer or further away of threshold levels (Stengl, 2010; Nolte *et al.*, 2016).

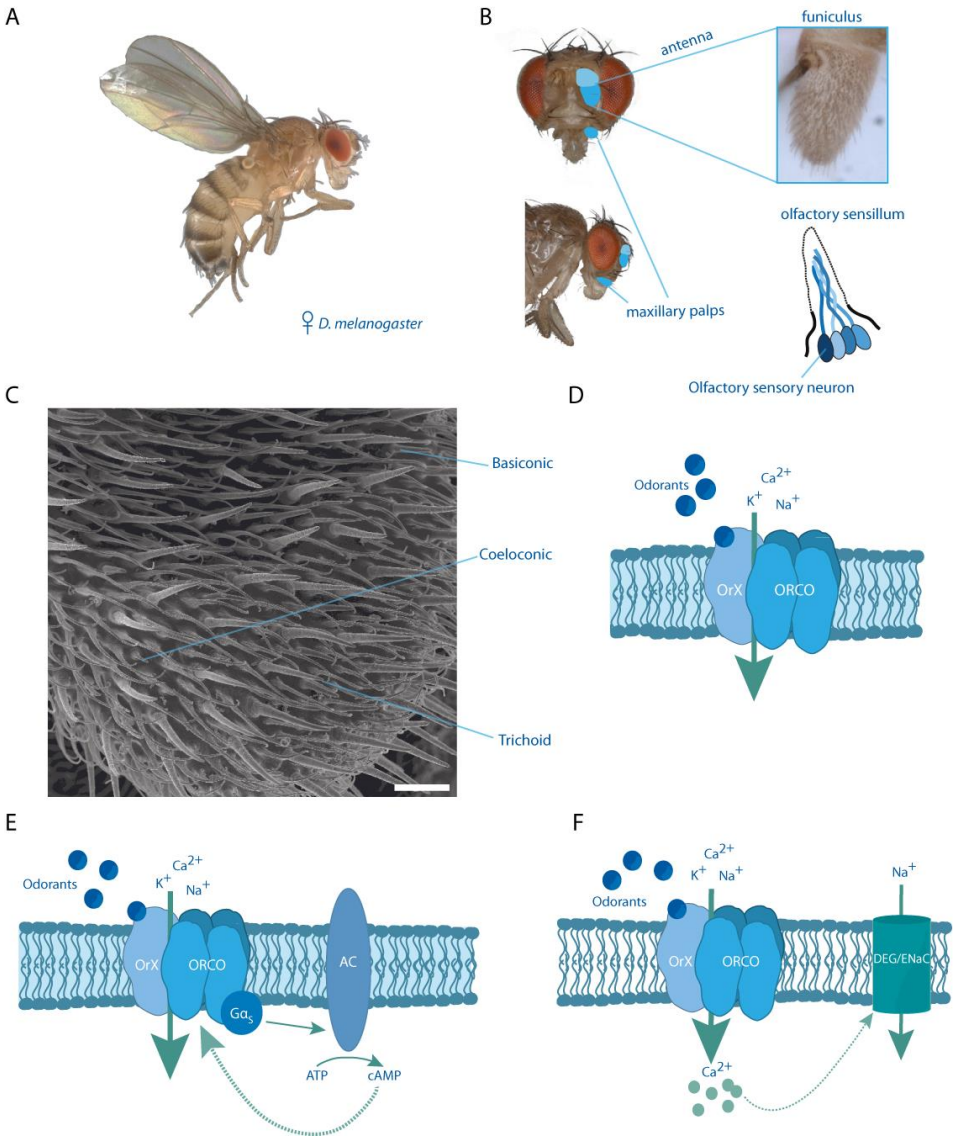

Figure 1 | Overview of the peripheral olfactory system in the vinegar fly *D. melanogaster*. (A) Lateral view of the whole body of a female *D. melanogaster* adult. (B) Lateral and frontal view of the head of an adult *Drosophila* fly including a magnification of the third antennal segment, the funiculus. Additionally, the general architecture of an olfactory sensillum with its housed olfactory sensory neurons (OSNs) is depicted. The two main olfactory organs, antennae and maxillary palps, are highlighted in blue (images provided by Ian W. Keesey and modified from Keesey et al. 2019). (C) Scanning electron image of the funiculus of a male *D. melanogaster* fly. Representative basiconic, coeloconic and trichoid sensilla are highlighted. Scale bar 10 µm (image Jürgen Rybak). (D) Model of an exclusively ionotropic olfactory signal transduction. Odorant binding to the OrX subunit of olfactory receptors is followed by an opening of the ion channel and a subsequent cation influx (Sato et al. 2008). (E) Model of a mixed ionotropic-metabotropic signal transduction. Odorant binding at the olfactory receptor leads directly to an ionotropic cation influx and activates a metabotropic signal transduction that enhances the original signal through the involvement of G-proteins. AC: adenylyl cyclase, cAMP: cyclic AMP, $G\alpha_S$: G_S-protein (Wicher et al. 2008). (F) Model for a metabotropic signal cascade that does not require an involvement of G-proteins. Odorant stimulation causes receptor activation and is followed by a Ca^{2+} influx into the OSN. Increased Ca^{2+} levels in turn activate DEG/ENaC channels and as second messengers amplify the original signal (Ng et al. 2019).

III. *D. melanogaster* as a model organism for chemoreception research

At the beginning of the 20s century, when William Ernest Castle and Thomas Hunt Morgan decided to work with the vinegar fly *D. melanogaster* as an inexpensive model organism, which is easy to rear and has a short generation time, to study genetic and developmental processes (Castle, 1906; Morgan, 1910), they paved the way for *D. melanogaster* to become the central model organism for genetics, neuroethology, physiology and developmental research. In fact, as mentioned earlier, *D. melanogaster* has been the model organism in which insect Ors have first been described and characterized. Since then, much more fundamental knowledge has been gained about insect olfaction using *D. melanogaster* as a model organism including the mapping of entire neural circuits in adult flies and larvae (Schneider-Mizell *et al.*, 2016; Takemura *et al.*, 2017; Thum and Gerber, 2019). Furthermore, *D. melanogaster* is the only insect model organism with a comprehensive database assigning the detection of chemosignals to certain olfactory receptors (Münch and Galizia, 2016). For no other insect species there exists a comparable, in depth knowledge, about the excitatory or inhibitory interaction of ligands with their corresponding interacting olfactory receptors. In *D. melanogaster* the functional characterization of chemosensory receptors, also referred to as deorphanization, was achieved through genetical manipulations in the live fly and Single Sensillum Recordings (SSR) from odorant stimulated sensilla (**Figure 2 A-C**). Here, scientists profited from the availability of a variety of different genetic tools in *D. melanogaster,* which includes binary expression systems where any target gene can be ectopically expressed in any tissue of choice, such as the Gal4/UAS- or LexA/LexAoP expression system (Brand and Perrimon, 1993; Lai and Lee, 2006). With the help of binary expression systems, target Ors were expressed in antennal OSNs that lack their endogenous Or ("empty" neuron system, "decoder systems", (Dobritsa *et al.*, 2003; Prieto-godino *et al.*, 2016)) and in extensive odor screens excitatory and inhibitory ligands for these receptors were identified (de Bruyne, Clyne and Carlson, 1999; de Bruyne, Foster and Carlson, 2001; Hallem and Carlson, 2004, 2006; Mansourian and Stensmyr, 2015; Dweck *et al.*, 2018). For these odor screens in SSR experiments, the sensillum potential of targeted sensilla was measured by inserting a reference tungsten electrode into the fly's compound eye and a recording tungsten electrode into the shaft of individual sensilla. Recorded signals were amplified, digitally converted and analyzed with specialized computer software (**Figure 2 A-C**).

The deorphanization of olfactory receptors in insect models other than *D. melanogaster* is more complicated but possible and has made great progress especially in the mosquito *Anopheles gambiae* (Wang *et al.*, 2010) and the moth *Spodoptera littoralis* (De Fouchier *et al.*, 2017). Unfortunately, the processes for olfactory receptor deorphanization outside of

D. melanogaster are very time-consuming and hampered by the lack of genetic manipulation *in vivo* in non-*melanogaster* insects. Likely, the introduction of the ground-breaking genome-editing CRISPR/Cas9 technique to the scientific community will allow for the introduction of genetic tools into the genome of more insect model species and potentially help the characterization of not yet known ligand-receptor interaction partners. However, the use of CRISPR/Cas9 requires a sequenced, annotated genome and non-model insect species without this requirement will still rely on time-consuming deorphanization in heterologous expression systems.

In 2015 von der Weid and colleagues described a correlation between long-time exposure to high odorant concentration with changes in mRNA levels of the interacting Ors, in both the mouse model and *D. melanogaster* (von der Weid *et al.*, 2015). With the discovery of the so-called DREAM method (**D**eorphanization of **r**eceptors based on **e**xpression **a**lterations of **m**RNA levels) it finally became possible to deorphanize chemosensory receptors in insects without access to genetic tools such as those available in *D. melanogaster*, with just transcriptomic data of chemosensory receptors on-hand (von der Weid *et al.*, 2015; Koerte *et al.*, 2018). Furthermore, if reliable, the DREAM-method could enable a high-throughput deorphanization of ligand interaction partners instead of a time-consuming identification of single ligand-receptor. Manuscript III addresses and discusses the applicability of the DREAM-technique as a tool for the deorphanization of chemosensory receptors in *D.* melanogaster and possible other model insect species.

IV. The genus *Drosophila* as a model for evolutionary neuroethology and chemical ecology

As mentioned earlier, for over a century, the vinegar fly *D. melanogaster* has been a model organism for various research areas, including neuroethology, however since the last decade the focus from *D. melanogaster* as a model species has been slowly shifting towards the entire genus *Drosophila* as a model (Prieto-godino *et al.*, 2016; Jezovit, Levine and Schneider, 2017; O'Grady and DeSalle, 2018; Auer *et al.*, 2019; Keesey, Grabe, Gruber, *et al.*, 2019; Markow, 2019). The genus *Drosophila* represents an extreme species-rich genus with around 1200-1500 different species and many individual species being commercially available in fly stock centers around the world (**Figure 2 D, E**). *Drosophila* flies populate an enormous range of diverse habitats from forests, over mountains as well as caves, to islands and deserts or even the excretory glands of land crabs (Gilbert, 1980; Markow and O'Grady, 2008). In these habitats

Drosophila flies can be found on a multitude of different hosts such as slime fluxes (Carlson, Knapp and Phaff, 1951; Cooper, 1960), leaves (Goldman-Huertas *et al.*, 2015), mushrooms (Grimaldi, 1985; Jaenike and James, 1991; Bunyward, 2003), cacti (Heed, 1978; Fogleman, Duperret and Kircher, 1986), fruits (Begon, 1975; Mansourian *et al.*, 2018), flowers (Brncic, 1983) or bat guano (Tosi *et al.*, 1990). In *Drosophila* flies a dietary shift and specialization to new hosts has evolved numerous times (Chandler *et al.*, 2011; Markow, 2019). A considerable amount of these hosts includes substrates with toxic and harmful contents that are inhospitable for other non-adapted insect species, such as the “noni” fruit of *Morinda citrifolia* plants, host to *Drosophila sechellia* (David and Van Herrewege, 1983; Fogleman, Duperret and Kircher, 1986; Legal, Chappe and Jallon, 1994; Spicer and Jaenike, 1996; Markow, 2019). However, the habitats and hosts of different *Drosophila* species are not always highly diverse but can also overlap and, in some cases, closely related species can in fact be found in the exact same host substrate (Carracedo, Casares and Miguel, 1989; Durisko *et al.*, 2014; Matavelli *et al.*, 2015).

Both, the closely related *Drosophila* species as well as the distantly related species make this genus highly attractive for research deciphering the mechanisms underlying genetic changes and adaptations in the involved neural circuits that lead to variations in host navigation, preference and acceptance. Research on these topics has made great progress in recent years and is particularly profiting from the introduction of different genetic tools into these *Drosophila* species, such as available in *D. melanogaster*, via for example CRIPR/Cas-9 mediated genome-editing (Auer *et al.*, 2019). Two prominent examples for advances on research for *Drosophila* specialization and speciation are the aforementioned specialist *D. sechellia* and the agronomic pest species *Drosophila suzukii*. In comparative studies to their closely related *Drosophila* species, including *D. melanogaster*, for *D. sechellia* and *D. suzukii*, scientists were able to pinpoint changes in ecological niche preference and acceptance to differences in the olfactory system, down to amino acid variations in chemosensory receptors, or plasticity in the wiring of controlling neuronal circuits as well as to shifts in courtship and mating behavior or oviposition preference (Dekker *et al.*, 2006, 2015; Keesey, Knaden and Hansson, 2015; Auer and Benton, 2016; Prieto-godino *et al.*, 2016; Karageorgi *et al.*, 2017; Auer *et al.*, 2019). In manuscript IV, the visual and olfactory system of 62 species of the genus *Drosophila* are compared regarding trade-offs between these two modalities as a consequence of possible developmental constraints. Furthermore, manuscript IV examines the impact of an olfactory or visual bias on the ecology of the analyzed *Drosophila* species with a focus on sexual behavior and host localization.

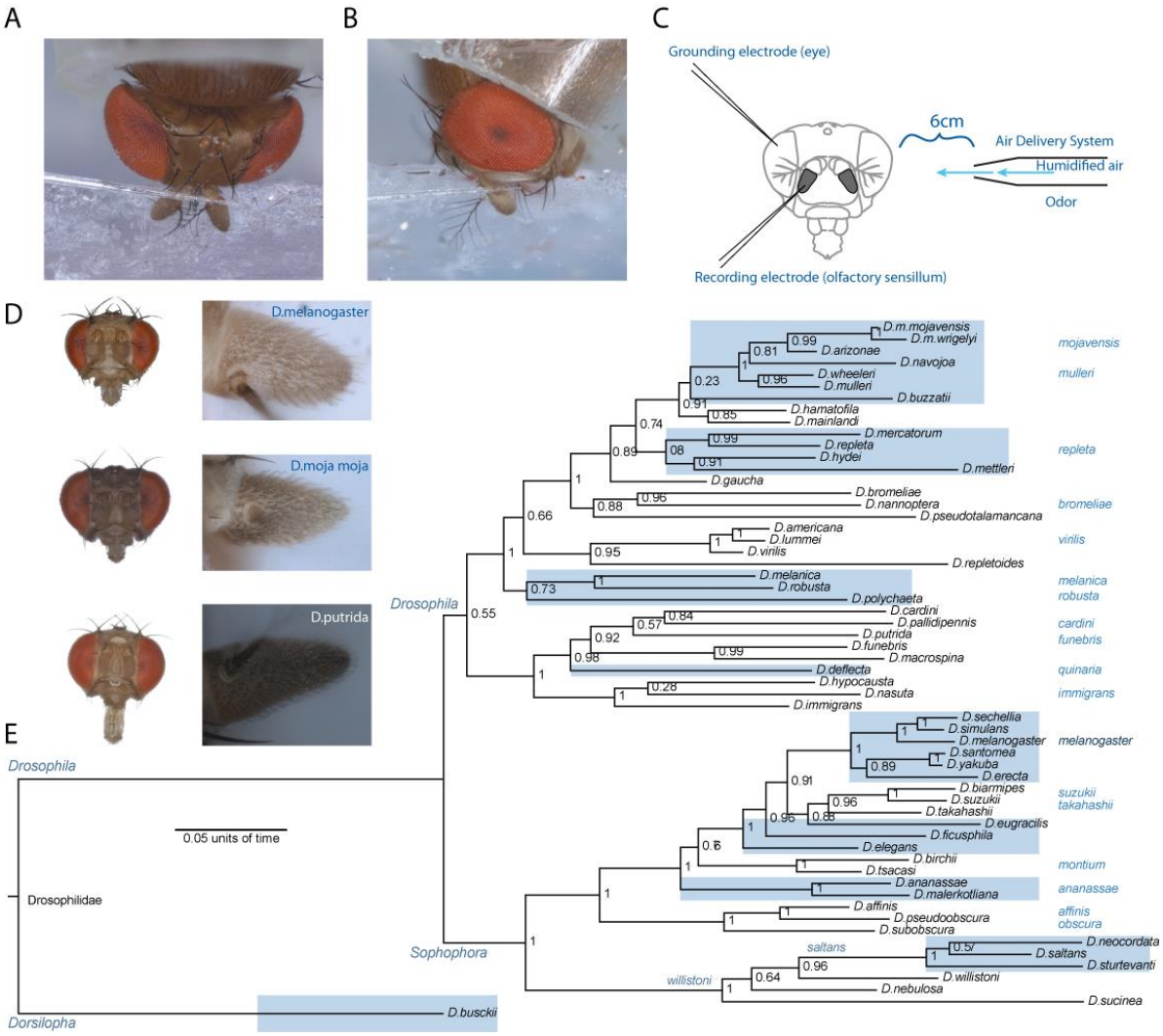

Figure 2 | Schematics of single sensillum recordings and phylogeny of *Drosophilidae* flies. (A-B) Dorsal and lateral view of a female *D. suzukii* fly in a cut pipette tip, prepared for single sensillum recording (SSR) measurements. A glass capillary fixates the third antennal segment (funiculus) to keep it stationary during recordings (images provided by Ian W. Keesey). (C) Schematic drawing of the experimental set-up for SSR studies in *Drosophila* flies (modified from manuscript V). (D) Frontal view of *Drosophila* heads and detailed views of the corresponding funiculus from species tested in manuscript V of this thesis (images provided by Ian W. Keesey). (E) Phylogeny of *Drosophilidae* flies with a focus on the genus *Drosophila* with 62 species analyzed total. Selected species are representative of a variety of different subgroups and show diverse genetic diversity. Blue boxes aim to visually separate the different subgroups (figure modified from Keesey et al. 2019).

Important stereotypic behavior of *D. melanogaster*

While *D. melanogaster* is capable of associating odorants or odor bouquets with other kinds of stimuli, the vinegar fly also has a repertoire of innate behaviors following the detection of specific olfactory stimuli. A considerable amount of these hardwired, developmentally preprogrammed behaviors of *D. melanogaster* are mediated by a small subset of narrowly tuned olfactory

receptors or often even by single olfactory channels. This predictable, innate behavior and its associated olfactory circuits are also referred to as ecologically labeled lines (Haverkamp, Hansson and Knaden, 2018). In *D. melanogaster*, OSNs that are predicted to mediate a certain behavior and are suggested to be an ecologically labeled line can be silenced or artificially activated without their natural ligands through various genetic tools (Lin *et al.*, 2015; Tanaka *et al.*, 2017; Haverkamp, Hansson and Knaden, 2018). This allows scientists to characterize the role of a neuronal circuit to be necessary and/or sufficient for the control of a stereotypic behavior (Haverkamp, Hansson and Knaden, 2018).The following two sections will highlight some prominent examples of stereotypic behavior in the vinegar fly in response to certain special general odors (section V.I) or pheromones (section V.II).

V.I General odors

In *D. melanogaster* a number of food related odorants induce innate attraction behavior and for many of these odorants their corresponding olfactory channels have been characterized (Stensmyr *et al.*, 2012; Mansourian and Stensmyr, 2015). Some general odorants that hold specific relevance to the vinegar fly and that innately trigger attraction behavior with upwind flight are chemicals produced in alcoholic fermentation or volatiles which indicate the presence of beneficial yeasts and bacteria (Mansourian and Stensmyr, 2015). Odorants that are generated in alcoholic fermentation processes indicate to *D. melanogaster* locations of sugar-rich substrates which are the habitat of the vinegar fly's predominate food source, yeasts (Mansourian and Stensmyr, 2015). Two of these volatiles associated with alcoholic fermentation are diacetyls and acetals, which are detected by Or92a and Or42b respectively (de Bruyne, Foster and Carlson, 2001; Mathew *et al.*, 2013).

Besides odorants that inform *D. melanogaster* about sites of alcoholic fermentation and that are by-products of the metabolic activity of microorganisms, predominantly of yeast species, yeasts further produce acetate esters that are equally highly attractive to the flies. Examples of these acetate esters are ethyl acetate, isoamyl acetate and amyl acetate, which all have a fruity smell and are predicted to be produced by the yeast for the sole reason to attract insects including *D. melanogaster* (Becher *et al.*, 2012, 2018; Christiaens *et al.*, 2014). The vinegar fly detects acetate esters with a set of Ors that have a rather broad ligand spectrum, such as Or43b, Or47a and Or85b (Hallem and Carlson, 2004; Christiaens *et al.*, 2014; Dweck, Ebrahim, Farhan, *et al.*, 2015; Mansourian and Stensmyr, 2015). Two more yeast-produced attractive odorants are the volatiles 4-ethylphenol and 4-ethylguaiacol that serve as antioxidants and presumably help *D. melanogaster* to minimize oxidative stress through entomopathogenic

microbes (Dweck, Ebrahim, Farhan, *et al.*, 2015; Mansourian and Stensmyr, 2015). Detection of these antioxidants is mediated through a highly specialized olfactory channel, Or71a, which not only triggers attraction but also activates neural circuits for feeding and oviposition behavior upon ligand binding (Jimenez-Del-Rio, Guzman-Martinez and Velez-Pardo, 2010; Dweck, Ebrahim, Farhan, *et al.*, 2015). In manuscript V the role of yeast emitted volatiles in the interaction of *Drosophila* flies with different yeast species is further analyzed and their ecological implications in the fly-microbe association are discussed.

Odorants emitted by bacteria can likewise act as attractants if released by beneficial bacterial species, such as the amines putrescine and spermidine that are the end products of bacterial amino acid degradation of arginine and which are detected by Ir41a (Silbering *et al.*, 2011; Min *et al.*, 2013). However, *D. melanogaster* also dedicates a single olfactory channel, Or56a, entirely to the detection of the repellent compound geosmin (Stensmyr *et al.*, 2012; Mansourian and Stensmyr, 2015). This chemical is emitted by harmful microbes and, upon activating Or56a, is rigorously avoided by the flies while suppressing feeding behavior and oviposition (Stensmyr *et al.*, 2012).

In female vinegar flies besides the localization of attractive food sources and the avoidance of harmful microbes, oviposition behavior is mediated by a few specific odorants and their corresponding, highly selective Ors. When *D. melanogaster* females detect valencene via Or19a at an oviposition site, they will preferably lay their eggs at this location (Dweck *et al.*, 2013). Interestingly, valencene and limonene at oviposition sites grant the flies protection from endoparasitoid wasps (Dweck *et al.*, 2013). In turn, the sex pheromone (iridomyrmecin) of exactly these endoparasitoid wasps is exclusively detected by Or49a in *D. melanogaster* and leads to avoidance of oviposition behavior upon detection (Ebrahim *et al.*, 2015).

While most odorants keep their valence (attraction or aversion) over a range of different concentrations, some chemosignals can change their valance depending on the odorant concentration. Two examples are the odorant acetic acid (Ir75a, (Silbering *et al.*, 2011)) and CO_2 (Gr21a and Gr63a) that are both attractive at low concentration but induce avoidance at higher concentrations. In both cases the opposing behavior is controlled by separated neural circuits (Jones *et al.*, 2007; Kwon *et al.*, 2007; Joseph *et al.*, 2009; Semmelhack and Wang, 2009; Turner and Ray, 2009).

V.II Pheromones

In *D. melanogaster* mate choice and courtship behavior are two more behaviors of particular importance that are controlled by hardwired neural circuity. Chemosensory cues play a prominent role in the mediation of sexual behavior in both sexes of the vinegar fly. Olfactory receptors involved in pheromone detection of *D. melanogaster* are tuned to the detection of very few and often even single compounds. The main sex pheromone of the vinegar fly, 11-cis-vaccenyl acetate (cVA), is male-produced, transferred during copulation, enhances female receptivity towards the courting male but mediates male-male repulsion (Greenspan, 2000; Benton, Vannice and Vosshall, 2007; Kurtovic, Widmer and Dickson, 2007). The detection of cVA takes place through Or67d, which is an olfactory channel that only interacts with this compound. Besides cVA, the pheromone methyl laurate (ML) plays a role in courtship behavior by governing male copulation success. This sex pheromone is detected by Or47b, which is activated by ML and presumably by the fatty acids myristic acid, palmitoleic acid, and palmitic acid (Dweck, Ebrahim, Thoma, *et al.*, 2015; Lin *et al.*, 2016). Palmitic acid and myristic acids are the fatty acid precursors of the sex pheromones, methyl palmitate (MP) and methyl myristate (MM), which likewise have an important role in the sexual behavior of *D. melanogaster*, albeit in a more indirect manner by attracting males and females to a common location as an aggregation cue (Dweck, Ebrahim, Thoma, *et al.*, 2015; Auer and Benton, 2016). The corresponding Or for the detection of MP and MM is Or88a, which is expressed in an OSN in the same trichoid sensilla as the Or47b expressing OSN and is also activated by ML (at4 sensilla, (Dweck, Ebrahim, Thoma, *et al.*, 2015).

Adult insect feces, frass, often mirror the odor profiles of the adult animal, including sex-specific pheromones produced by the insect. As such, frass provides a substrate that allows for the identification of novel pheromones, which may be otherwise difficult to isolate as shown on the boll weevil and the western pine beetle (Bellas, Brownlee and Silverstein, 1969; Tumlinson *et al.*, 1969). Insect frass itself has often relevance for pheromone-based behavior, such as aggregation, due to its specific odor profiles. Manuscript I describes the pheromone contents of the frass of different *Drosophila* species, including *D. melanogaster*, and assesses the role of adult insect feces in fly behavior.

V. *Drosophila*-microbe interactions

Microorganisms play a prominent role in the life of insects by impacting essential aspects of an insect's physiology and behavior including development, digestion, reproduction (fecundity and pheromone production), immunity and longevity (Anagnostou, Dorsch and Rohlfs, 2010; Guo and Kim, 2010; Wong, Dobson and Douglas, 2014; Sansone *et al.*, 2015; Fischer *et al.*, 2017; Téfit and Leulier, 2017; Bellutti *et al.*, 2018; Grangeteau *et al.*, 2018; Murgier *et al.*, 2019; Qiao *et al.*, 2019). The interactions of microorganisms with animals are very complex and the ubiquity of microbial-animal associations are only starting to be understood (Chandler *et al.*, 2011). Studying processes underlying the interaction of microbes and insects that populate the same habitat can advance our knowledge about the evolution of mutualism and the adaptation to new environments.

Pathogenic interaction partners of animals have been of extraordinary interest for microbe-animal interaction research. However, pathogens represent only a minority of microorganisms that animals are in contact with. Microbes contribute also in a beneficial, often essential manner to the life of their hosts and *D. melanogaster* acts as an important model for research on non-pathogenic insect-microbe interactions (Chandler *et al.*, 2011). In the vinegar fly especially bacteria of the gut microbiome have been studied for their role in different physiological processes of the insect such as the provision of nutrition (Newell and Douglas, 2014; Wong, Dobson and Douglas, 2014; Leitão-Gonçalves *et al.*, 2017; Téfit and Leulier, 2017), their impact on larval development or adult phenotypic traits (Shin *et al.*, 2011; Storelli *et al.*, 2011; Ridley *et al.*, 2012; Téfit and Leulier, 2017; Qiao *et al.*, 2019) and their involvement in resistance to pathogens (Sansone *et al.*, 2015). The gut microbiota has also been shown to be of specific importance for ecological adaptations to the insects' hosts (Brune, 1998; Hosokawa *et al.*, 2006; Janson *et al.*, 2008) and have an influence on chemosensory-guided foraging behavior including microbial preference in *D. melanogaster* (Wong *et al.*, 2017; Qiao *et al.*, 2019). Experiments on *D. melanogaster* involving genetic manipulations were able to successfully identify some of the genes that play a role in maintaining the gut microbiome community composition in this insect (Lhocine *et al.*, 2008; Ryu *et al.*, 2008).

While a considerable number of bacteria interacting with *D. melanogaster* have a positive impact on the fly's life, symbionts and pathogens have also evolved to manipulate their host in order to increase dispersal and transmission rates. These manipulations in the best case only influence the evolution of the host population by for example changing mate choice, but might also be ultimately detrimental for the flies (Arbuthnott, Levin and Promislow, 2016). In the life of insects sexual behavior and reproduction play a very prominent role and these processes

are controlled by hardwired neuronal circuits that lead to stereotypic behavior. Consequently, the reproductive system has been targeted and interfered with by symbionts as well as pathogens to their own benefit (Arbuthnott, Levin and Promislow, 2016). In manuscript II my colleagues and I show how pathogens exploit pheromone communication channels in *D. melanogaster* to increase rates of pathogen transmission and enhance the pathogens' chances to infect new hosts.

Besides beneficial and harmful bacteria, yeast communities have particular importance in the life of *Drosophila* species. As described in section V.I, a large portion of the olfactory receptor repertoire of *D. melanogaster* is devoted to the detection of odorants emitted by yeasts. Not only do yeasts represent a major food source for *Drosophila* flies by providing essential nutritional factors such as amino acids, antioxidants, fatty acids, sterols and B vitamins (Loeb and Northrop, 1916; Tatum, 1939; Becher *et al.*, 2012; Dweck, Ebrahim, Farhan, *et al.*, 2015), but also yeasts can detoxify secondary metabolites in *Drosophila* host material (Fogleman, Duperret and Kircher, 1986) and suppress the growth of pathogens and fungal molds at *Drosophila* breeding sites (Goddard, 2008; Becher *et al.*, 2012). For example, the well-known fruit-associated yeast species, *Saccharomyces cerevisiae*, hinders the growth of competitive microbes, which could also be potentially deleterious for *Drosophila* adults and larvae, in its surroundings through the release of ethanol, heat and CO_2 as byproducts of the yeast's metabolic activity and the fermentation of sugar in ripe fruits (Goddard, 2008).

The specific significance of yeasts in the life of vinegar flies is mirrored in the exceptional attractiveness of yeast-produced volatile for these flies. In fact, it was shown that yeast-emitted odorants are the major factor in the attraction of *D. melanogaster* towards food sources and breeding sites and host odors have only a secondary role in foraging behavior and host preference (Becher *et al.*, 2012; Scheidler *et al.*, 2015). The production of attractive chemosignals by yeast co-evolved with the development of the corresponding chemosensory receptors in insects that detect these volatiles (Engel and Grimaldi, 2004; Dujon, 2006, 2012; Nel *et al.*, 2013; Scheidler *et al.*, 2015; Becher *et al.*, 2018). The odor-mediated interactions between insects and yeasts can be found across the class Insecta (Davis *et al.*, 2013; Andreadis, Witzgall and Becher, 2015; Madden *et al.*, 2018) and are associated with mutual benefits for both interaction partners. Here, yeasts profit from an association with *Drosophila* flies, not only by vectoring through the flies to new substrates (Ganter, 1988; Reuter, Bell and Greig, 2007; Coluccio *et al.*, 2008) but also by growth promotion through *Drosophila* species (Stamps *et al.*, 2012). Yeast growth is promoted via physical modifications of the host substrate structure through *Drosophila* larvae as well as through the deposition of frass that not only

contains a pre-selected microbial community but also nutritional factors (Stamps *et al.*, 2012). Furthermore, after digestion by the flies and passage through the oral-fecal route, yeast spores are released from their tetrads and can sexually reproduce, which allows the yeasts to rapidly adapt to environmental changes via outbreeding (Reuter, Bell and Greig, 2007).

In manuscript V the preference of *Drosophila* flies towards yeasts from shared or unfamiliar ecological niches is assessed. In addition, the role of *Drosophila*-yeast associations in local adaptation processes and host range of the flies are discussed.

VI. Objectives of this thesis

Even though *D. melanogaster* has long been a powerful model organism in a wide range of research areas, including neuroethology and chemical ecology, and scientists have begun to take advantage of the whole genus *Drosophila* for comparative studies in these research fields, much is still to learn about the ecology of these flies, their interaction within each and between different *Drosophila* species as well as their interaction at various trophic levels.

Countless *Drosophila* species are commercially available in fly stock centers, but little information can be found about non-*melanogaster Drosophila* species. What food sources do they use? Do their adults and larvae feed on similar resources and how do individuals within a species communicate with each other? What enemies and pathogens do the flies have to avoid? Do *Drosophila* flies from overlapping habitats interact with each other and if so, what communication channels do they use? What are factors that explain the immense species richness of the genus *Drosophila* and what principles underlie speciation events of *Drosophila* flies, which are populating similar ecological niches? Finally, what is the role of *Drosophila*-associated yeast communities in host localization and host acceptance of these flies? These open questions were the motivation for the projects of my thesis, and I aimed to expand our knowledge about the evolution of *Drosophila* host navigation as well as intra- and interspecies communication.

Within this thesis I explored an overlooked or forgotten mode of communication in *D. melanogaster*, being adult frass. From other insect species it was already known that frass can play a role in social behaviors, such as aggregation, however in *Drosophila* flies frass had not yet been analyzed for a possible involvement in intra- and interspecies communication. In manuscript I, I contributed to the characterization of pheromone contents found in *Drosophila* frass and to the elucidation of the behavioral relevance of adult frass in feeding and aggregation

behavior. Furthermore, my colleagues and I were able to show that *Drosophila* flies can discriminate between the frass of distantly related *Drosophila* species and we had a look at the role of chemosensory systems in the detection of frass-associated chemosignals.

After realizing that adult frass was mediating social behaviors in *Drosophila* flies and that frass resembled the sex-specific pheromone composition of the depositing fly, we wondered whether flies would be able to recognize frass to be originating from diseased flies and avoid areas with the risk of potential infection (manuscript II). We were curious to find out if there were chemosignals found in frass and emitted from flies that were indicative of an infected individual. Opposite to our initial expectations, flies did not avoid frass from infected conspecifics or infected conspecifics themselves but rather were seeking the presence of these sick individuals and preferred to feed on infected over healthy frass. In interacting with infected flies, the attracted healthy flies would often contract their pathogens. In manuscript II, we traced these astonishing observations back to the fact that some pathogens manipulate the pheromone communication channels of *D. melanogaster* by increasing the production of sex pheromones in order to enhance their own dispersal.

From literature research and our findings in manuscript II, we learned that insect frass contains a magnitude of viable microorganisms and that the frass can inoculate new substrates with these microbes. In manuscript V, I wanted to investigate whether *Drosophila* flies might be farming species-specific yeast communities by depositing their frass at food sources and breeding sites. I predicted that *Drosophila* flies would prefer yeast species from a shared host over yeast species from unfamiliar habitats and that this would be linked to an increased larval and adult performance on diets containing familiar yeast species. Furthermore, I was suspecting that associated yeast communities play an essential role in local adaptation events of *Drosophila* flies. However, I found that *Drosophila* flies do not always necessarily prefer yeasts from a known habitat but may also be attracted to yeast species from new environments. Additionally, I observed that yeast presence and diet affected adult and larvae performance differently, making larval constraints restricting factors for yeast acceptance. Interestingly, I found that female flies would prefer yeast species at oviposition sites that were not always beneficial for larval development and performance.

In our work with the different *Drosophila* species of our lab, my colleagues and I often observed that, especially between closely related species, eyes or antenna were enlarged in direct comparison and we continued to compare 62 *Drosophila* species to verify whether this trend would hold true on a large scale. In manuscript IV, I contributed to the identification of an

inverse resource allocation between the olfactory and visual system in the genus *Drosophila*. We wanted to investigate why there was an olfactory or visual bias and why the flies did not invest in both sensory modalities equally. Here, we found evidence that one explanation for the bias between these two sensory modalities is the developmental genetic constraint of a shared imaginal, eye-antennal disc. Moreover, we propose that this trade-off allows a relaxed competition between *Drosophila* species, which are sharing the same habitat, and enables them to coexist.

For ecological relevant behaviors of insects, the identification of odorants that are involved in the control of these behaviors is highly interesting and could be of use for the control of agricultural pest species and disease vectors. However, the deorphanization of these ligand-receptor interaction pairs is time-consuming and often impossible without the sophisticated genetic tools available in *D. melanogaster*. In manuscript III, I worked on establishing a previously described methodology (DREAM technique) for the high-throughput deorphanization of chemosensory receptors without the requirement of genetic tools and based only on the availability of transcriptomic data for chemosensory receptors in insects. We wanted to use this technique to identify the ligands involved in mediating behaviors of *Drosophila* flies observed in other projects of this thesis. However, I found that the DREAM technique had severe limitations and was not reliable enough to fulfill this purpose without further improvement. Consequently, there is yet no faster option besides the pairwise deorphanization of ligand-receptor interaction partners in “decoder systems” or heterologous expression systems.

Overview of manuscripts

Manuscript I

Adult frass provides a pheromone signature for *Drosophila* feeding and aggregation

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‡ These authors share senior authorship

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Manuscript I shows that adult *Drosophila* frass plays a role in intraspecies communication between adult flies and possibly even in the communication between different *Drosophila* species. Pheromones found in the insect feces resemble the pheromone profile of the corresponding adult flies, including sex-specific compounds, and act as intraspecies aggregation cues. In behavioral experiments with wild-type *D. melanogaster* and mutant flies we provide evidence that the attraction of flies to *Drosophila* fecal spots is in part mediated by the olfactory system. Furthermore, we found that frass functions as a feeding stimulus, which is controlled to a majority by chemosensory receptor other than odorant receptors.

Author contributions

Built on an idea conceived by: IWK, MK, BSH and SK (10 %)

Experimental design: IWK, MK, BSH and SK (10 %)

Behavioral assays: IWK, TR and SK (15 %)

Wrote the manuscript: IWK, MK, BSH and SK (15 %)

Manuscript II

Pathogenic bacteria enhance dispersal through alteration of *Drosophila* social communication

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Published online August 16th, 2017

Can *Drosophila* flies recognize conspecific flies infected with pathogens based on the odor profile of the diseased flies and do flies then avoid infected individuals? In manuscript II, the odor profile of *D. melanogaster* flies that were infected with selected pathogens was characterized. Contrary to our initial hypothesis, we found that healthy flies do not avoid sick conspecifics but are in fact attracted to these individuals and to the frass of these flies. We provide evidence that pathogens manipulate pheromone-based communication channels in *D. melanogaster* through immune and insulin signaling pathways which resulted in an immensely increased release of fatty-acid pheromones. Healthy flies would become infected by aggregating with sick conspecifics and by consumption of contaminated frass and ultimately contributed to the dispersal of the corresponding pathogen.

Author contributions

Built on an idea conceived by: IWK, MK, NB and BSH
Experimental design: IWK, MK, NB, BSH and SK (20 %)
Behavioral assays: IWK, MAK, SK (5 %)
Drosophila mutant and RNAi experiments: IWK, NB, AG, SK (60 %)
Chemical analyses and SSRs: IWK and SK (10 %)
Wrote the manuscript: IWK, EG, MK, NB, BSH and SK (20 %)

Manuscript III

Evaluation of the DREAM technique for a high-throughput deorphanization of chemosensory receptors in *Drosophila*

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In *D. melanogaster* a very extensive dataset is available for the interacting odorants of olfactory receptors, including information on the mode of interaction (excitatory or inhibitory). The deorphanization of olfactory receptors allowed scientist to decipher entire neural circuits involved in the control of ecologically relevant behavior in the vinegar fly. In other non-*melanogaster* insect models progress has been made in identifying ligand-receptor pairs but is not comparable to the data available in *D. melanogaster* and these studies were very time-consuming. In manuscript III, I evaluated the applicability of a technique for the deorphanization of olfactory receptors that is based on odorant-exposure induced changes in interacting chemosensory receptor mRNA-levels. This so-called DREAM-technique (**D**eorphanization of **r**eceptors based on **e**xpression **a**lterations of **m**RNA levels) does only require a sequenced and annotated genome and no other genetic tools in the model insect. However, in my experiments I found that without further improvements the DREAM technique can not be used for a reliable deorphanization of chemoreceptors in non-*melanogaster* insects yet and traditional methods like the “empty” neuron technique or heterologous expression systems are still needed in conjunction with the DREAM technique to identify ligand-chemoreceptor pairs.

Author contributions

Built on an idea conceived by: SK, BSH and MK
DREAM application: SK (100 %)
qPCR experiments: LC, EG, MK and SK (80 %)
Chemical analyses and SSRs: IWK, MAK and SK (60 %)
Data analyses: LC and SK (90 %)
Wrote the manuscript: BSH, MK and SK (100 %)

Manuscript IV

Inverse resource allocation between vision and olfaction across the genus *Drosophila*

Ian W. Keesey, Veit Grabe, Lydia Gruber, Sarah Koerte, George F. Obiero, Grant Bolton, Mohammed A. Khallaf, Grit Kunert, Sofia Lavista-Llanosm Dario Riccardo Valenzano, Jürgen Rybak, Bruce A. Barrett, Markus Knaden‡ & Bill S. Hansson‡

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The diverse genus *Drosophila* allows to compare neural architecture between closely and distantly related fly species. Especially when comparing *Drosophila* species found in an overlapping habitat it becomes apparent that one species has either enlarged eyes or antenna but not both. In manuscript IV, the visual and olfactory system, including the corresponding neural circuits, of 62 *Drosophila* species were studied and an inverse resource allocation between these two sensory modalities was identified. We found that across the selected *Drosophila* species independently but repeatedly either a visual or olfactory bias was selected for in evolutionary processes. The one-sided selection for one of these two sensory modalities we traced back at least in parts to a developmental genetic constraint through a shared developmental structure, the eye-antennal imaginal disc. Furthermore, we provide evidence that the trade-off between olfactory and visual system leads to relaxed competition between *Drosophila* species that populate overlapping habitats and allows the different species to co-exist.

Author contributions

Built on an idea conceived by: VG, BSH, MK and IWK
Experimental design: IWK, VG, BAB, DRV, BSH, MK and SK (10 %)
Imaginal disc preparation and staining: IWK and SK (60 %)
Confocal microscopy scans: VG and SK (70 %)
Wrote the manuscript: IWK, BSH, MK and SK (10 %)

Manuscript V

Variable dependency on associated yeast communities influences host range in *Drosophila* species

Sarah Koerte*, Ian W. Keesey*, Michael L.A.E Easson, Jonathan Gershenzon, Bill S. Hansson‡ and Markus Knaden‡

*These authors contributed equally
‡These authors share senior authorship

In preparation for submission to
Functional Ecology and bioRxiv

What is the extent of the influence that associated yeast communities have on host localization and preference in *Drosophila* flies? Given the particular important role yeasts have in the life of *Drosophila* species by providing nutrition and by detoxifying secondary metabolites found in host material, are yeasts possibly the major factor in local adaptation and host acceptance of *Drosophila* flies? In manuscript V, we examined the interaction between three different *Drosophila* species and yeasts found in their natural habitat. We analyzed the preference of adult *Drosophila* flies for yeasts from a common environment or unfamiliar habitat as well as larval performance in presence of these yeast species. Here, we found that larval development and survivorship act restricting on host acceptance, while adult flies have a broader acceptance and preference for different yeast species that not always correlate with benefits to larval performance. Our results let us predict that indeed associated yeast communities seem to have a significant influence on local adaptation processes in *Drosophila*. Furthermore, we observed that *Drosophila* flies and yeast species both actively modify their shared host substrate, which in turn leads to benefits for both interaction partners.

Author contributions

Built on an idea conceived by: IWK, BSH, MK and SK (50 %)
Experimental design: IWK, MK and SK (70 %)
Microbial work: SK (100 %)
Measurements of phenotypic traits and survivorship tables: IWK and SK (80 %)
Behavioral assays: IWK and SK (70 %)
Statistics and figure design: IWK and SK (80 %)
Sugar measurements: MLAEE, JG and SK (50 %)
Wrote the manuscript: IWK, MLAEE, BSH, MK and SK (90 %)

Manuscript I

Adult frass provides a pheromone signature for *Drosophila* feeding and aggregation

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Adult Frass Provides a Pheromone Signature for *Drosophila* Feeding and Aggregation

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Abstract Adult *Drosophila melanogaster* locate food resources by using distinct olfactory cues that often are associated with the fermentation of fruit. However, in addition to being an odorous food source and providing a possible site for oviposition, fermenting fruit also provides a physical substrate upon which flies can attract and court a potential mate. In this study, we demonstrate that *Drosophila* adults are able to recruit additional flies to a food source by covering the exposed surface area with fecal spots, and that this recruitment is mediated via olfactory receptors (Ors). Analyses of the deposited frass material demonstrates that frass contains several previously studied pheromone components, such as methyl laurate (ML), methyl myristate (MM), methyl palmitate (MP), and 11-*cis*-vaccenyl acetate (cVA), in addition to several cuticular hydrocarbons (CHCs) that are known to be behaviorally active. Moreover, this study also demonstrates that adult feeding is increased in the presence of frass, although it appears that Ors are less likely to mediate this phenomenon. In summary, the frass deposited by the fly onto the fruit provides both pheromone and CHC cues that lead to increased feeding and aggregation in *Drosophila*. This research is the first step in examining *Drosophila* frass as an important chemical signature that provides information about both the sex and the species of the fly that generated the fecal spots.

Keywords Olfactory · Gustatory · Chemical ecology · *Drosophila* · Frass · Feces · Pheromones · Insect behavior

Introduction

The pheromone system of *Drosophila* has been extensively studied, and previous research provides detailed information on the chemical identity of behaviorally relevant compounds that are generated by male and female flies (Auer and Benton 2016). This broad area of research also delves deeply into the neuronal mechanisms for both the detection and the decision-making of the fly in response to the presence of these pheromones, including the governance of complex multi-modal phenomena such as mate recognition and courtship. Recently, several important olfactory receptor ligands were uncovered, including methyl laurate (ML), methyl myristate (MM), and methyl palmitate (MP), which are some of the best known ligands for pheromone receptors Or47b and Or88a (Dweck et al. 2015). In addition, work by Lin et al. (2016) also suggests that myristic acid, palmitoleic acid, and palmitic acid could also act as important ligands as well. These two new studies provide olfactory ligands that fit nicely into the already established model for the neuronal activation of these circuits; however, the origin and production site of these fatty acid derived ligands has not yet been determined.

Feces collected from various insects has been previously studied for several attributes such as chemistry, shape, and color (Kuhns et al. 2012; Shao et al. 2012; Tumlinson et al. 1969; Wayland et al. 2014). In the case of the boll weevil, the examination of frass provided the behavioral relevance and eventually the identification of specific pheromone components that were

Hansson and Knaden shared seniority and last authorship

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otherwise difficult to isolate from adult odor collections or from the associated chemical analyses of courtship (Tumlinson et al. 1969). More recently the importance of fecal pheromones in aggregation behavior also was demonstrated in the German cockroach, *Blattella germanica*, where researchers showed that this insect emits highly attractive carboxylic acids in healthy adult feces (Wada-Katsumata et al. 2015). It also has been noted that frass can provide behaviorally relevant cues to parasitoids, such as wasps that target larvae of the diamondback moth (Reddy et al. 2002). Thus, frass across the order Insecta already has been established as a well-known substrate for behaviorally relevant odor cues.

Previous examination of *Drosophila melanogaster* frass has yielded information concerning the physical properties such as shape, size, and optical density of fecal droplets. These studies provided interesting differences in frass that depend on mating status and sex of each *D. melanogaster* fly that was tested (Wayland et al. 2014). In addition, researchers also have examined frass in regard to the quantification of fecal production, as well as the concentration of fecal material, in order to generate data on total excretion and water reabsorption (Linford et al. 2015; Urquhat-Cronish and Sokolowski 2014; Wayland et al. 2014). These studies showed the importance of frass in non-invasive studies of *Drosophila* metabolism and suggested that frass could be used as a metric for assessing general health, especially as it pertains to either nutrient or microbial stress. However, no previous studies have examined *Drosophila* frass in regard to its chemical properties or tested this digestive byproduct for any behavioral relevance. Here, we first document strong attraction of *Drosophila* adults towards frass, as well as demonstrate the presence of several CHCs and pheromones. We also provide a protocol for the collection of fecal material, as well as potential procedures for the examination of sex- and species-specific differences between fecal collections across this genus of flies.

Materials and Methods

Fly Stocks All wildtype fly lines, including *D. simulans* (14,021–0251.195), *D.erecta* (14,021–0224.01), *D. mauritiana* (14,021–0224.01), *D. virilis* (15,010–1051.00), *D. suzukii* (14,023–0311.01), *D. biarmipes* (14,023–0361.10), and *D. pseudoobscura* (14,021–0121.94) were obtained from the UCSD Drosophila Stock Center (www.stockcenter.ucsd.edu). All experiments with wild-type *D. melanogaster* were carried out with Canton-S (WTcs, stock #1), which were obtained from the Bloomington *Drosophila* Stock Center (www.flystocks.bio.indiana.edu). Stocks were maintained according to previous studies, and for all behavioral experiments we used 2–5 d-old flies of both sexes.

Stimuli and Chemical Analysis All of the synthetic odorants that were tested and confirmed were acquired from commercial sources (Sigma, www.sigmaaldrich.com and Bedoukian, www.bedoukian.com) and were of the highest purity available. Stimuli preparation and delivery for behavioral experiments followed previously established procedures, and any headspace collection of volatile odors was carried out according to standard procedures (Keesey et al. 2015). Blueberries were selectively used for fruit experiments since *D. melanogaster* could not penetrate or oviposit through the hardened surface of the berries. In addition, the small size of the blueberry allowed the use of intact, completely sealed fruit, which further prevented *D. melanogaster* from gaining any access beneath the surface or skin of the berry. GC-MS analyses were performed on all volatile and insect body wash collections as described previously (Dweck et al. 2015). The NIST mass-spectral library identifications were confirmed with chemical standards where available.

Frass Collections The sides of rearing vials that contained 100 adult flies were scraped after 1 wk. with a flat, rounded-end micro spatula. Each rearing vial could be separated into distinct zones of pupation as well as frass deposition (Supplemental 3), and thus no larvae or pupal cases were included in these frass collections. After scraping was completed, 150–200 mg of frass were added to either 1 ml of water, methanol, or hexane solvent. After 24 h, collected material was filtered through sterilized paper disks to remove large particles, and then these frass infused solvents were used in behavioral trials with the addition of mineral oil.

Behavioral Assays Trap assays were performed with 2–5 d-old flies as previously described (Keesey et al. 2015; Knaden et al. 2012), but with an additional 200 μl of light mineral oil (Sigma-Aldrich, 330,779–1 L) that was added to capture and drown flies upon contact with the treatment or control within the container. All behavioral traps consisted of 60 ml plastic containers (Rotilabo sterile screw cap, Carl Roth GmbH, EA77.1), with one trap used as a solvent control and the other containing the treatment (Fig. 3f). All trap experiments were repeated using water, methanol, or hexane as solvents for the frass collections. While all solvents generated significant attraction towards frass when compared to the control, water was the best solvent for behavior, but it could not be used for further GC-MS analyses, thus methanol was utilized instead for all additional experiments with *Drosophila* frass, as it had the closest polarity to water. Flywalk trials also were conducted as described previously (Steck et al. 2012; Thoma et al. 2014; Supplemental Fig. 5). In short, 15 flies were placed individually into parallel glass tubes. During the experiment, flies were exposed continuously to a humidified airflow of 20 cm/s (70 % relative humidity, 20 °C). Flies were presented repeatedly with air pulses from the head space of frass solved

in water, or to pulses of water alone, at an interstimulus interval of 90 s for 8 h. The 500 ms pulsed air stimuli were added to the continuous airstream and thus traveled through the glass tubes at a constant speed. The individual flies' movements before and after stimulus arrival were monitored under red-light conditions using advanced video-tracking software (Steck et al. 2012; Thoma et al. 2014).

Feeding Assays All tested flies were 2–5-d-old, included both males and females, and were starved beforehand for 18–20 h with constant access to water. Flies then were cooled for 5 min at -20 °C to assist in their transfer to the petri dish arena. Basic feeding solutions consisted of water with 5 % sucrose and 5 % baker's yeast, and experiments were conducted with or without colored dye markers (red and blue). Frass was added to treatment solutions, and included 150–200 mg of material per 1 ml of sugar water. After the 20 flies entered the arena, observations of fly feeding behavior were made at 2 min intervals for 30 min. Flies that fed on dye markers then were frozen at -20 °C, and images were taken for counting and additional analyses. The capillary feeder (CAFÉ) assays utilized glass micropipettes with liquid media that were filled by capillary action, and then inserted through pipette tips into the container holding the adult flies (modified from Ja et al. 2007). One capillary contained the control (5 % sucrose), while the other contained the treatment (5 % sucrose plus frass), and the volume consumed from each side was measured after a set duration of fly feeding.

Results

Fecal Deposits on Fruit *Drosophila* adults that had access to fruits, deposited fecal spots directly onto the fruit surface area using randomly spaced, often non-overlapping droplets (Fig. 1a, b). Surface washings of the fruit with and without deposited fecal spots, and solvent extractions of frass material alone revealed that several behaviorally important compounds were present in association with these fecal droplets, including the recently described pheromone components methyl laurate (ML), methyl myristate (MM), and methyl palmitate (MP), as well as their corresponding acids (lauric acid, myristic acid, palmitoleic acid, and palmitic acid). In a trap assay, when *Drosophila* adults were allowed to choose between the odor of fruit alone, and the odor of fruit that had been in contact with other *Drosophila*, the majority of flies selected the fruit with previous exposure to conspecifics (Fig. 1c). To ascertain the chemical profile of the frass alone, the fecal deposits were collected along the sides of the clear plastic rearing vials and placed into three solvents, which included water, methanol and hexane (Fig. 1d; Supplemental Fig. 3). Although water and methanol extracts were the most consistently attractive, all three fecal solvent extractions produced attraction in WT flies (i.e., wildtype flies of the Canton S strain) and w1118 control flies (i.e., white eye flies that carry the same genetic background as the other tested mutant fly lines). It also was noted that water completely dissolved the fecal material while hexane did not, suggesting that the frass contains predominantly polar compounds.

Differences between Male and Female Frass To test for any differences between male and female frass, newly emerged virgin flies were collected and placed into separate rearing vials based on sex. Subsequent fecal collection was completed as described previously (Supplemental Fig. 3), and this sex-specific frass material was added to methanol for further chemical analyses. By comparing adult body washes to these sex-specific fecal profiles by using GC-MS, it was demonstrated that frass contains information regarding the sex of the fly (Fig. 1d; Supplemental 6 A, B), and moreover, that the chemical signature of the frass matches most closely the *Drosophila* adult that produced it (Fig. 1d). More specifically, the GC-MS data showed that feces of both sexes contain the recently described pheromones ML, MM, and MP, while male feces contains a large amount of 11-*cis*-vaccenyl acetate (cVA) and 7-tricosene (7 T), and that female feces contains higher amounts of (7Z-11Z)-heptacosadiene (7,11-HD) and (7Z,11Z)-nonacosadiene (7,11-ND), which matches previously reported adult pheromone and adult CHC profile differences between the two sexes (Auer and Benton 2016; Dweck et al. 2015).

Attraction Towards Frass To test the behavioral relevance of frass, trap assays were used to compare the solvent control against the fecal collections. For water, methanol and hexane solvents, the frass was significantly more attractive than the evaporated solvent controls (Fig. 2a; WT, Canton S and w1118, white eyes; methanol data shown). Next, to examine the importance of odorant receptors, mutant flies lacking a functional olfactory co-receptor (Orco) were tested for their attraction towards frass. These mutant flies displayed a significantly reduced but still significant behavioral preference for frass, suggesting that at least part of the attraction towards frass was mediated by olfactory sensory neurons expressing odorant receptors, but also that other types of receptors were involved. To further address the importance of previously identified pheromone components in the attraction towards frass, multiple mutant fly lines were utilized that were only deficient in specific pheromone receptors, including Or47b (detecting ML), Or67d (detecting cVA), and Or88a (detecting ML, MM, and MP). All three of these mutant fly lines demonstrated reduced attraction towards frass, and all three were significantly different from the two control fly lines (WTcs and w1118); moreover, these mutant fly lines were not statistically different from the ORCO mutant line, further suggesting the important role of olfactory pheromone receptors in the

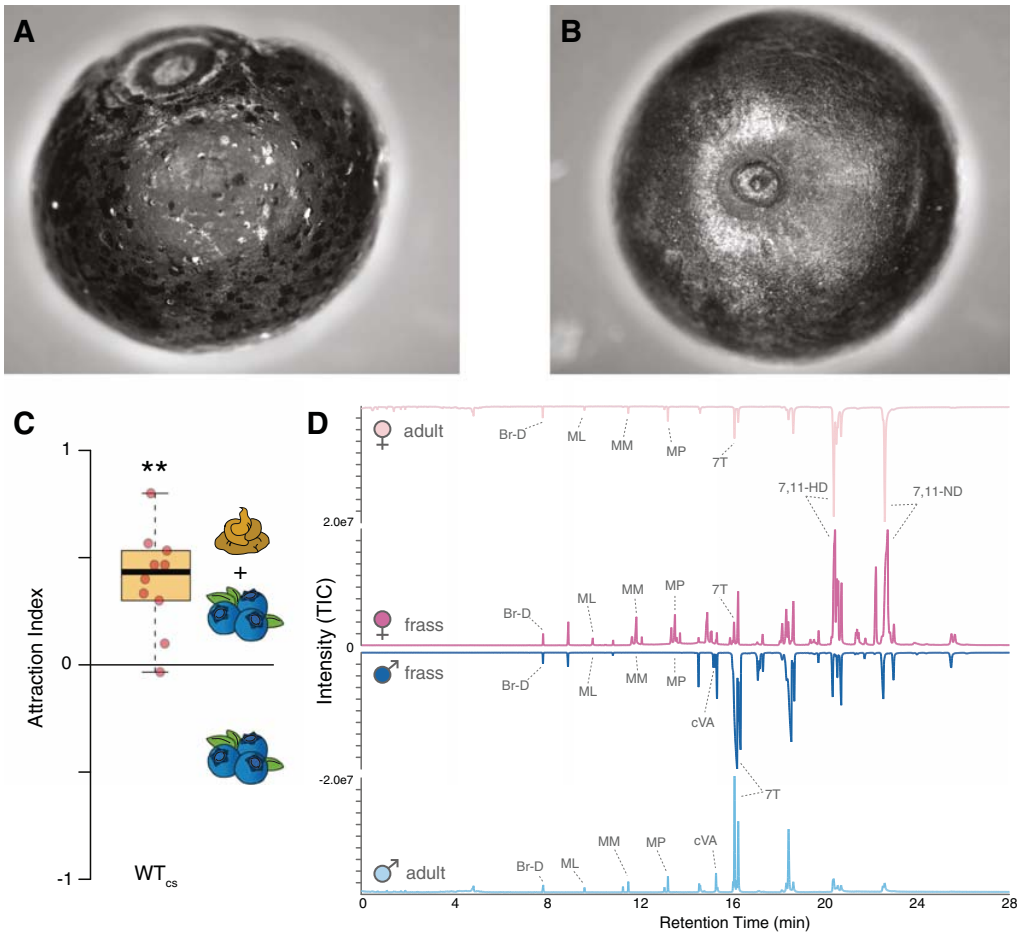

Fig. 1 **a** Image of a blueberry that was exposed to *Drosophila melanogaster* flies for 24 h, where the flies randomly distribute droplets of feces to cover the entire exposed surface area of the fruit. **b** Blueberry without exposure to flies. **c** Trap assays using fruit with and without previous fly contact (i.e., with and without fecal spots), where the fruit with *Drosophila* frass was preferred over the fruit alone. Attraction indices were calculated as (O-C)/T, where O is the number of flies observed in the treatment trap, C is the number of flies in the control trap, and T is the total number of flies used in the trial. **d** Adult male and female chemical profiles were established via short body washes in solvent, and the same procedures were used for GC-MS analyses of frass. Both male and female frass contained significant amounts of previously identified pheromone components, and each frass sample most closely resembles the sex of the adult that produced it. (Br-D, bromodecane [internal standard]; ML, methyl laurate; MM, methyl myristate; MP, methyl palmitate; 7 T, (Z)-7-tricosene; cVA, cis-vaccenyl acetate; 7,11-HD, (7Z, 11Z)-heptacosadiene; 7,11-ND, (7Z, 11Z)-nonacosadiene)

behavioral attraction of adult flies towards frass material (Fig. 2a). To test that all mutant lines (Or47b, Or67d, Or88a) were still behaviorally functional, additional trap assays were conducted with vinegar, which is a general attractant that does not rely on pheromone receptors for attraction (Fig. 2b). While Orco mutant flies were still deficient in their attraction towards vinegar, the three pheromone receptor mutants (Or47b, Or67d, Or88a) all displayed the same level of attraction to vinegar as both control lines, suggesting that these mutant flies exhibited normal behavior towards attractants that do not rely on pheromone detection. Therefore we conclude that the reduced response to frass by these three pheromone mutant lines is due to their loss of specific pheromone Ors. To further test the role of frass in aggregation and attraction, the Flywalk was utilized as well (Thoma et al. 2014; Supplemental Fig. 5D). Using this behavioral paradigm it was demonstrated that the odor of frass was indeed more attractive than the water control for both virgin and mated males ($P < 0.01$), as well as for both virgin and mated females ($P < 0.01$) (Fig. 2c), with flies reaching walking speeds towards frass odor that exceeded those previously published with some of the best *Drosophila* attractants such as ethyl acetate and ethyl butyrate (Thoma et al. 2014). There was no significant difference between mated and virgin males ($P > 0.05$), nor was there any significant difference between mated and virgin females ($P > 0.05$). However, mated males were significantly more attracted than

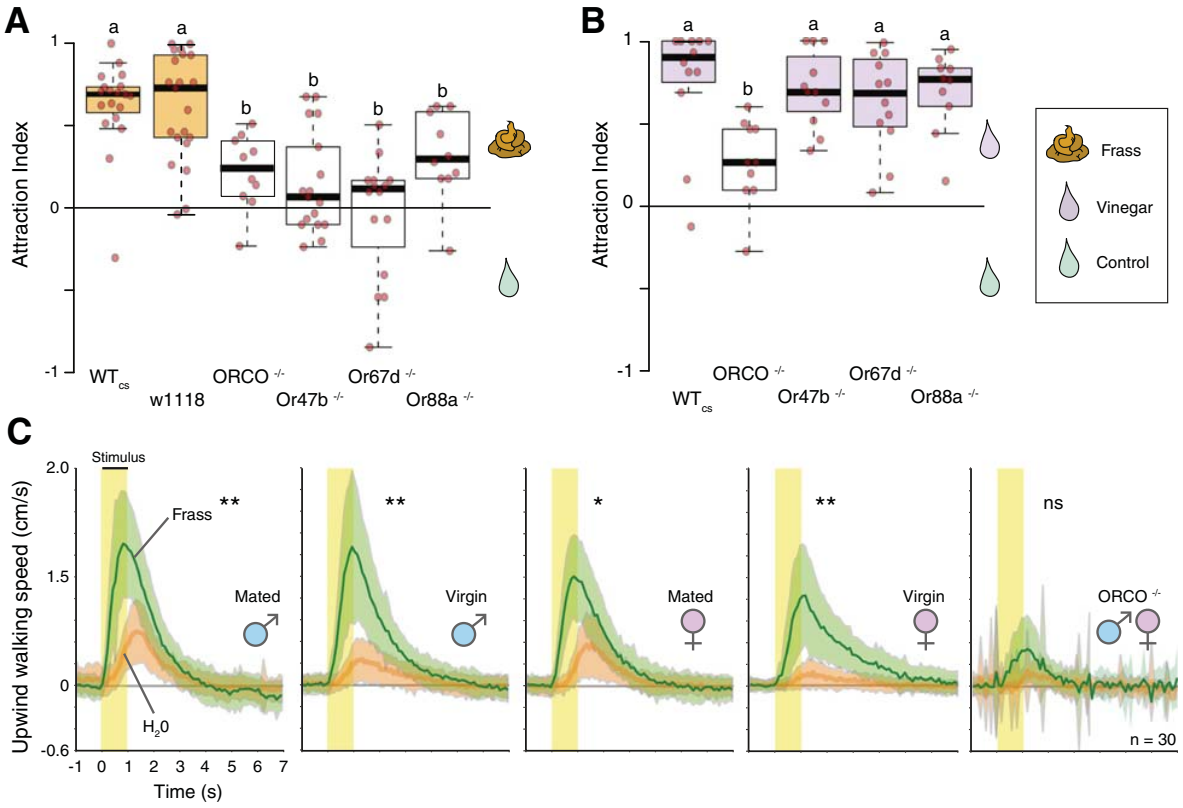

Fig. 2 Attraction indices from trap assays containing either **a** frass or **b** vinegar. Data includes flies deficient in either Orco or pheromone-specific Ors, and also shown are the corresponding responses of wild type (WTcs, *Drosophila melanogaster* Canton S) and other transgenic control flies (w1118, white eye). Attraction indices were calculated as (O-C)/T, where O is the number of flies observed in the treatment trap, C is the number of flies in the control trap, and T is the total number of flies used in the trial. **c** Responses to frass vs. the water control in the Flywalk, which includes behavioral response data from mated and virgin, as well as male and female adults. Both males and females are significantly attracted towards frass at all time intervals ($P < 0.01$). Males were significantly more attracted than females, regardless of mating status ($P < 0.01$). Tests with Orco flies did not produce any significant attraction towards frass

mated females towards frass ($P < 0.01$), and virgin males were more attracted than virgin females ($P < 0.01$). As was shown with the previously reported trap assays, the Orco mutant line again was significantly less attracted to frass than either WT males or females (Fig. 3c). In addition, behavioral trials were conducted with either virgin female or virgin male frass vs. a solvent control, and each trial produced statistically identical attraction, with both male and female frass being behaviorally attractive in trap assays (Supplemental 6C). In summary, the data show that frass is a strong attractant across several tested behavioral paradigms for *Drosophila* attraction and aggregation, and that both male and female frass is attractive.

The Effect of Frass on Feeding Behavior We conducted three sets of feeding trials, first using food dye to determine the preference of *D. melanogaster* for feeding on substrates infused with frass (Fig. 3a). Regardless of whether red or blue dye was used, flies preferred to feed from solutions containing frass (Fig. 3a; Supplemental Fig. 4). To confirm that flies were feeding in addition to aggregating at the solution, images of the colored dye were taken after the feeding trials were completed (Supplemental Fig. 4). In a second feeding trial, in this case without dye and during 30 min of direct observation with starved flies, the feeding solution containing frass again was significantly preferred over the control solution (Fig. 3b). In addition, we conducted a third set of feeding trials using CAFÉ assays, which compared 5 % sugar water (control) to the same solution with the addition of fecal material (Fig. 3c). In these trials, WT control flies fed more from the treatment containing frass; however, we also observed that ORCO flies preferred to feed from the capillary that contained frass (Fig. 3c), suggesting that while feeding is enhanced by fecal material, that this increase is perhaps not directly influenced by odorant receptors.

Examination of Frass from Different Species Having shown that frass from *D. melanogaster* contains a sex-specific

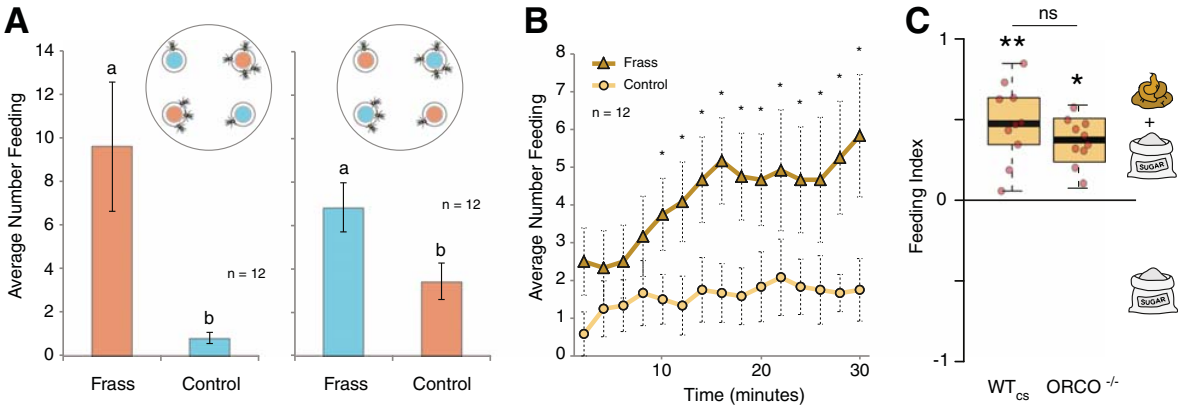

Fig. 3 Assays comparing feeding on sugar solution alone vs. sugar solution that also contain frass. Trials were conducted with both red and blue dye. Feeding behavior of each fly was documented based on the amount of red or blue dye in the abdomen after a 30 min exposure to the food (see also Supplementary Fig. 4). **b** Numbers of flies that were observed feeding at the frass-containing and the control food sources during 2 min intervals of direct observation for a total of 30 min. Flies contacted and fed upon frass-containing sugar solutions significantly more than the controls. **c** Feeding indices of wildtype and Orco mutant flies using a CAFÉ assay with 5 % sucrose solution either with or without frass. Significant differences are denoted by letters or asterisks (ANOVA followed by Tukey's test; $P < 0.05$). Error bars represent SEM

combination of CHCs and pheromones, our next interest was determining whether different *Drosophila* species contained notable differences in their frass. To test this we examined eight species of *Drosophila* flies, and compared the male and female adult body washes of each species to their corresponding fecal collections. We examined GC-MS data from 600 s onward, which included a total of 69 distinct compounds across the 8 fly species, and the data were normalized to the total amount of peak area in each total ion chromatogram (TIC). Data were log transformed to ensure normality, which was checked by the Shapiro-Wilk test. We used open-source XCMS implemented into the statistical program R to align the raw total ion traces (Smith et al. 2006), which we then used for the PCA, with PCA1 explaining 28 % and PCA2 explaining 16 % of the total variance. In the case of the *melanogaster* clade, all species that we examined produced remarkably similar chemical profiles, not just in the adult body washes, but also in their frass (Fig. 4a; Supplemental 1, 2). While the *melanogaster* relatives (*D. erecta, D. mauritiana, D. simulans*) all produced similar levels of ML, MM, and MP in their frass to that of *D. melanogaster*, there were small differences regarding both cVA content as well as other specific CHCs.

When our analyses was expanded to include more distant relatives of the family Drosophilidae, we were able to demonstrate species-specific differences in fecal deposits (Fig. 4a) in addition to the differences that were observed between adult males and adult females of each species (Fig. 4a; Supplemental 1, 2). Thus, frass appears to provide a chemical signature for each species, and provides species-specific markers to identify as well as leave behind information about the flies that were previously present. In general, the frass that was generated appeared to mirror the adult CHC and pheromone profile. While all examined species and their frass contain pheromone components such as ML, MM, and MP, many species and their corresponding frass appears to be deficient in cVA, further confirming that this compound and other male-produced compounds may be more indicative of species differences than other behaviorally relevant odors. For example, we were able only to identify a minuscule amount of cVA that was generated by *D. suzukii* or *D. virilis*, which had been suggested previously (Dekker et al. 2015), but other species such as *D. biarmipes* appeared to contain larger amounts of this pheromone component in adult male male body washes as well as in collected male frass.

Attraction of Frass from Different Species To test for behavioral differences between the frass collected from different *Drosophila* species, we again utilized the Flywalk. Here we tested the response of *D. melanogaster* adult males towards odor pulses from the frass collected from several different species. While *D. melanogaster* adults were equally attracted to 45 mg of frass from closely-related species (*D. melanogaster*, *D. mauritiana, D. simulans, and D. erecta*), they were significantly less attracted to the odor pulses from more distantly related fly species such as the fecal collections from *D. virilis* (Fig. 4b).

Discussion

In this study, we showed that *Drosophila* frass is behaviorally attractive, and that it provides chemical cues for aggregation in *Drosophila*. Our data also demonstrate that this attraction is predominantly due to the presence of pheromone compounds within the fecal droplets, specifically, the ligands that activate Or47b, Or88a, and Or67d (ML, MM, MP, and cVA,

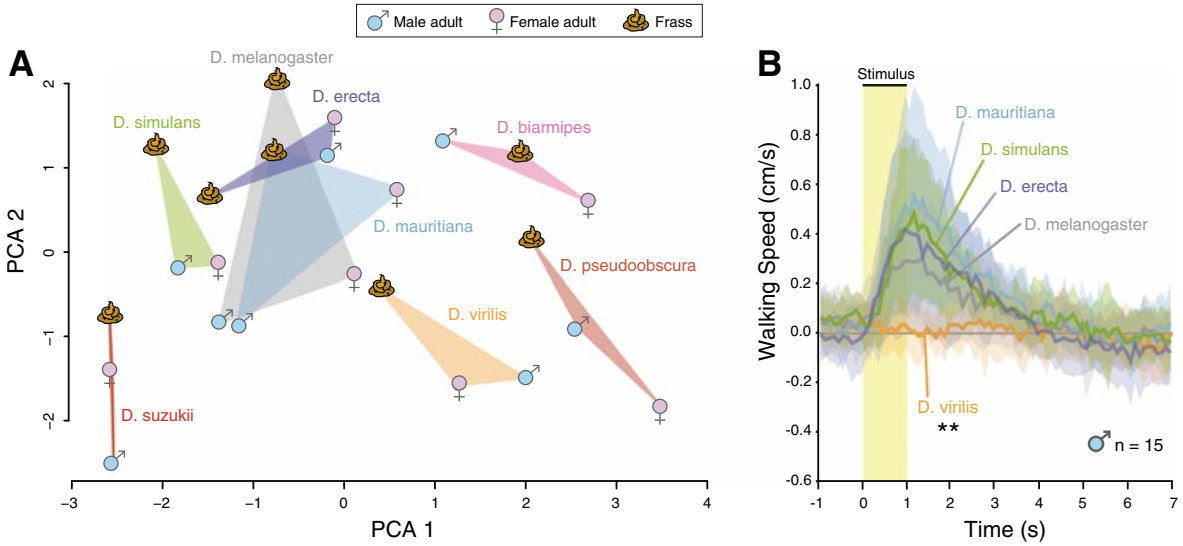

Fig. 4 **a** PCA (variance–covariance matrix) of normalized and quantified major peaks within the GC-MS profiles for 8 species of *Drosophila* flies, including adult male, adult female, and adult frass collections. Several species differ significantly from each other (one-way ANOSIM; Bray–Curtis distance; $R = 0.78$; $P < 0.001$), with the melanogaster clade clustering together without significant differences (*D. simulans*, *D. melanogaster*, *D. erecta,* and *D. mauritiana*; $P > 0.05$). The frass samples collected from *D. suzukii*, *D. biarmipes*, *D. pseudoobscura,* and *D. virilis* were all significantly different from each other, and from the *D. melanogaster* clade ($P < 0.05$). **b** Behavioral trials using *D. melanogaster* adults in the Flywalk that were given the choice between frass collected from several different *Drosophila* species. Flies showed no difference in attraction for closely-related species within the same clade, but were not attracted to the frass from more distant relatives such as *D. virilis*

respectively). Moreover, the importance of MM, ML, and MP and their role in aggregation and courtship already has been demonstrated (Dweck et al. 2015). Recent work by Lin et al. (2016) has suggested that several fatty acids (i.e., myristic acid, palmitoleic acid, and palmitic acid) also strongly activate Or47b, and our analyses has shown that these compounds are also all found in high abundance in the frass. It also has been previously established that 7-T and 9-T inhibit mating between species and contribute to aggregation (Fan et al. 2013), and our current study confirmed that these CHC compounds were found in high abundance within the fecal droplets as well. Numerous studies have shown that cVA has roles in aggregation, in mating deterrence, in male-male aggression, and that this compound is passed from males to females as an anti-aphrodisiac during mating (Auer and Benton 2016). Given all this information, our data suggest that frass also could achieve these same behavioral outcomes through the activation of the same neuronal circuits, due to the presence of the before mentioned chemistry (ML, MM, MP, and cVA, as well as their corresponding acids), and thus that frass is to a great extent a general aggregation signal that is composed of robust gustatory and olfactory cues. However, future work is necessary to examine the importance of frass in other *Drosophila* behaviors beyond attraction, such as mate recognition, courtship, male-male aggression, and oviposition.

In subsequent experiments we also generated evidence that the presence of frass increases feeding behavior. Given that this increase in feeding appears to not be mediated by olfactory receptors, as demonstrated by the use of Orco mutants (Fig. 3c), future studies will target the possible role of gustatory (Gr), as well as ionotropic (Ir) and PPK receptors. Since 7-T is detected by gustatory neurons expressing Gr32a (Wang et al. 2011), this receptor might be a candidate in mediating the increased feeding. It also is worth noting that while the contents of *Drosophila* frass have not yet been analyzed specifically for microorganisms, it is likely that this fecal material contains both yeast and bacteria in addition to the described pheromone components. It recently has been shown that specific Grs and Irs are responsible for the increased feeding and mating receptivity afforded by the presence of yeast (Gorter et al. 2016). Therefore, the increased feeding on solutions containing frass is most likely at least partially linked to these same taste receptors, although more work is needed to test this hypothesis, and to further examine the presence of potential microorganisms in *Drosophila* frass.

The frass collected from each sex and each species of fly appears to match the odor profile of the adult that produced it (Fig. 4a). This similarity between adult and frass chemistry is not surprising given that the alimentary canal consists of a cuticular material similar to that which forms the outer epi- and exocuticle. It is thus reasonable that frass content positively correlates to the exterior pheromone and CHC profile of the adult fly (Fig. 4a). The data reported here support the current literature that *Drosophila* can discriminate between species-

related chemical differences among adults, but our data go one step further and also support the notion that *Drosophila* can discriminate between the frass or fecal deposits left behind by distantly related species at a food source (Fig. 4a, b). While it has not been shown previously that frass from *Drosophila* contains behaviorally relevant chemical stimuli, this has been demonstrated repeatedly for other insect orders, including Coleoptera and Blattodea (Symonds and Gitau-Clarke 2016; Wada-Katsumata et al. 2015). In research with other insects, frass has also been shown to provide a substrate that can be used to identify novel pheromone components from several agricultural and economic pests, such as the boll weevil and the many destructive species of pine beetle (Bellas et al. 1969; Hall et al. 2002; Symonds and Gitau-Clarke 2016; Tumlinson et al. 1969).

While previous work has identified the presence of pheromones as part of the fecal signature in these insects, it has not been shown that *Drosophila* frass also contains sex-specific and species-specific markers. Therefore, our current investigation of frass chemistry provides several avenues for future application, such as the identification of novel pheromone components from additional insect species, especially in cases where the induction of calling behaviors or where the release of pheromones is difficult to stimulate in the laboratory. Examination of *Drosophila* frass also provides novel approaches to the studies of economically important species within this genus, such as *D. suzukii*, where the loss of cVA might have been replaced by another behaviorally relevant male-generated pheromone component that could be more easily identified from fecal studies. It also is likely that certain chemical components of *D. suzukii* frass could provide species-specific attraction and aggregation cues that in turn may benefit current IPM strategies.

While frass from otherwise healthy adults is behaviorally attractive, it is not yet determined whether diet or other external influences can modify the chemical signature of feces. It would be interesting to address whether the chemistry of frass changes in regard to food resources, such as in *Drosophila* reared upon different food substrates (e.g., food deficient in amino acids or sugars) or by rearing the flies upon the same fruit at different stages of decay. Moreover, it would be interesting to ascertain whether the frass itself changes after exposure to or ingestion of different healthy or pathogenic microbes that have been incorporated into the diet, such as different yeast or bacteria strains. It is possible that frass can provide a signature or snapshot of individual insect health, or perhaps insect population health, especially as it relates to mid- and hindgut metabolism (Kuhns et al. 2012; Newell and Douglas 2014). Additional work is also required to ascertain whether the frass itself affects the substrate that it is deposited onto, namely the fruit or food resource utilized by each *Drosophila* species. While it is clear that frass contains pheromone components, and that frass is involved in the attraction or recruitment of other *Drosophila* to a food source, it still is open for debate whether the frass itself is an active substance that plays any role in breaking down food resources, such as through the utilization of gut microbes, including yeasts or bacteria, or through the use of enzymatic and digestive substances that are potentially deposited along with or within the fecal spots. In the present study, we showed that flies deposit frass in a rather random, but often non-overlapping distribution across the entire exposed surface area of potential food substrates (Fig. 1a). Therefore frass may aid in the decay or fermentation of nutrient resources through the recruitment or deposition of microorganisms. It has already been demonstrated that ingested microbes such as yeast spores can survive the digestive tract of *Drosophila* (Coluccio et al. 2008; Erkosar and Leulier 2014). Thus, it is likely that different species of *Drosophila* produce frass that contains different strains of microorganisms that could in turn be distributed through fecal spots to assist or accelerate the breakdown of species-specific food resources (e.g., cacti, mushrooms, or fruit) (Wong et al. 2013, 2014). This scenario would potentially benefit both the fly and the microorganisms that they in turn vector to each new host plant.

It is clear from the present study that frass contains relevant chemical information for each *Drosophila* species and that fecal deposits appear to play a role in both feeding and aggregation. However, it is not yet clear whether frass plays any additional roles in aspects of courtship, or whether frass affects oviposition decisions, such as site selection. It has been demonstrated that some species of flies such as Tephritids leave oviposition marks that ward off other females (Arrendondo and Diaz-Fleischer 2006). Thus, it is possible that some species of *Drosophila* might utilize similar fecal deposits to mark fruit after oviposition, especially in cases when eggs are either laid singly or where they are laid in tight clusters. A recent study has examined sperm plugs containing cVA that are deposited by mated *Drosophila* females that enhance oviposition (Dumenil et al. 2016). Potentially, we could have overlooked sperm plugs when collecting mated female feces for examination. However, as feces from virgin females and virgin males were both significantly attractive to adult flies (Supplementary Fig. 6C), we can conclude that additional cues besides cVA are involved in fly attraction towards frass. Nevertheless, specific studies examining the effects of frass on oviposition also are still required, and future studies will need to separate the contributions of cVA from the other pheromone cues found in frass. Currently, one of the more economically important *Drosophila* species, *D. suzukii*, would be a prime candidate for a more extensive study of frass in regard to attraction, avoidance or oviposition, as any attractive or deterrent chemistry from frass may aid in IPM strategies towards the control of this pest insect. While we were able to show the presence of cVA in *D. suzukii* for both adult extractions and within male frass, albeit greatly reduced compared to

D. melanogaster, it is possible that another male-produced compound is still passed from males to females during copulation in this species, and thus frass material may provide an avenue for the identification of such novel chemistry. In summary, as growing evidence continues to support an intimate association between *Drosophila* and distinct microorganisms, it is clear from our study that additional research should be conducted to examine *Drosophila* frass and its role in the chemical ecology of this genus of fly.

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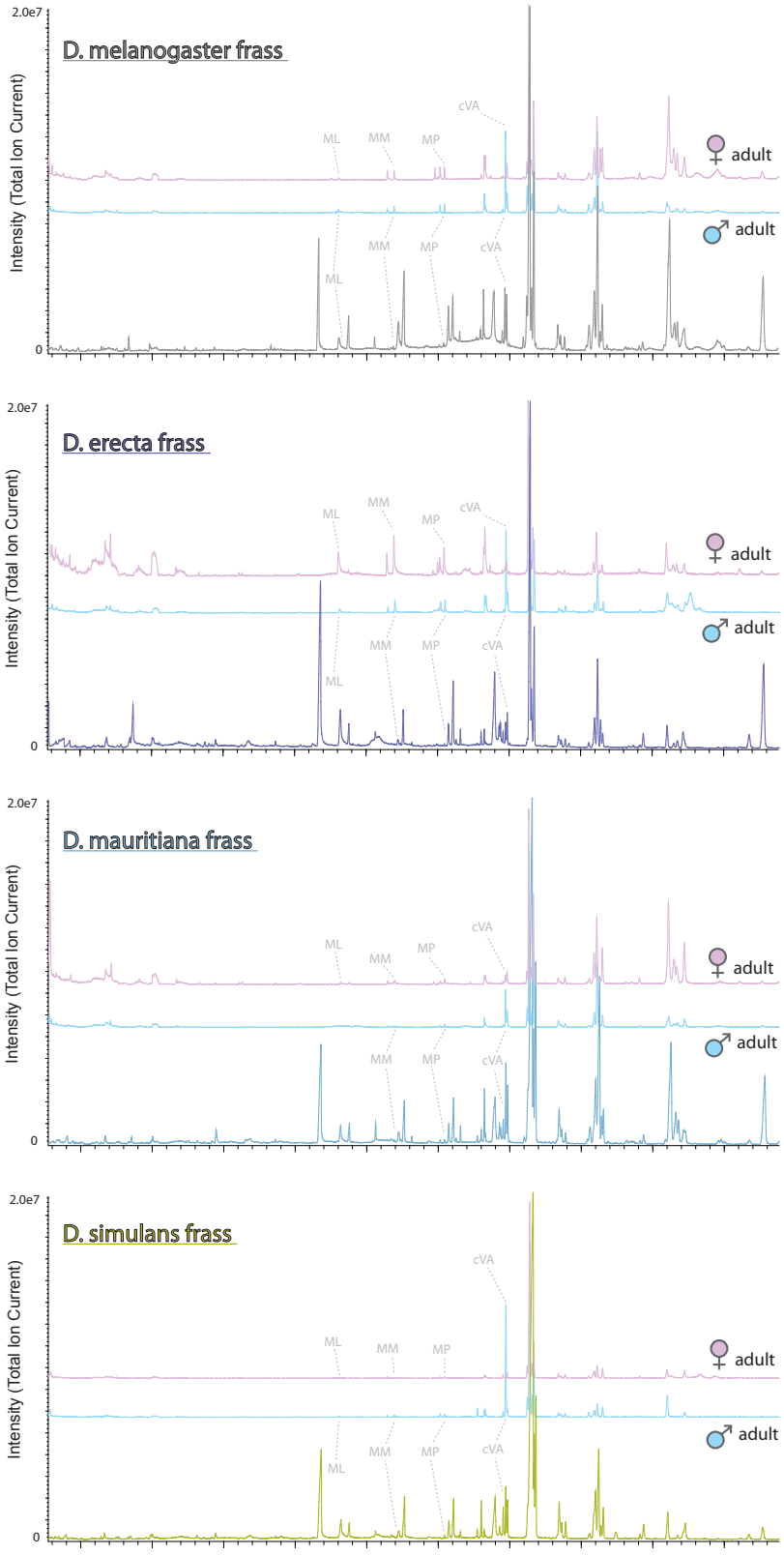
Supplemental Figure 1.



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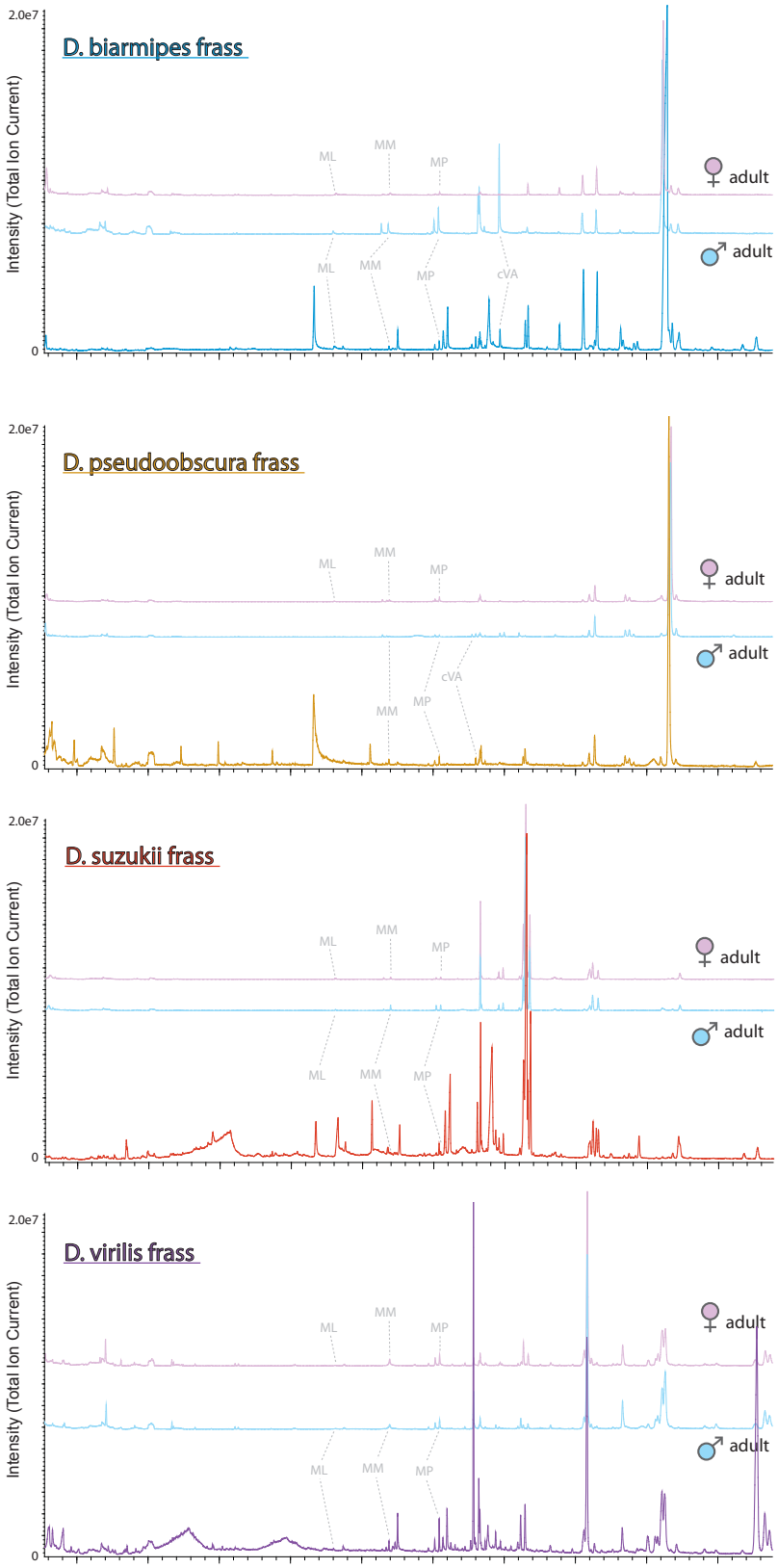
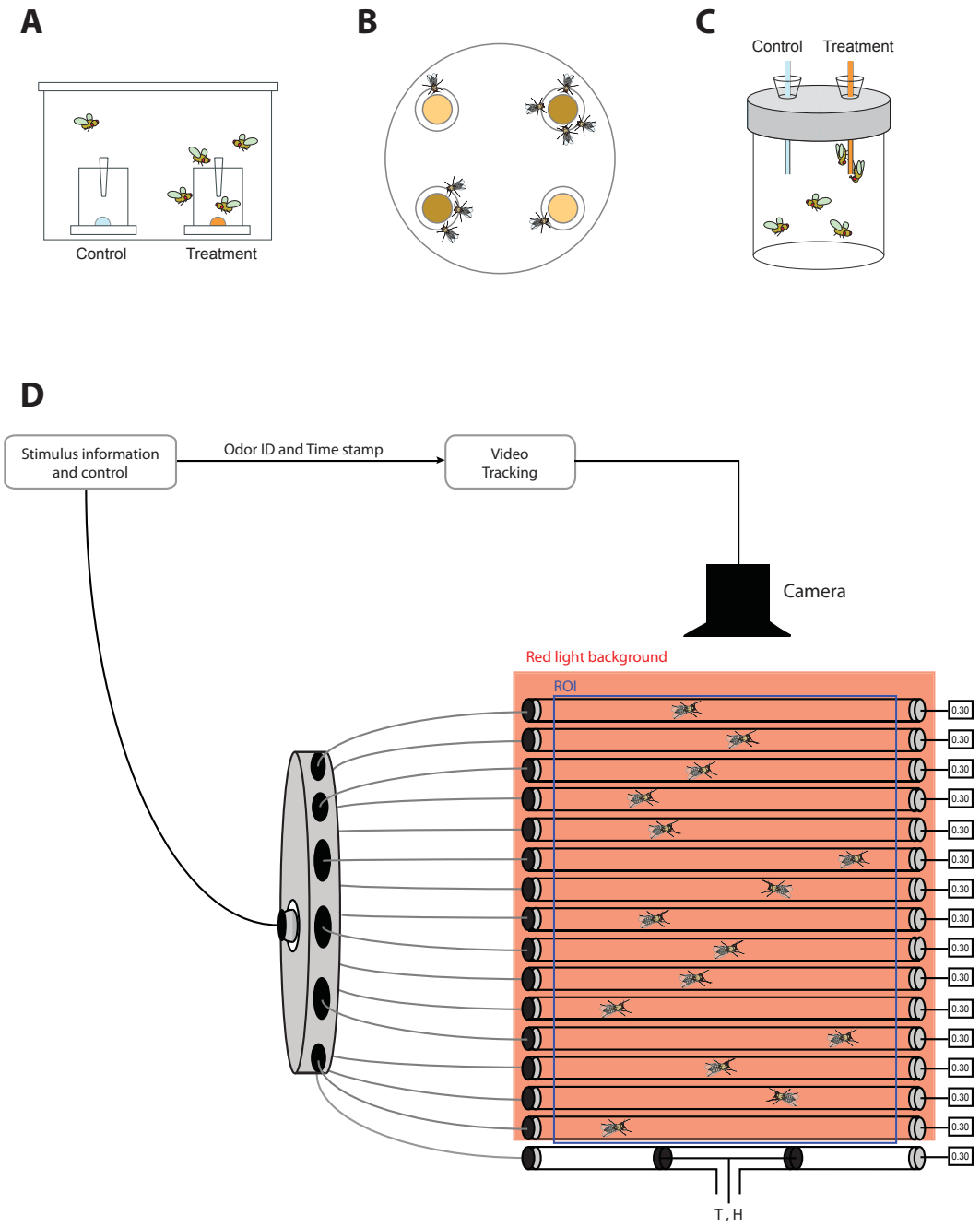


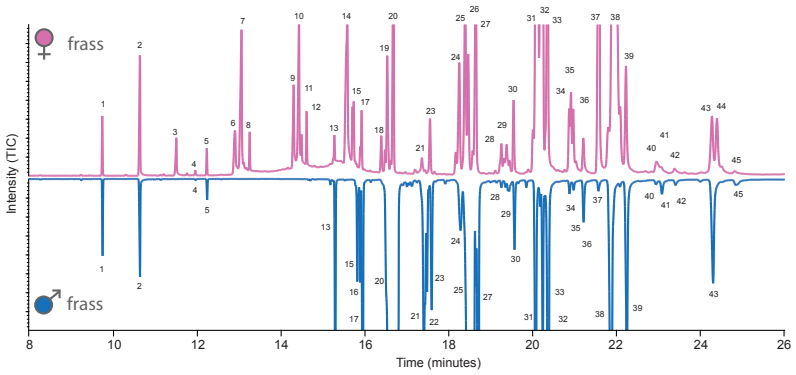
Supplemental Figure 3.

Supplemental Figure 5.



Supplemental Figure 6.

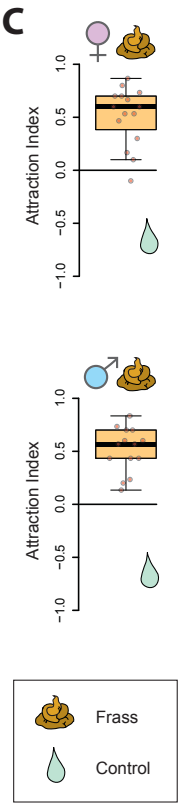
A



B

Peak No.	Kovats Index	Compound Name	Male	Female
1	1329	Bromodecane (internal standard)	+	+
2	1365	Methylparaben	+	+
3	1498	Lauric Acid	-	+
4	1527	Ethyl Laurate	+	+
5	1545	N-Pentyl-decanamide	+	+
6	1723	Myristoleic Acid	-	+
7	1724	Myristic Acid	-	+
8	1726	Ethyl Myristate	-	+
9	1925	Methyl Palmitoleate (Palmitoleic Acid)	-	+
10	1926	Palmitic Acid	-	+
11	1928	E-9-Hexadecenoate	-	+
12	1931	Ethyl Palmitate	-	+
13	2165	Heneicosane	+	+
14	2188	Linoleic Acid	-	+
15	2190	Ethyl Oleate	+	+
16	2194	(Z)-11-Vaccenyl Acetate (cVA)	+	-
17	2195	Heneicosane	+	+
18	2272	7(Z),11(Z)-Heptacosadiene	-	+
19	2274	(Z)-9-Tricosene	-	+
20	2275	Heneicosane	+	+
21	2279	Cyclotetracosane	+	+
22	2281	(Z)-7-Tricosene	+	-
23	2284	Tetracosane	+	+
24	2481	(Z)-14-Tricosenyl Formate	+	+
25	2487	(Z)-12-Pentacosene	+	+
26	2493	(Z)-12-Pentacosene	-	+
27	2498	Octacosane	+	+
28	2541	11-Hexacosyne	+	+
29	2543	9-Hexacosene	+	+
30	2547	Hexacosane	+	+
31	2665	7(Z),11(Z)-Heptacosadiene	+	+
32	2669	1-Heptacosanol	+	+
33	2772	Heptacosane	+	+
34	2791	Unknown	+	+
35	2799	Unknown	+	+
36	2804	Tetratetracontane	+	+
37	2821	Squalene	+	+
38	2832	7(Z),11(Z)-Nonacosadiene	+	+
39	2837	Hexatriacontane	+	+
40	2901	1,30-Triacontanediol	+	+
41	2902	Unknown	+	+
42	2912	Unknown	+	+
43	2934	Hentriacontane	+	+
44	2936	Tetracontane-1,40-diol	+	+
45	2951	Tetratriacontane	+	+

C



Manuscript II

Pathogenic bacteria enhance dispersal through alteration of *Drosophila* social communication

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ARTICLE

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Pathogenic bacteria enhance dispersal through alteration of *Drosophila* social communication

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Pathogens and parasites can manipulate their hosts to optimize their own fitness. For instance, bacterial pathogens have been shown to affect their host plants' volatile and non-volatile metabolites, which results in increased attraction of insect vectors to the plant, and, hence, to increased pathogen dispersal. Behavioral manipulation by parasites has also been shown for mice, snails and zebrafish as well as for insects. Here we show that infection by pathogenic bacteria alters the social communication system of *Drosophila melanogaster*. More specifically, infected flies and their frass emit dramatically increased amounts of fly odors, including the aggregation pheromones methyl laurate, methyl myristate, and methyl palmitate, attracting healthy flies, which in turn become infected and further enhance pathogen dispersal. Thus, olfactory cues for attraction and aggregation are vulnerable to pathogenic manipulation, and we show that the alteration of social pheromones can be beneficial to the microbe while detrimental to the insect host.

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Certain pathogens, parasites, and viruses possess the ability to manipulate their host, including examples in vertebrates[1–3], invertebrates[4–7] as well as in plants[8–10]. For instance, bacterial pathogens use several strategies to hijack plant host physiology to their own benefit while often to the detriment of their host plant, including alterations of volatile and non-volatile host metabolites and immune-related proteins. This change in volatile release after host–plant infection can also lead to an enhanced attraction of insect vectors to the infected plant, and can therefore lead to increased pathogen dispersal by insect vectors[8, 9, 11]. It has also been shown that a pathogenic bacterium, *Pseudomonas syringae*, is able to alter the physiology of its plant host, *Arabidopsis*, in order to enhance bacterial growth and to help the bacterium avoid defensive responses within the host by altering hormone signaling as well as host susceptibility[10]. In the case of the parasitic flatworm, *Leucochloridium paradoxum*, it infects land snails and the parasite congregates in the eye stalks, where it pulsates different colors and patterns in a display to make the snail more noticeable to bird predators, which are the primary host of this flatworm[6]. Similarly, rats and mice lose their fear of cats upon infection with the parasite *Toxoplasma gondii* and subsequently become more likely to be killed and consumed by a cat, again the primary host of the parasite[12]. This fearless or suicidal behavior in mice has subsequently been shown to be due to an impairment of the olfactory receptors that usually trigger aversion to feline urine, and that this olfactory impairment is caused directly via the infection by the *Toxoplasma* parasite[1, 13]. Other systems for the study of pathogenic alteration of behavior include several examples within insect hosts, such as ants[5, 14], crickets[4], and leafhoppers[11]. Thus, in both plants and animals, microorganisms have been shown to alter the behavior and physiology of a host in order to provide a benefit to the pathogen. However, especially in animal systems, the specific mechanisms for host alteration by pathogens and parasites are not well understood.

Drosophila has been a powerful model to study bacterial infection as it pertains to immune, hormonal, and metabolic responses mounted by the insect host[15–18]. Several strains of pathogenic bacteria, including *Erwinia carotovora* sp. *carotovora* 15 (Ecc15), *Serratia marescens Db11*, and *Pseudomonas entomophila*, have been well characterized in regard to the immune responses elicited by *Drosophila melanogaster* following infection[15, 19–22], and thus these bacteria have arisen as a part of a model system for the study of insect immunity. Although *D. melanogaster* does not possess an adaptive immune system, their innate immune defense has proven to be efficient against most bacteria that are ingested or injected into the fly, perhaps an evolutionary result of living and breeding in high-density, and within microbe-rich food substrates such as rotten and decaying fruit[17, 23]. The *Erwinia* bacterium we use in this study is a member of the Gram-negative Enterobacteriaceae family, several species of which are phytopathogenic, often causing soft rots on fleshy fruits, vegetables, and ornamental crops[24, 25]. This bacterial pathogen has developed sustained plant-to-plant infection cycles, usually via insect vectors such as Hymenopterans and Dipterans[24, 25]. This bacterium also overlaps with the preferred host range of *D. melanogaster*, an insect that has a strong preference for decaying or rotting substrates. Moreover, *D. melanogaster* has been previously shown to be a natural vector for *Erwinia carotovora carotovora* and *E. carotovora atroseptica*, both of which cause potato blackleg disease. *Drosophila* are found naturally carrying these strains of bacteria in potato fields, and, at least under greenhouse conditions, it has been established that the vinegar fly is able to vector blackleg disease between potato plants[26, 27]. Similarly, *P. entomophila* was originally described from field-collected *Drosophila*[20]; thus, fly infection by this bacteria is also thought to be naturally occurring. In addition, the strain of *S. marcescens* we use is highly pathogenic to *D. melanogaster*, and one which has been described from these insects[21]; moreover, bacterial community surveys in natural field conditions have demonstrated that Enterobacteriaceae, including the genus *Serratia*, are found naturally in the wild and within naturally occurring populations of *Drosophila*[28]. Therefore, we can hypothesize that the activation of the *Drosophila* immune response by certain strains of bacteria indicates that these bacteria have some natural interaction with the fly, and that these bacteria can perhaps exploit *Drosophila* as a potential intermediate host as well as a vector between fruits, vegetables, or other plants. We also tested other naturally occurring, non-pathogenic bacteria, such as *Acetobacter pomorum* and *Lactobacillus plantarum*, neither of which have been shown to induce substantial immune responses, and are the dominant bacteria strains within the midgut and hindgut of *D. melanogaster* adults and larvae[29].

In previous studies, the ability of *Drosophila* to detect and avoid potentially harmful microorganisms in their environment has been elucidated, such as for pathogenic fungi and bacteria[30–33]. These studies have outlined two olfactory (geosmin, Or56a; phenol, Or46a) and a single gustatory avoidance pathway (lipopolysaccharides, Gr66a) that allow the fly to avoid certain pathogens when presented alone. Conversely, and counter to our initial hypotheses, here we show for the first time that flies become strongly attracted toward conspecifics that have become infected by specific pathogenic bacteria. Moreover, we demonstrate that the increased attraction toward infected flies is due to amplified aggregation pheromone emission by infected flies and their feces, and that this increase is mediated by pathogen-induced alterations to immune, hormonal, and metabolic response cascades following infection.

Results

Behavioral response toward sites of infection. We first tested the behavioral response of *Drosophila* in attraction, feeding, and oviposition toward a natural pathogen, the bacterium *P. entomophila* (Fig. 1a–c and Supplementary Fig. 1A–G). While flies did not respond to the odor of *P. entomophila* in an attraction assay (Fig. 1a, b), we could confirm previous findings from Soldano et al. that flies avoid feeding and ovipositing on food sources containing Gram-negative bacterial pathogens (Supplementary Fig. 1A, C). However, we were also interested in whether *Drosophila* can identify and avoid infected conspecifics as these individuals could be another potential source of infection within the population. Therefore, we repeated the behavioral assays but did not present the pathogen alone, but instead tested infected flies or their feces (Fig. 1c). While both oral and systemic infection generated similar results, for consistency, and to ensure similar levels of infection, all flies were systemically infected along the pleural suture line along the mesothorax with growth media containing bacteria or mock infected with growth media only as a control (Fig. 1j). Contrary to our initial expectation, *Drosophila* strongly preferred the odor of infected flies (or feces of infected flies) over that of healthy flies (or their feces) in the attraction assays (Fig. 1c). We repeated these tests of attraction using an alternative behavioral paradigm, and again we were able to observe that flies were significantly more attracted toward the odors from infected flies when compared to those of healthy controls (Supplementary Fig. 1E). In tests with Orco mutant flies, this preference for infected conspecifics and their feces was lost; thus, we concluded that this attraction was due to olfactory cues (Fig. 1c). We gained similar results when we tested the body

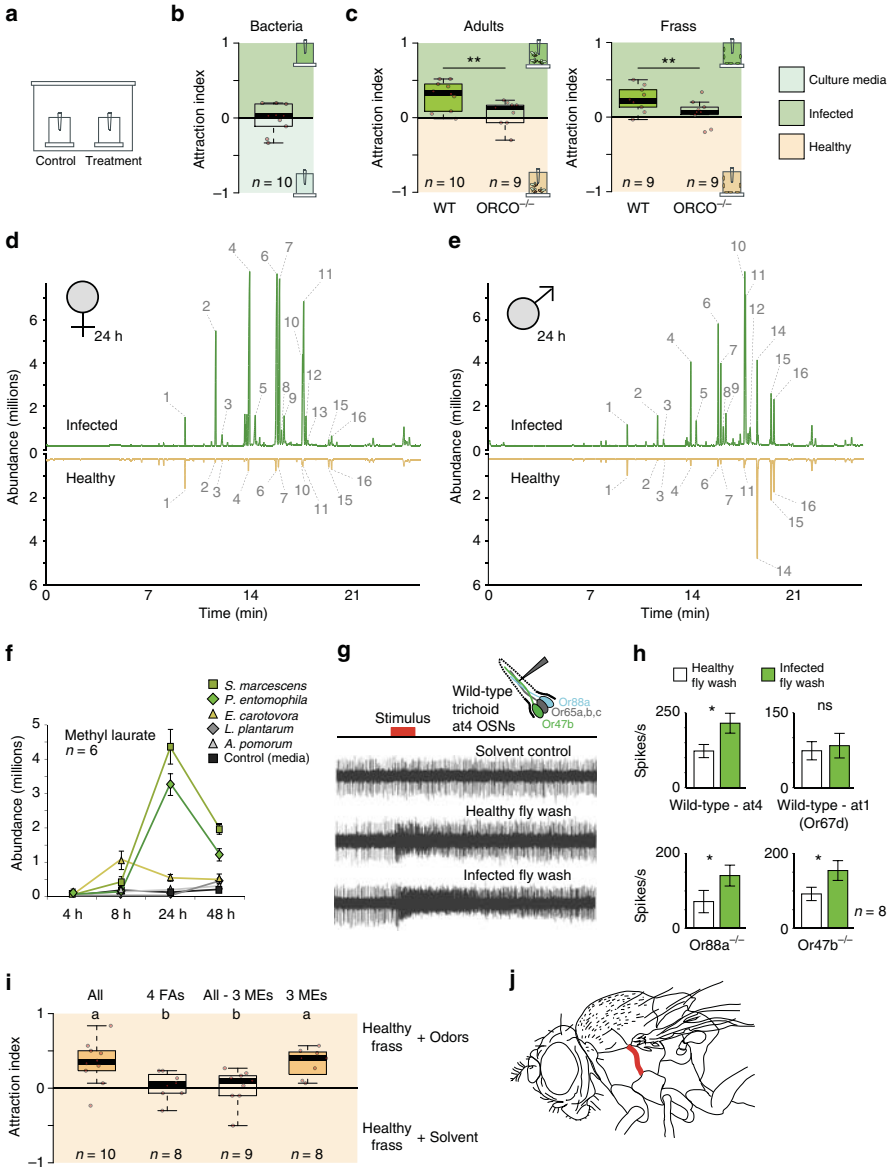

Fig. 1 Effects of infection on *Drosophila* attraction and odor profile. **a** Experimental design of attraction assays. **b** Attraction index of adult *Drosophila* toward the olfactory cues from *Pseudomonas* bacteria or from growth media control. **c** Attraction indices or naive wild type or Orco mutant flies given the choice between other adults with and without *Pseudomonas* infection or between frass of flies with or without infection. Attraction index: ((no. of flies in treatment trap) − (no. of flies in control trap)) / total no. of flies. **d**, **e** GC-MS profile of female **d** and male **e** *Drosophila* adults either infected with *Pseudomonas entomophila* bacteria or mock-infected with growth media (healthy control). Numbers from GC-MS refer to FID peaks: (1) bromodecane (internal standard); (2) methyl laurate; (3) lauric acid; (3) methyl myristate; (5) myristic acid; (6) methyl palmitoleate; (7) methyl palmitate; (8) palmitoleic acid; (9) palmitic acid; (10) methyl linoleate; (11) methyl oleate; (12) methyl stearate; (13) oleic acid; (14) Z-11-cis-vaccenyl actetate (cVA); (15) 7-Z-tricosene; (16) heneicosane. **f** Amount of methyl laurate produced over time, from 4 to 48 h after infection with several strains of bacteria (for time courses of other compounds see Supplementary Fig. 2D). **g** Example of SSR responses of healthy *Drosophila* antennal trichoid (at4) neurons to body washes of infected or healthy *Drosophila*. Stimulus: 0.5 s. **h** Quantified SSR responses toward healthy or infected fly body washes, including recordings from wild-type at4 and at1 neurons, as well as from fly mutants for Or47b and Or88a pheromone OSNs. **i** Attraction indices of adult *Drosophila* toward healthy frass perfumed with treatment odors or solvent control. Frass was perfumed either with all odors (All) that were increased after infection or with a subset. 4FAs: mixture of fatty acids increased after infection that were reported to act as pheromones (lauric acid, myristic acid, palmitoleic acid, and palmitic acid, Lin et al.[36]); 3 MEs: methyl esters (ML, MM, and MP) increased after infection and reported to act as pheromones (Dweck et al.[35]). More details in Supplementary Fig. 2A. **j** Schematic of septic or systemic infection location for both bacterial and mock infection. *Filled boxes* denote significance from zero

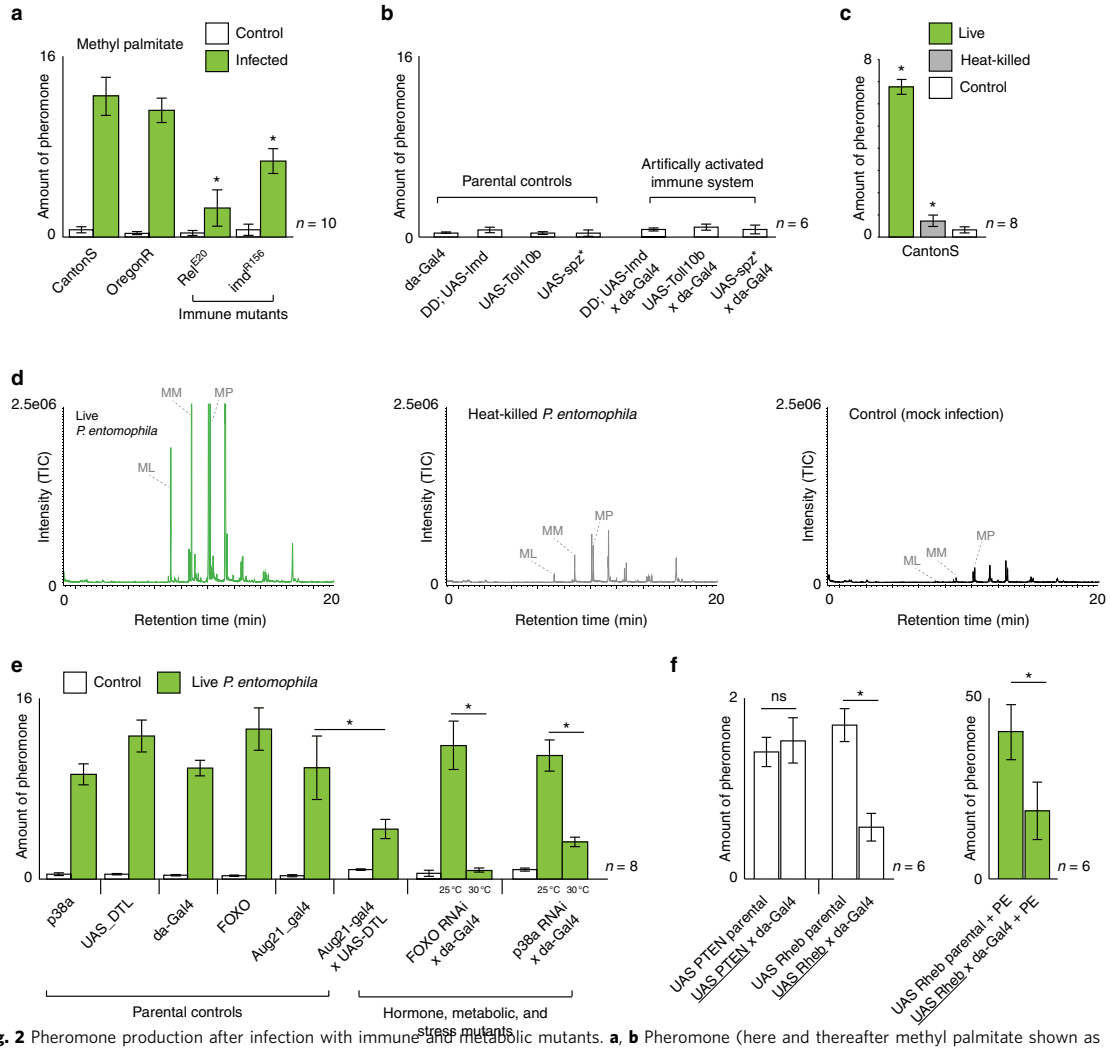

Fig. 2 Pheromone production after infection with immune and metabolic mutants. **a**, **b** Pheromone (here and thereafter methyl palmitate shown as example) production of CantonS or OregonR wild-type flies, and flies deficient in the Imd immune response pathway (RelE20 and IMD^{156}) with *Pseudomonas entomophila* infection (*green*) and without (*white*; **a**), or of flies, where the IMD (Imd flies) or the Toll (Toll flies and spz* flies) response pathway were artificially activated (**b**). **c** Pheromone production after infection with live (*green*) or heat-killed (*gray*) bacteria. **d** GC-MS profiles of live bacteria, heat-killed bacteria, and mock-infected control flies. **e** Pheromone production of flies with either decreased juvenile hormone (Aug21-gal4 x UAS-DTI flies), insulin metabolism (FOXO flies), or stress responses (p38a flies) and their parental control lines. RNAi lines tested at the non-active 25 °C served as further controls. **f** Pheromone production of flies with or without artificial activation of Rheb (an inhibitor of the FOXO pathway) with infection (*green*) or without (*white*)

washes of infected flies or their feces in feeding and oviposition assays (Supplementary Fig. 1b, d). In both cases the flies avoided the bacterium when it was presented alone; however, the flies did not avoid sites of infection and instead preferred infected individuals and material generated by the infected flies (Fig. 1c and Supplementary Fig. 1B, D). Interestingly, the oviposition-related attraction of infected flies was time-sensitive and peaked between 16 and 24 h after infection, while the infected flies were still alive, but dropped after their death (i.e., 48 h after infection, Supplementary Fig. 1D). Thus, it seems that the repulsive behavioral effect of pathogenic bacteria when presented alone can be overcome by the attractive odors generated by infected flies and their feces.

Insect-derived odor emission following infection. In order to examine any odor-derived differences between healthy and infected *Drosophila*, we performed extensive gas chromatography mass spectrometry (GC-MS) analyses of the volatile and non-volatile chemical cues associated with *Drosophila* following systemic infection with pathogenic and non-pathogenic strains of bacteria. While infection with the non-pathogenic *L. plantarum* or *A. pomorum*, or with the facultative endosymbiont *Wolbachia*[34], did not generate any significant difference in the odor profile of the fly (Supplementary Fig. 2C), infection with three strains of natural bacterial pathogens, including *S. marcescens*, *E. carotovora carotovora* (*Pectobacterium carotovora*), and *P. entomophila*, each induced large changes in the chemical profile of

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both sexes of infected flies (as compared to mock-infected controls) (Fig. 1d–f; Supplementary Fig. 2C, D). This increase in fly odors after infection included in total 12 compounds (Supplementary Fig. 2A, B). Interestingly, after infection, many of the 12 compounds for which emission increased significantly have been previously identified as *Drosophila* pheromones that modulate courtship and aggregation[35, 36], including methyl laurate (ML), methyl myristate (MM), methyl palmitate (MP), and palmitoleic acid (PA). However, notably, *cis*-vaccenyl acetate (cVA), the male-specific pheromone produced by the male accessory glands, was not affected by any tested bacterial infection (Fig. 1e).

To further examine the increase in pheromone production after infection, we next quantified the amount released over time (Fig. 1f and Supplementary Fig. 2D). After systemic infection with *E. carotovora*, pheromone production peaked around 8 h post infection and returned thereafter to normal levels comparable to those found in control or mock-infected flies. Infection with this strain of bacteria is non-lethal, as the vinegar flies are able to mount a successful immune response to thwart the infection[15]. However, in the case of both *P. entomophila* and *S. marcescens*, pheromone production continued to increase dramatically until the death of the fly, usually around 24 h post infection, with pheromone levels in dead flies then decreasing rapidly toward control levels (Fig. 1f and Supplementary Fig. 2D).

Olfactory response to odors from healthy and infected flies. After having established that pheromone production was highly upregulated in live flies following infection with specific pathogenic bacteria, we proceeded to investigate differences in olfactory responses to this increase in the odor profile of the fly. Using single sensillum recordings (SSRs), we could demonstrate that healthy *D. melanogaster* flies show an increased olfactory response to body washes from infected flies when compared to that of healthy flies (Fig. 1g). We could also show that this response is elicited from olfactory sensory neurons (OSNs) present in the at4 but not in the at1 sensillum (Fig. 1h), and, more specifically, elicited by ligands of the olfactory receptors Or47b and Or88a (i.e., ML, MM, and MP[35]; Fig. 1g, h and Supplementary Fig. 3A–D). Notably, despite PA and several other fatty acids being increased for flies infected with *P. entomophila*, these suggested Or47b ligands[36] did not activate any of the tested OSNs within the at4 sensillum (Supplementary Fig. 3A–D), nor did any of these fatty acids generate a preference in *Drosophila* behavior (Fig. 1i). Together, these results match our previous GC-MS analyses that showed increases after infection for fatty-acid-derived ligands (detected in at4 trichoid sensillae) but not in cVA (detected in at1 sensillum). Moreover, we could show that three fatty-acid methyl esters (ML, MM, and MP) were necessary and sufficient to account for the increased behavioral attraction and electrophysiological response following infection of *Drosophila* with *P. entomophila* bacteria (Fig. 1I and Supplementary Fig. 3A–D).

Pheromone changes with immune and metabolic cascades. Since the pheromone production over time closely matches the published timeline of the immune response to infection for *E. carotovora* and *P. entomophila*[15, 20], we next focused on repeating the GC-MS experiments with immune, hormonal, and metabolic *D. melanogaster* mutants in order to identify any involvement of these pathways in the increased production of pheromones following infection by these bacterial pathogens. Healthy flies with a reduced immune induction (e.g., Rel^{E20} and Imd^{R156} flies)[37] produced normal amounts of pheromones relative to Canton S, but following infection, the same flies produced significantly less pheromones compared to infected wild type (WT) and other control flies (Fig. 2a). This suggests that a functional Imd pathway is necessary for the increase in pheromone production following infection. Moreover, we found that impairment of either the Imd or the Toll immune response pathway resulted in a lower maximum amount of pheromone production after infection with *P. entomophila* (Supplementary Fig. 4A). However, when we tested flies that had either their Imd or Toll immune response pathways artificially activated in the absence of bacteria, we could not induce this increase in pheromones (Fig. 2b), suggesting that the immune system is necessary but not sufficient to account for the change in pheromone production following *P. entomophila* infection. Infection with dead, but intact bacteria can still result in an immune response, including the increase of antimicrobial peptides (AMPs) such as diptericin and drosomycin[15, 19, 38]. We therefore tested whether an infection with heat-killed *P. entomophila* was sufficient to yield AMPs (Supplementary Fig. 4B). Although heat-killed bacteria resulted in the production of two different AMPs and a smaller but significant increase in pheromone production, infection with heat-killed bacteria never reached the degree of pheromone production observed in flies infected with living bacteria (Fig. 2c, d). This suggests that ongoing bacterial growth and subsequent damage by the pathogen are required to induce the large increases in pheromone production.

In addition to the immune response, the fly hormonal system as well as metabolic and stress responses are also affected by bacterial infection, especially in relation to the utilization of the fat body, inflammation, and the mobilization of energy to combat infection, which primarily results in a decrease in adult fat body content[16, 39]. With this in mind, we next focused on the potential origin of these fatty-acid pheromone odors (ML, MM, and MP) by using transgenic fly lines that were deficient in their ability to synthesize juvenile hormone (Aug21-Gal4 > UAS-DTI), flies that were deficient in the transcription factor FOXO (a transcription factor related to insulin signaling and induced in response to stress, pathogenic damage, and starvation), as well as flies deficient in the stress response pathway regulator p38a. Alterations of some of these pathways can be lethal during fly development; thus, in these cases we took advantage of RNA interference (RNAi) inducibility to pass fly development and still test the function of otherwise lethal genes in adult *Drosophila*. The reduction of juvenile hormone through the UAS-Gal4 system, or FOXO via RNAi, caused a significant decrease in pheromone production after infection when compared to the parental lines or to the genetically identical RNAi controls that had not been activated by temperature (Fig. 2e). As p38 directly phosphorylates FOXO[40], these two mutants have already been shown to be linked in their function. Hence, by repeating the experiments with p38a RNAi flies, we were able to confirm the involvement of FOXO in the increased pheromone production after infection. As inhibiting the FOXO transcription factor (either directly through FOXO RNAi or indirectly through p38a RNAi) revealed the most drastic reduction in pheromone production after infection (Fig. 2e), we next activated the *Rheb* gene (part of the target of rapamycin signaling pathway, and which is an inhibitor of the product of FOXO)[17, 41, 42]. As we expected, activating Rheb (and by that indirectly decreasing the product of FOXO), we again found a significant decrease in pheromones, even in the absence of any infection, as well as a strong decrease in infected flies relative to the infected controls (Fig. 2f), thus reconfirming the involvement of FOXO in the pathogen-induced pheromone production. However, when we tested flies in which we increased the expression of PTEN, a factor that is only distantly related to the FOXO transcription factor within the insulin receptor pathway (InR), we did not find any effect on pheromone production (Fig. 2f). Hence, it appears that

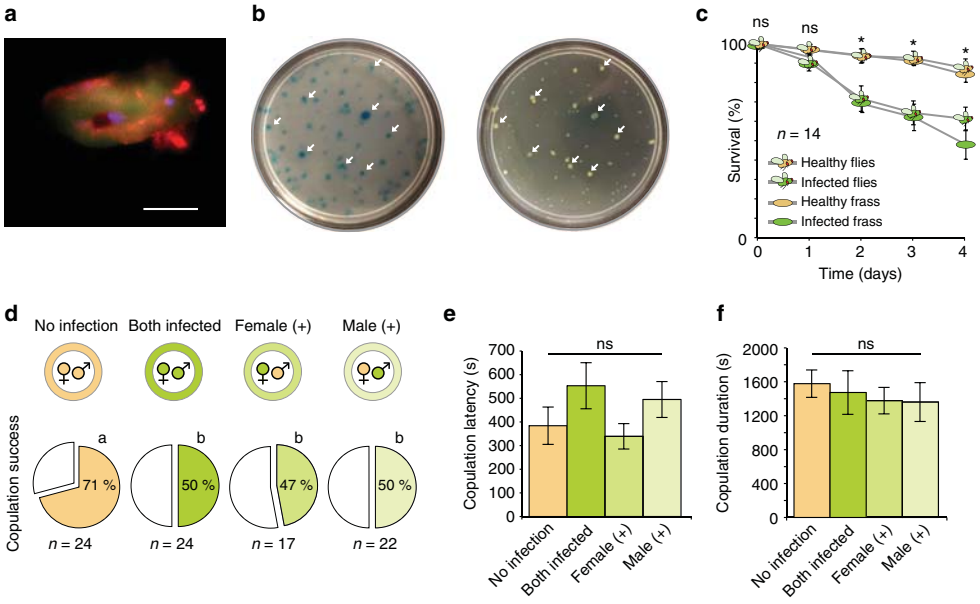

Fig. 3 Ecological impact of preference for infected flies and frass. **a** Frass droplet from a fly that was fed green fluorescent protein (GFP)-labeled bacteria, showing live bacteria (*green*) and dead bacteria (*red*) present in the feces. *Scale bar* depicts 10 µm. **b** Flies fed with a solution containing bacteria and blue dye were allowed to deposit frass onto an agar plate (*left*), and bacterial colony growth was assessed from the fecal deposits (*right*), demonstrating that bacteria can survive the digestive tract and be transferred via feces to new locations. **c** Survival over time of cohorts of flies reared in containers that held either healthy or infected fly adults or their frass (see Supplementary Fig. 5). **d**–**f** Copulation success (**d**), latency (**e**), and duration (**f**) of single pairs of flies following all combinations of infection

several but not all genes related to this metabolic cascade may be influenced by *P. entomophila* infection. When testing oviposition with body washes of flies that were either deficient in their immune response (Relish) or metabolic response (FOXO), we observed a reduced preference for infected flies (Supplementary Fig. 1H). As both immune (Relish) and insulin response pathway mutants (FOXO) resulted in reduced pheromone production after infection, and a corresponding decrease in behavioral preference following infection (compared to WT-infected flies), we conclude that both of these general signaling cascades (immunity and insulin metabolism) are required for *P. entomophila* to alter the fatty-acid pheromone production of *D. melanogaster* adults.

Ecological effects of pheromone changes after infection. We next examined the potential costs and benefits of increased pheromone production for both the insect and the bacteria. Our analyses of fecal material using green fluorescent protein-labeled bacteria revealed that ingested bacteria can survive the digestive tract (Fig. 3a), which was similar to studies that confirmed that yeast can survive ingestion by *Drosophila* and be passed through fecal deposits[43]. In addition, by using blue dye in feeding solutions, we could show that frass deposited on agar plates by infected flies (Fig. 3b, *left*) resulted in new bacterial colonies at the same locations (Fig. 3b, *right*), providing further support that pathogenic bacteria can survive passage through the *Drosophila* digestive system and be transferred to new locations via the oral–fecal route. To study the transmission of bacteria through infected frass material, we introduced healthy flies to containers that held infected conspecifics or to containers in which flies were removed but their frass remained (Supplementary Fig. 5A–D). In both cases we could observe an acute increase in the mortality of the introduced flies when in the presence of infected conspecifics or infected frass (Fig. 3c). Thus, the *P. entomophila* pathogen survives the *Drosophila* gut and potentially profits from increased contact and dispersal through increased attraction of healthy flies toward infected flies or their frass, material that has been previously shown to be attractive for *Drosophila* adults[44]. Moreover, this attraction to infected flies has a high cost for the arriving flies, as they run an increased risk of becoming infected and dying. Conversely, the same attraction could be beneficial for the infected flies, as it could increase their chances of finding a mate and reproducing before death. Thus, we conducted mating assays where all combinations of healthy and infected flies were tested (Fig. 3d). When we presented a healthy and an infected female to two males, preliminary experiments indicated increased orientation and courtship displays toward the infected female; however, in single-pair mating experiments, infection always resulted in lower copulation success, irrespective of whether the female, the male, or both flies were infected (Fig. 3d–f). We furthermore found that infected females were less likely to accept any male for copulation, as they were less likely to extend their abdomen or separate their wings during the male courtship song. We thus found no benefit to the infected fly with regard to successful copulation, even given the robust increase in pheromone production, perhaps due to other confounding behavioral alterations after infection, such as lethargy, cell damage, or another byproduct of pathogen growth. Although the increased pheromone emission did not result in the hypothesized higher mating success of infected flies, we cannot exclude that without this increase infected flies would even have less copulation. It is also possible that different degrees of infection may result in increased courtship success, although additional work is

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required to address this hypothesis. Therefore, our current data suggest that the increased pheromone emission of infected flies mainly results in attracting more *Drosophila* to sites of infection, promoting contact and dispersal benefits for the bacterial pathogens, while not providing any direct courtship benefit to the infected fly.

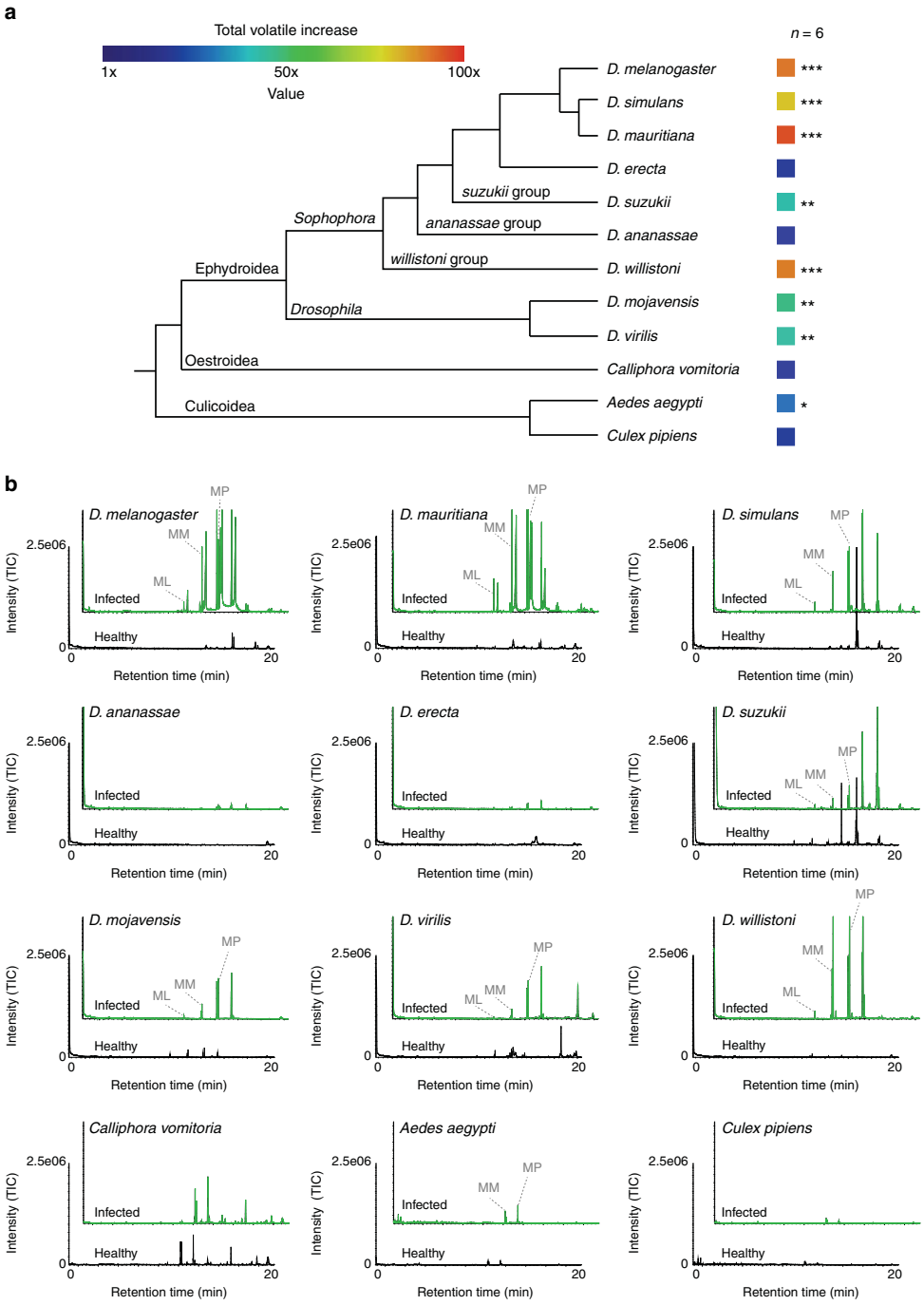

Fig. 4 Infection of *Drosophila* and Diptera species with *P. entomophila* bacteria. **a** Phylogenetical relationship and color-coded relative increase of odor emissions after infection for all tested species. **b** Example of GC-MS traces for each species before and after infection with *P. entomophila* bacteria with those methyl esters identified that were behaviorally relevant in *D. melanogaster*

Pathogenic infection with other Dipterans. To augment our screening of *D. melanogaster*, we also tested *P. entomophila* infection with eight other Drosophilids and three other Dipterans, including the blue bottle fly, *Calliphora vomitoria*, as well as two mosquitoes, *Aedes aegypti* and *Culex pipiens* (Fig. 4a, b). While infections were lethal for all tested insect species, we found significantly increased emissions of potential fatty-acid pheromones in seven out of nine *Drosophila* species as well as in *A. aegypti* (but no increase in *Calliphora* nor in *Culex*), suggesting that the manipulation of the insect's volatile emission by the pathogen *P. entomophila* is a more general phenomenon.

Discussion

We conclude that specific pathogenic bacteria can overcome the avoidance mechanisms of *D. melanogaster* flies[32] by taking advantage of, or hijacking, a chemosensory circuit related to social communication[35, 36, 45]. This preference and attraction toward infected individuals is due to pheromone signals and cannot be avoided by conspecific flies, as these chemical cues are vital for both aggregation and courtship in *D. melanogaster*. While previous research has documented viral or parasitic alterations in pheromone production for *Helicoverpa zea* and *Apis mellifera*[7, 46], the ecological impact as well as physiological and neural mechanisms for this shift have not been previously addressed. Here we assert that both the immune response pathway and the InR pathway are necessary for this increase in fatty-acid-derived pheromone release after infection by *P. entomophila* bacteria. This linkage between the *Drosophila* immune system, insulin signaling, and the fat body has been previously noted[39], as has the connection between the Rheb, FOXO, and damage response pathways[41]. However, our data show for the first time a pheromone change in *Drosophila* after infection, and show a mechanistic connection between the pathogen and the alteration of the pheromone communication system of the insect host. In addition, our data also reveal for the first time the associated ecological ramifications for both the pathogen and for the insect following infection.

This increase in pheromone production after infection might just be a byproduct of the bacterial growth and the associated damage to the insect[16, 39]; however, this insect–microbe interaction results in a potential evolutionary advantage for the bacterium by increasing its chances for contact and dispersal through enhancing several aggregation pheromones of a potential host and insect vector. Previously, it has been suggested that humans infected with malaria are more attractive to the *Anopheles* vector and that mosquito vectors carrying Malaria are also more likely to take additional bloodmeals, both of which result in increased dispersal benefits for the *Plasmodium* protozoan[47–50]. Our data may be pertinent for not only the study of insect-transmitted human diseases, but also studies related to insect-vectored plant pathogens, such as those similar to the *Drosophila*-transmitted plant pathogen *E. carotovora* used in this study. In addition, the application of species-specific pathogens may be useful as a tool in identifying novel pheromones from other infected host organisms, such as *D. suzukii* or *A. aegypti*.

Therefore, in summary, it is our assertion that specific pathogenic bacteria alter the lipid metabolism of *Drosophila* during infection through both immune and insulin signaling pathways, which results in increased fatty-acid pheromone release by the adult insect after infection. Moreover, this increase in pheromone release attracts more adult flies to sites of infection and contributes to the potential uptake and dispersal of the pathogenic bacteria toward new fruit, vegetable, or insect hosts. Thus, our data begin to generate a better understanding of how microorganisms can alter insect host physiology as well as alter insect host behavior, and, moreover, our findings might provide future tools or novel strategies to combat insect-transmitted human and plant diseases.

Methods

Drosophila stocks. WT fly lines included the *D. melanogaster* Canton-S and OregonR strains. Flies were raised on standard diet at 25 °C with a 12 h light/dark cycle. Transgenic lines related to immunity, hormones, and insulin signaling were obtained where possible from the Bloomington Drosophila Stock Center (flystocks.bio.indiana.edu), and include: *p38a RNAi*, Rel^{E20}, *DD; UAS-imd*, *UAS-Toll10b*, *FOXO RNAi*, IMD^{R156}, *UAS-Rheb* (BL 9690), *Aug21-Gal4*, *UAS-DTI*, *UAS-spz**, and *da-Gal4* (Gaia). Other transgenic lines include: *Or88a* mutant (Leslie Vosshall; E4365-181) and *Or47b*[3] mutant (BL 51307). All fly lines have been described previously[15, 19, 35, 37, 41]. *Drosophila* RNAi lines after crossing were kept at 30 degree (treatment) or 25 degree (as negative controls) prior to subsequent testing with infection.

Bacterial strains and infection experiments. Bacterial strains were kept in long-term storage at −80 °C in 70% glycerol or 70% dimethyl sulfoxide (DMSO). Fresh bacterial cultures were generated daily and cultured overnight in 1000 µl lysogeny broth (LB) growth medium and grown at 29 °C and 70% humidity[51]. Adult flies between 4 and 7 days of age were pricked with a sharpened tungsten needle that had first been sterilized with ethanol and then inoculated by dipping the needle into a concentrated bacterial pellet[52]. Control flies were also pricked in the same manner, but with only LB culture medium. Flies were maintained for set time intervals at 29 °C following infection with either the bacteria or the mock control and then later used for subsequent behavioral experiments or body wash collections. To generate heat-killed samples, fresh 1 ml bacterial cultures were placed into Eppendorf tubes and then allowed to float in a water bath that was heated to 90 °C for 1 h. After cooling to room temperature, these heat-killed bacteria were then used following the previously described pricking procedures to infect the adult flies. Bacteria were also confirmed to be dead by plating them without observing any growth.

Trap assays and FlyWalk. Trap assays were performed with 2–5-day-old flies as previously described[44, 53]. Briefly, test chambers (transparent yoghurt cups (500 ml) with 50 ventilation holes in the lid) contained a treatment and a control trap made from small transparent plastic vials (30 ml) with a cut micropipette tip (tip diameter 2 mm) inserted into a hole of the vial. Thirty flies (males and females, ratio about 1:1, 4–5 days old, starved for 24 h before the experiment) were placed in each test box. Experiments were always started at the same time of day and carried out in a climate chamber (25 °C, 70% humidity, 12-h-light:12-h-dark cycle). The number of flies in and outside the traps was counted after 24 h. Valence of the tested cuess was quantified with an attraction index (AI), calculated as: $AI = (O - C)/(30)$, where O is the number of flies in the odorant trap, C the number of flies in the control trap, and 30 the sum of all flies tested. The resulting index ranges from −1 (complete avoidance) to 1 (complete attraction). A value of zero characterizes a neutral or non-detected odorant. FlyWalk trials were also conducted as described previously[54, 55]. In short, 15 individual flies were placed in glass tubes (0.8 cm i.d.). The glass tubes were aligned in parallel, and flies were monitored continuously by an overhead camera. *xy* positions were recorded automatically at 20 fps using Flywalk Reloaded v1.0 software (Electricidade Em Pó; flywalk.eempo.net). Experiments were performed under red LED light (peak intensity at λ, 630 nm). During the experiments, flies were continuously exposed to a humidified airflow of 20 cm/s (70% relative humidity, 20 °C). Flies were repeatedly presented with pulses of various olfactory stimuli at interstimulus intervals of 90 s. Stimuli (i.e., headspace of either 100 healthy or infected adult flies (50 males and 50 females)) were added to the continuous airstream and thus traveled through the glass tubes at a constant speed. The paradigm allows us to measure the stimulus-induced change of upwind speed of the tested flies.

Feeding assays. Flies were collected and tested between the ages of 2–5 days, and included both males and females that were starved beforehand for 18–20 h with constant access to water. Flies were then cooled for 2 min at −20 °C to assist in their transfer to the behavioral arena. The capillary feeder (CAFÉ) assays utilized glass micropipettes with liquid media that were filled by capillary action and then inserted through pipette tips into the container holding the adult flies, modified from Ja et al.[56]. One capillary contained the control (5% sucrose with LB media), while the other contained the treatment (5% sucrose plus LB media and either bacteria or frass), and the volume consumed from each side was measured after a set duration of feeding. Feeding indices were calculated as $(T - C)/(T + C)$, where T is the amount of food consumed from the treatment solution and C is the amount of food consumed from the control solution.

Chemical analyses and SSRs. All of the synthetic odorants that were tested and confirmed were acquired from commercial sources (Sigma, www.sigmaaldrich.com, and Bedoukian, www.bedoukian.com) and were of the highest purity available. Stimuli preparation and delivery for behavioral experiments followed

previously established procedures, and collection of volatile and non-volatile compounds was carried out according to standard procedures[35, 44]. GC-MS (HP5 and HP-Innowax) and TDU-GC-MS analyses were performed on all odor collections and insect body washes as described previously[35]. The NIST mass-spectral library identifications were confirmed with chemical standards where available, and the internal standard bromodecane was utilized for quantification and statistical comparisons between analyzed samples. SSR experiments were conducted as described previously[35, 44].

Oviposition experiments. Virgin flies were collected and separated by sex 4–5 days prior to the experiments. Before the trials, male and female virgins were allowed to mate for 4 h, and then separated again. Cohorts of 20 recently mated females were added to small container (10 × 10 × 20 cm) that was equipped with two Petri dishes (diameter, 5 cm) containing agar (1%), of which one was loaded with the odor in solvent, and the other with solvent only (or with another odor, if, e.g., when odors of infected vs. healthy flies were tested). Experiments were carried out in a climate chamber (25 °C, 70% humidity, 12 h light:12 h dark cycle). We added 50 µl of body wash extracts collected from either healthy (mock infection with LB media) or body washes from flies infected with *P. entomophila* for sequential time intervals. Flies were allowed to lay eggs for 3 days, after which eggs were counted to generate the oviposition indices (which were calculated as $(T - C)/(T + C)$ where T is the number of eggs on the treatment plate and C is the number of eggs on the control plate).

Courtship and mating experiments for single pairs. Adults were collected as newly emerged virgins, where males were kept in individually separated vials and females were reared in groups of 20–30 flies. Courtship was conducted with virgin flies that were 4–5 days old, and the behavioral experiments were conducted as described previously within the lid of an Eppendorf that was covered by a plastic slide[35]. Mating and courtship behaviors were recorded for 20 min and then analyzed. Copulation latency refers to the time delay until the successful physical coupling of the male and female, while copulation success refers to the percentage of total pairs that mated within the 60 min timespan. Copulation duration was the time that the male and female were conjoined during mating.

Statistics and figure preparation. Statistical analyses were conducted using GraphPad InStat 3 (https://www.graphpad.com/scientific-software/instat/), while figures were organized and prepared using R Studio, Microsoft Excel, and Adobe Illustrator CS5. The Wilks–Shapiro test was used to determine normality of each data set. Normally distributed data were then analyzed using two-tailed, paired *t*-tests and one-way analyses of variance. Nonparametric distributed data were assessed using Kruskal–Wallis with Dunn's post hoc test for multiple comparisons for selected pairs. An asterisk denotes statistical significance between two groups ($*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$). Courtship data were analyzed and confirmed by an additional blind observer who was not aware of the treatments being viewed. Boxplots represent the median (bold black line), quartiles (boxes), as well as the confidence intervals (whiskers). Whiskers in barplots represent the standard error.

Data availability. Additional supplementary information and extended data including methodology, courtship videos, and other raw data are available with the online version of the publication. All data supporting the findings of this study are available within the article and its Supplementary Information files.

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Author contributions

I.W.K., M.K., N.B., and B.S.H. contributed to the design of this study. I.W.K. conducted the behavioral trials including trap and CAFÉ feeding assays. I.W.K. and S.K. performed the oviposition and heat-killed bacteria assays, while S.K. and N.B. designed the *Drosophila* mutant and RNAi experiments. M.A.K. and I.W.K. completed the courtship and mating assays, as well as the SSR data sets. T.R. conducted the flywalk trials. I.W.K. conducted all the GC-MS experiments, while M.A.K. and I.W.K. completed the TDU-GC-MS experiments. I.W.K. prepared the original manuscript, and M.K., N.B., and B.S.H. contributed to the final manuscript and subsequent revision.

Additional information

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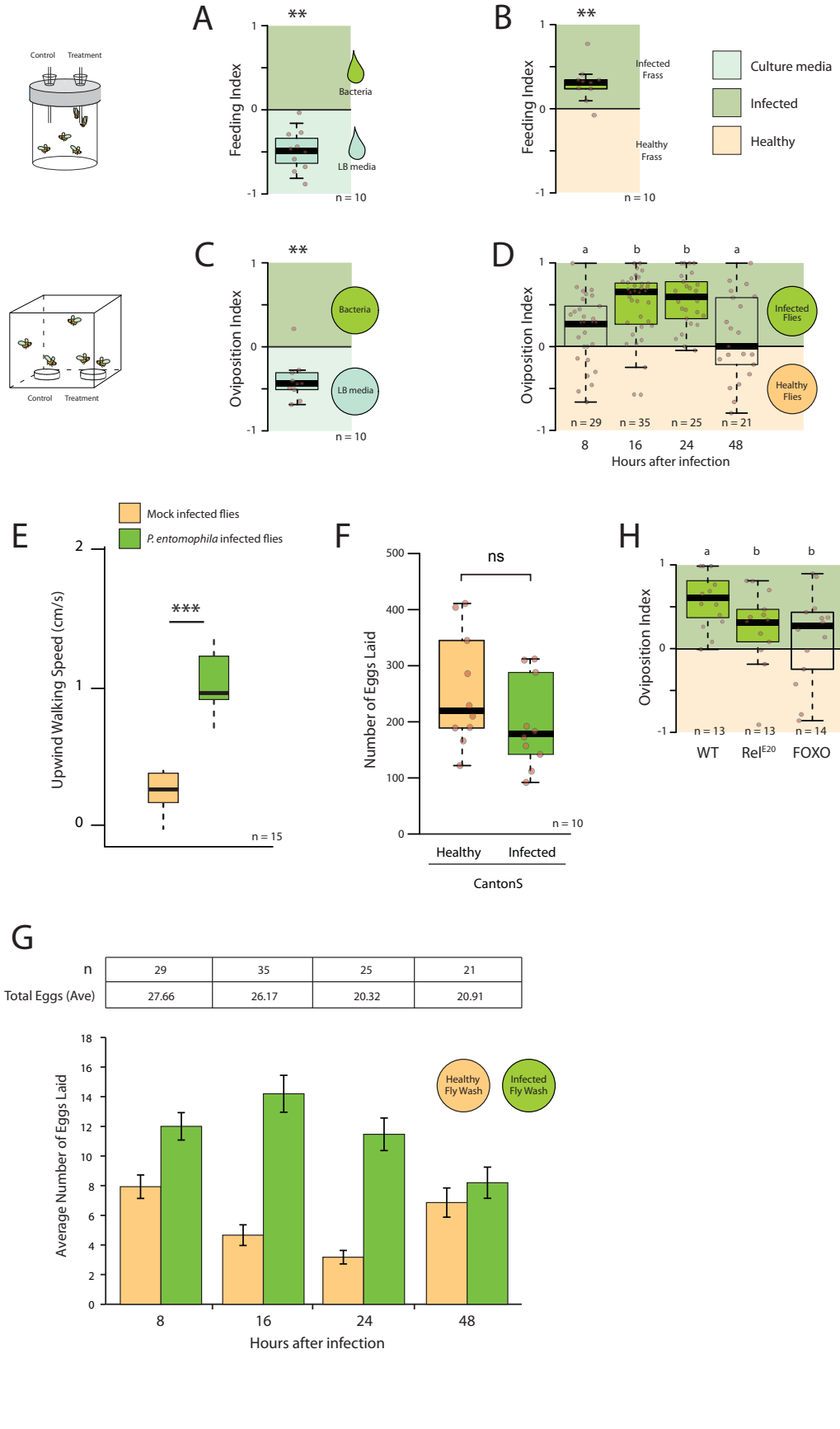
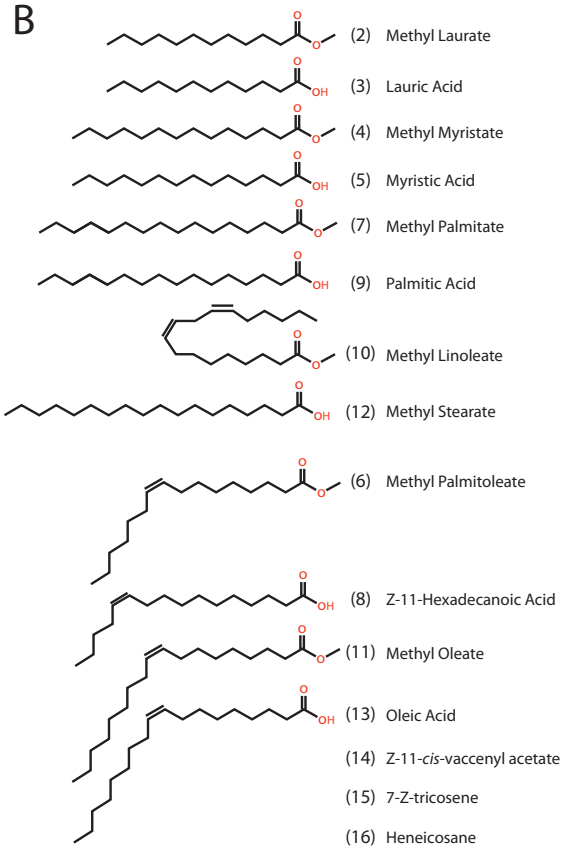

A
B
C
D
E
F
G
H
Control
Treatment
Feeding Index
Bacteria
LB media
n = 10
Infected Frass
Healthy Frass
Culture media
Infected
Healthy
Oviposition Index
Infected Flies
Healthy Flies
n = 29
n = 35
n = 25
n = 21
Hours after infection
Mock infected flies
P. entomophila infected flies
Upwind Walking Speed (cm/s)
n = 15
Number of Eggs Laid
ns
Healthy
Infected
CantonS
n = 13
n = 14
WT
Rel^E20
FOXO
n
29
35
25
21
Total Eggs (Ave)
27.66
26.17
20.32
20.91
Average Number of Eggs Laid
Healthy Fly Wash
Infected Fly Wash

Supplementary Figure 1. Feeding and oviposition preferences towards infected flies and their frass. A, Feeding (**A**)and oviposition (**C**) preference of flies when given the choice between sugar solutions or agar plates containing growth media, or the media plus *Pseudomonas entomophila* bacteria (for definition of indeces see Fig. 1 of the manuscript). Feeding (**B**)and oviposition (**D**) preference of flies when given the choice between sugar solutions or agar plates containing frass of healthy or infected flies. Whenever time course is not shown, data were collected 24 hours after infection. **E**, Average upwind speed of flies after being exposed to a 1s-pulse of headspace of healthy or infected flies. For details of Flywalk assay see method part. **F**, Average number of eggs laid by each 20 healthy or infected female *Drosophila* during 24 hours. **G**, Average number of eggs laid by wildtype flies given a choice between the body washes of healthy and infected flies collected at different time points after infection (see S.Fig. 1D). Only 16 and 24 hour post-infection body washes generated a difference in egg-laying preference, but no difference in total number of eggs. **H**, Oviposition indices of naïve females towards body washes from wildtpe (WT), immune mutants (RelE20) or towards metabolic mutants (FOXO) after infection. Filled boxes are significantly different from zero, boxes with different letters differ significantly. In all box plots, filled boxes denote significance from zero.

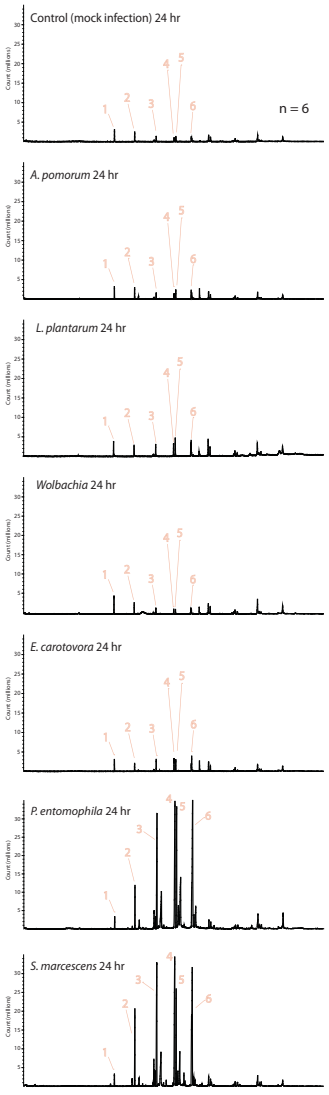
A

Peak No.	Kovats Index	Compound Name	Female		Male	
			Healthy	Infected	Healthy	Infected
1	1320	Bromodecane (internal standard)	4.3	4.1	3.4	3.8
2	1485	Methyl laurate (ML)	0.3	24.9	0.3	3.5
3	1500	Lauric Acid	0.2	2.5	0	0.8
4	1710	Methyl Myristate (MM)	0.9	35.1	1	9.5
5	1725	Myrisitic acid	0	6.5	0	2.9
6	1900	Methyl palmitoleate	0.85	35.5	1.6	13.9
7	1912	Methyl palmitate (MP)	0.59	35.3	0.8	9.7
8	1920	Palmitoleic acid	0	3.4	0	2.3
9	1930	Palmitic acid	0	6.7	0	3.6
10	1969	Methyl linoleate	0.52	19.9	1.9	18.8
11	1980	Methyl oleate	0.67	30.2	1.4	16.6
12	1995	Methyl stearate	0	6.3	0	1.5
13	1998	Oleic acid	0	1.2	0	2.1
14	2210	Z-11-*cis*-Vaccenyl actetate (cVA)	0	0	14.2	9.9
15	2284	9-Z-Tricosene	0.68	1.4	6.3	6.1
16	2295	Heneicosane	0.84	2.6	5.2	5.5
			n = 3	n = 3	n = 3	n = 3

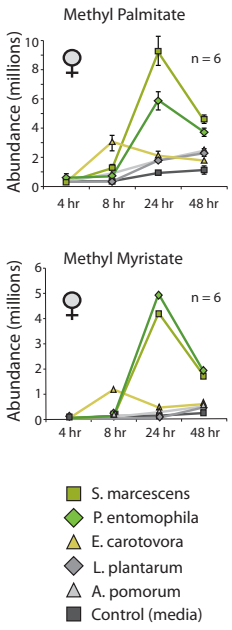
B



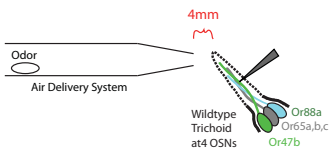
C

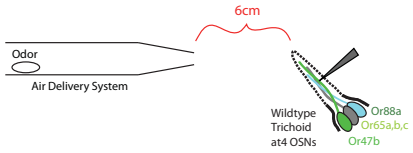


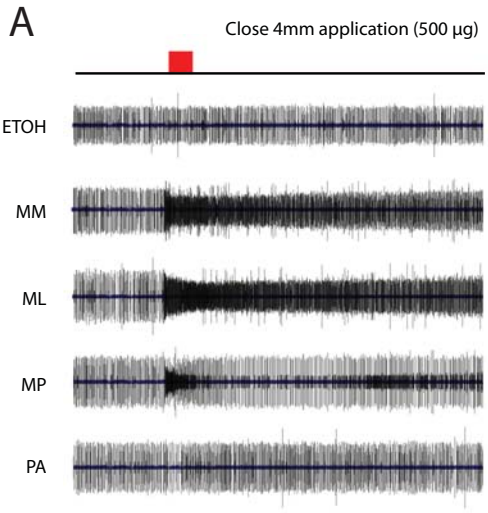
D

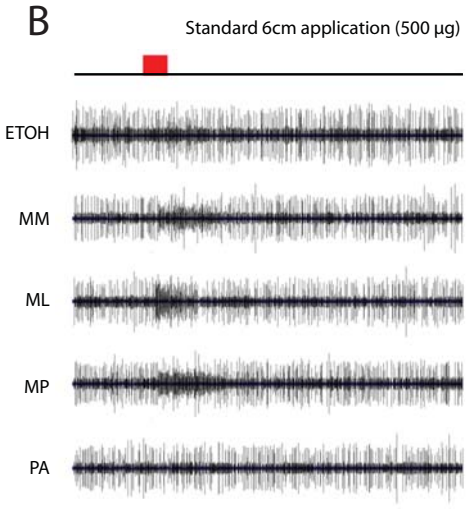


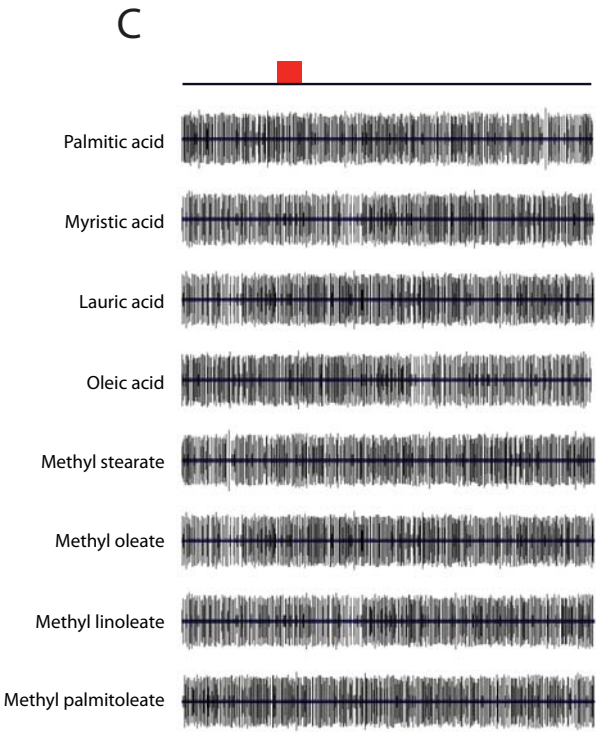
Supplementary Figure 2. GC-MS analyses of flies infected with pathogenic bacteria. **A**, Table corresponds to Fig. 1A, and shows the average amount of compounds identified from male and female flies after infection by *Pseudomonas entomophila* bacteria. The 12 colored compounds were those that were significantly different from healthy flies (Fig. 1I; ALL), while compounds in black were not different between healthy and infected flies. Red indicates the three methyl esters (3MEs), blue are the four fatty acids (4FAs) from Fig. 1I. **B**, Chemical structure of compounds identified from GC-MS data in Fig. 1A and B. **C**, Examples of raw total ion traces from adult female *Drosophila* after 24 hours of infection with several bacterial strains (Wildtype control (mock infected), *A. pomorum*, *L. plantarum*, *Wolbachia*, *E. Carotovora*, *P. entomophila*, *S. marcescens*). (1- bromodecane; 2- methyl laurate; 3- methyl myristate; 4- methyl palmitoleate; 5- methyl palmitate; 6- methyl oleate). **D**, Specific pheromone production over time for females infected with several strains of pathogenic and non-pathogenic bacteria.

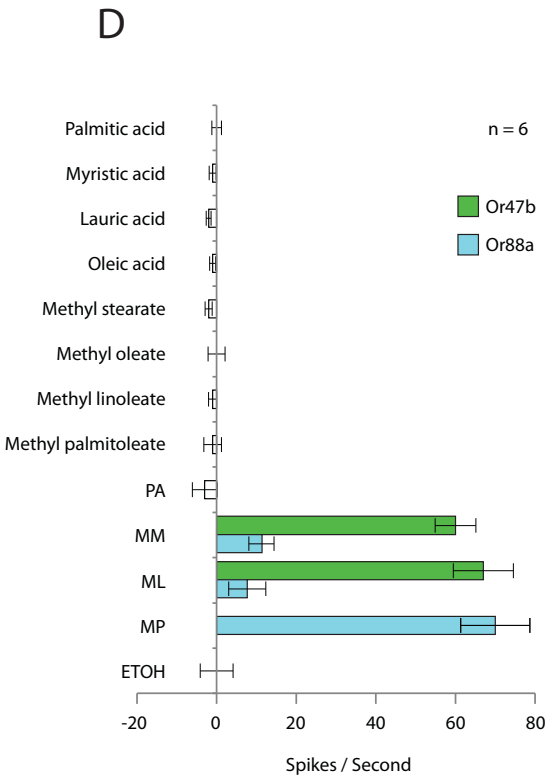

4mm
Odor
Air Delivery System
Wildtype Trichoid at4 OSNs
Or88a
Or65a,b,c
Or47b


6cm
Odor
Air Delivery System
Wildtype Trichoid at4 OSNs
Or88a
Or65a,b,c
Or47b


A
Close 4mm application (500 µg)
ETOH
MM
ML
MP
PA

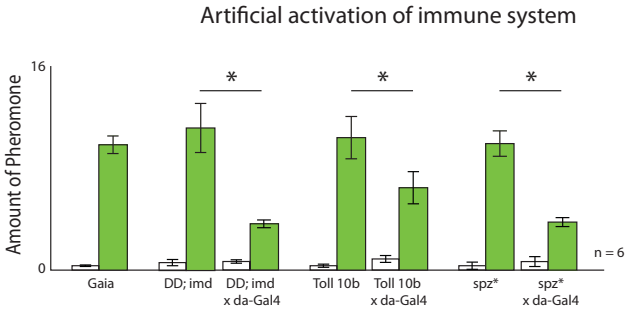

B
Standard 6cm application (500 µg)
ETOH
MM
ML
MP
PA


C
Palmitic acid
Myristic acid
Lauric acid
Oleic acid
Methyl stearate
Methyl oleate
Methyl linoleate
Methyl palmitoleate

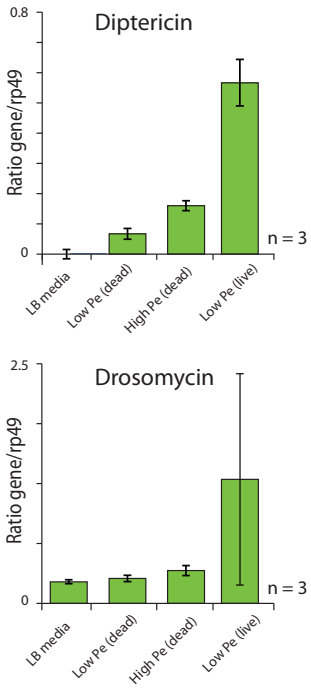

D
n = 6
Or47b
Or88a
Palmitic acid
Myristic acid
Lauric acid
Oleic acid
Methyl stearate
Methyl oleate
Methyl linoleate
Methyl palmitoleate
PA
MM
ML
MP
ETOH
-20
0
20
40
60
80
Spikes / Second

Supplementary Figure 3. Representative SSR traces for at4 responses to all odors that were increased after infection. A, *D. melanogaster* antennal trichoid 4 (at4) responses to the application of odors from 4mm cm distance (following the stimulus protocol of Lin et al. 2016), including ethanol solvent control (ETOH), and responses towards potential pheromone components that were upregulated after infection by *P. entomophila* (methyl myristate (MM), methyl laurate (ML), methyl palmitate (MP), and palmitoleic acid (PA). Large spike amplitudes correspond to the activation of the at4A neuron expressing Or47b, while smaller spikes correspond to the activation of the at4C neuron expressing Or88a. **B,** at4 responses to the application of odors from 6 cm distance (following the stimulus protocol of Dweck et al. 2015). **C**, SSR stimulation of at4 sensillum with other odors that increased after infection with *P. entomophila* bacteria. No responses were found for any of these odors. **D**, Quantified SSR responses towards 12 odors that were increased after infection and used in SSR testing of at4 OSNs.

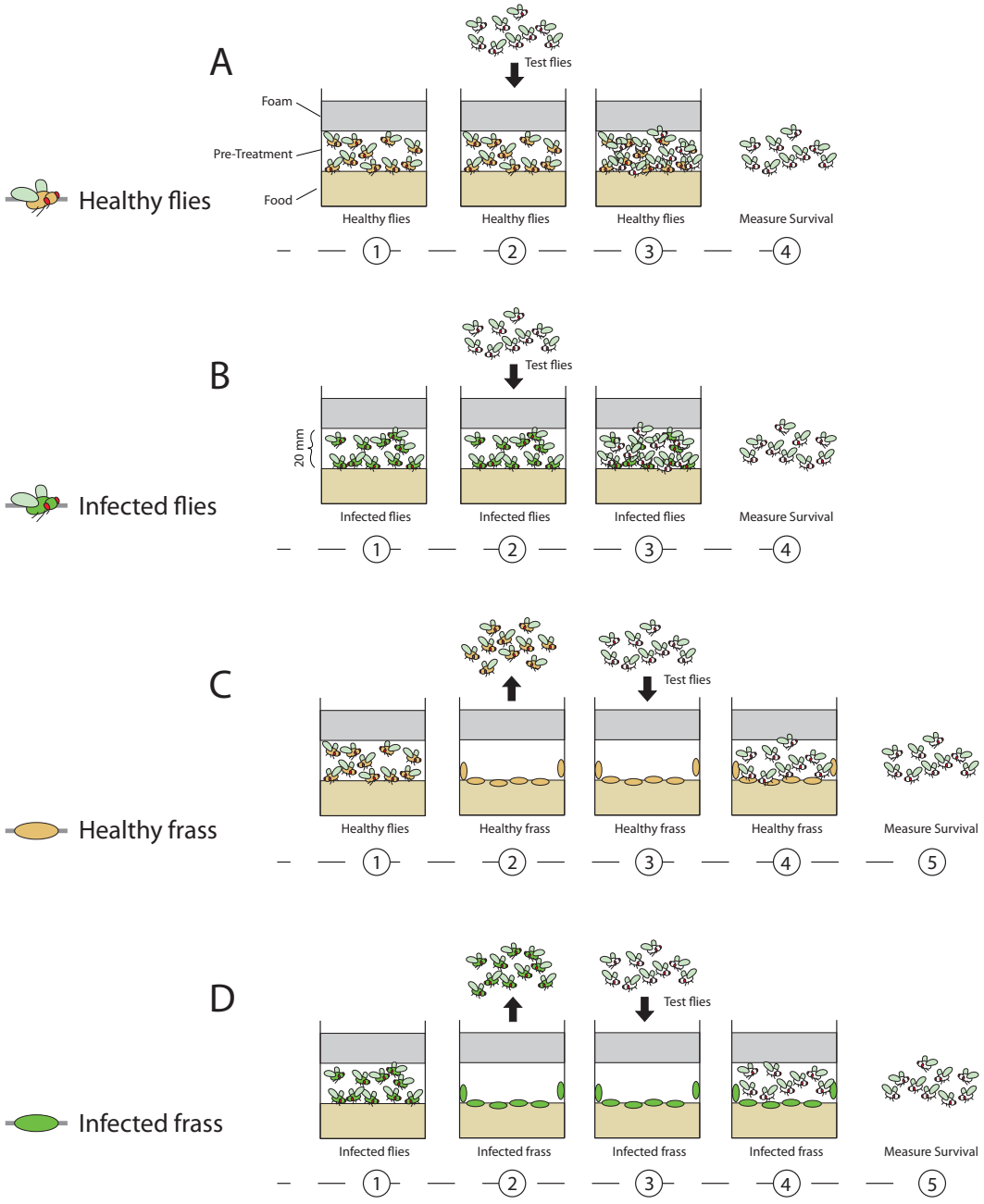
A


Artificial activation of immune system
Amount of Pheromone
16
0
*
*
*
n = 6
Gaia
DD; imd
DD; imd x da-Gal4
Toll 10b
Toll 10b x da-Gal4
spz*
spz* x da-Gal4

B


Diptericin
Ratio gene/rp49
0.8
0
n = 3
LB media
Low Pe (dead)
High Pe (dead)
Low Pe (live)
Drosomycin
Ratio gene/rp49
2.5
0
n = 3
LB media
Low Pe (dead)
High Pe (dead)
Low Pe (live)

Supplementary Figure 4. Antimicrobial peptide (AMP) production after infection.
A, Artificial activation of Imd, Toll and Spatzle transgenic fly lines, showing that both immune response pathways are necessary (decreased after *P. entomophila* infection) but not sufficient (activation without infection) to observe maximum pheromone production following infection. **B**, Levels of two antimicrobial peptides in mock infected flies as well as in flies that were infected with different amount of living and heat-killed *P. entomophila*.


A
Test flies
Foam
Pre-Treatment
Food
Healthy flies
Healthy flies
Healthy flies
Healthy flies
Measure Survival
1
2
3
4
B
Test flies
20 mm
Infected flies
Infected flies
Infected flies
Infected flies
Measure Survival
1
2
3
4
C
Test flies
Healthy flies
Healthy frass
Healthy frass
Healthy frass
Healthy frass
Measure Survival
1
2
3
4
5
D
Test flies
Infected flies
Infected frass
Infected frass
Infected frass
Infected frass
Measure Survival
1
2
3
4
5

Supplementary Figure 5. Ecological consequences of attraction to sites of infection. Diagrams of the methodology for measuring the survival of cohorts of naïve flies that were placed in rearing vials that had previously been exposed to either healthy or infected adults and/or frass. **A**, healthy fly exposure to healthy flies. **B**, healthy fly exposure to infected flies. **C**, healthy fly exposure to healthy frass. **D**, healthy fly exposure to infected frass.

Manuscript III

Evaluation of the DREAM technique for a high-throughput deorphanization of chemosensory receptors in *Drosophila*

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
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Evaluation of the DREAM Technique for a High-Throughput Deorphanization of Chemosensory Receptors in *Drosophila*

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In the vinegar fly *Drosophila melanogaster*, the majority of olfactory receptors mediating the detection of volatile chemicals found in their natural habitat have been functionally characterized (deorphanized) *in vivo*. In this process, receptors have been assigned ligands leading to either excitation or inhibition in the olfactory sensory neuron where they are expressed. In other, non-drosophilid insect species, scientists have not yet been able to compile datasets about ligand–receptor interactions anywhere near as extensive as in the model organism *D. melanogaster*, as genetic tools necessary for receptor deorphanization are still missing. Recently, it was discovered that exposure to artificially high concentrations of odorants leads to reliable alterations in mRNA levels of interacting odorant receptors in mammals. Analyzing receptor expression after odorant exposure can, therefore, help to identify ligand–receptor interactions *in vivo* without the need for other genetic tools. Transfer of the same methodology from mice to a small number of receptors in *D. melanogaster* resulted in a similar trend, indicating that odorant exposure induced alterations in mRNA levels are generally applicable for deorphanization of interacting chemosensory receptors. Here, we evaluated the potential of the DREAM (**D**eorphanization of **r**eceptors based on **e**xpression **a**lterations in **m**RNA levels) technique for high-throughput deorphanization of chemosensory receptors in insect species using *D. melanogaster* as a model. We confirmed that in some cases the exposure of a chemosensory receptor to high concentration of its best ligand leads to measureable alterations in mRNA levels. However, unlike in mammals, we found several cases where either confirmed ligands did not induce alterations in mRNA levels of the corresponding chemosensory receptors, or where gene transcript-levels were altered even though there is no evidence for a ligand–receptor interaction. Hence, there are severe limitations to the suitability of the DREAM technique for deorphanization as a general tool to characterize olfactory receptors in insects.

Keywords: *Drosophila*, olfaction, ligand–receptor interaction, DREAM, deorphanization

INTRODUCTION

Despite more than two decades of research, the mechanisms by which mammals as well as insects detect a seemingly unlimited amount of odorants with a comparably small set of chemosensory receptors remain elusive up to date. Clearly, a one-to-one relationship between volatile chemicals and chemosensory receptors is not plausible. Thus, the general consensus is that insects as well as mammals need a combinatorial code to allow for a differentiation of the plethora of diverse volatile compounds found in nature (Haverkamp et al., 2018). Here, the identity of an odorant would be defined by a pattern of interactions with various chemosensory receptors. One odorant would interact in an excitatory or inhibitory manner with several receptors, and the same chemosensory receptor may interact with a number of different odorants (Malnic et al., 1999).

The drastic discrepancy between the diversity of airborne chemo-signals and the amount of detecting receptors becomes quite prominent in insects. The adult vinegar fly *Drosophila melanogaster* possesses a repertoire of approximately 44 functional odorant receptors (ORs) including the OR co-receptor ORCO, expressed in the olfactory organs, antennae and maxillary palps, solely for the detection of odorants (Vosshall et al., 2000; Couto et al., 2005). Additionally, the olfactory system of the fly deploys 17 chemosensory receptors belonging to the receptor family of ionotropic receptors (IRs) including four co-receptors for the detection of airborne organic acids, aldehydes, and amines (Stocker, 2001; Yao et al., 2005; Benton et al., 2009; Silbering et al., 2011; Menuz et al., 2014) as well as the two gustatory receptors (GRs) Gr21a and Gr63a for carbon-dioxide sensing (Jones et al., 2007; Kwon et al., 2007). Chemosensory receptors involved in olfaction reside in the dendritic membrane of olfactory sensory neurons (OSNs), which are found in groups of two-to-four and are housed in hair-like structures called sensilla on the antennae or maxillary palps (Vosshall and Stocker, 2007; Su et al., 2009). In *D. melanogaster* four morphologically distinct sensillum types (Stocker, 1994; Yao et al., 2005; Su et al., 2009; Martin et al., 2013; Lin and Potter, 2015) take part in the discrimination of different chemical classes: basiconic, intermediate, and coeloconic sensilla house OSNs for detection of general odorants represented by esters, alcohols, aldehydes, amines and acids, respectively (Hallem and Carlson, 2006), while trichoid sensilla are exclusively mediating the perception of pheromones, which are predominantly long fatty acid chains (Clyne et al., 1997; Kurtovic et al., 2007; Dweck et al., 2015b). Summed up, *D. melanogaster* expresses a set of approximately 62 known chemosensory receptor types total on the antennae and maxillary palps conferring the detection of a multitude of different odorants in nature including odorants for the location of food sources (Zhu et al., 2003; Hallem and Carlson, 2006; Dweck et al., 2016) as well as oviposition sites (Dweck et al., 2013), avoidance of harmful microorganisms (Stensmyr et al., 2012) or natural enemies (Ebrahim et al., 2015), and finally for governing courtship behavior (Clyne et al., 1997; Dweck et al., 2015b). These chemosensory receptors can be categorized into two types of receptors, those which only interact with a small set of ligands or even just one odorant, referred to as narrowly tuned receptors, and those which have a broad spectrum of ligands, characterized as broadly tuned receptors.

For the vinegar fly, most chemosensory receptors involved in olfaction have been assigned a "best ligand," which is an agonist that already at low doses leads to a strong activity of OSNs expressing this receptor. The identification of a chemosensory receptor's best excitatory ligand is referred to as deorphanization. The deorphanization of chemosensory receptors playing a role in the olfactory system of *D. melanogaster* has been a time-consuming endeavor and has only been possible thanks to the extensive genetic tools available in this model organism, like "empty neuron" or "decoder" systems. These mutant OSNs are lacking their endogenous receptor gene but instead when Gal4/UAS targeted express a receptor of interest, which can thereby be functionally characterized via Single Sensillum Recordings (SSR; Dobritsa et al., 2003; Hallem and Carlson, 2004, 2006; Yao et al., 2005; Grosjean et al., 2011; Silbering et al., 2011; Ronderos et al., 2014; Gonzalez et al., 2016). However, recent findings suggest that not only the excitation of OSNs via their agonists or best ligands is behaviorally relevant for *Drosophila* but also inhibitory interactions of chemosensory receptors and antagonistic ligands seem to play an important role in the perception of odorants leading to a behavioral output (Cao et al., 2017; MacWilliam et al., 2018). The revelation of this phenomenon indicates a bidirectional code in addition to combinatorial coding allowing for an even greater odor-coding capacity by adding another level of complexity, as the excitation or inhibition of an OSN concurring with the activation or inhibition of a set of OSNs expressing other ORs confers different meanings (Cao et al., 2017).

While in *D. melanogaster* scientists have been able to work on understanding the principles underlying the function of the olfactory system, studies are hampered by the lack of genetic tools available in related non-*melanogaster* flies within the genus Drosophilidae. A great progress has been made in the deorphanization of chemosensory receptors in non-drosophilid insect models in the mosquito *Anopheles gambiae* (Wang et al., 2010) and the moth *Spodoptera littoralis* (De Fouchier et al., 2017), but the methods used (mosquito: ectopic expression of receptors in *Xenopus* oocytes; moth: *Drosophila* empty neuron system) were extremely time-consuming. Deorphanization of chemosensory receptors involved in detecting olfactory signals becomes even more time-consuming in insect species without a sequenced, annotated genome, and without an option for the application of genetic tools. Fortunately, in mammals and potentially also in *Drosophila* the discovery of a correlation between prolonged exposure to high odorant concentrations and regulations in mRNA levels of interacting chemosensory receptors (von der Weid et al., 2015) gave rise to a procedure potentially allowing for chemosensory receptor deorphanization in any species of interest without the requirement of genetic tools such as those available in *D. melanogaster*. Furthermore, this method, which is referred to as DREAM (**D**eorphanization of **r**eceptors based on **e**xpression **a**lterations of **m**RNA levels), has the potential to allow identification of all chemosensory receptors interacting with an odorant in a high-throughput manner instead of a deorphanization of single ligand-receptor pairs at a time.

In the present study, we evaluated the applicability of the DREAM technique for a high-throughput deorphanization of general ORs, pheromone receptors (PRs), and IRs utilizing previously established ligand-receptor combinations in *D. melanogaster*. Using RealTime quantitative polymerase chain reaction (qPCR), we were able to reproduce the described down-regulation of target genes by analyzing ligand–receptor combinations tested in the original study (von der Weid et al., 2015). Subsequently, we evaluated the general suitability of the DREAM technique for deorphanization of broadly and narrowly tuned ORs. Here, we were not able to consistently correlate ligand–receptor interactions with alterations in gene transcript-levels; only three out of six additionally tested ORs showed changed mRNA levels upon prolonged exposure to their best known ligand. Furthermore, we tested the applicability of the DREAM technique for the deorphanization of chemosensory receptor classes besides ORs, monitoring the effect of prolonged odorant exposure on the transcription of IRs. However, after odorant treatment, we did not observe any significant changes in the IR's gene transcription, implying that the DREAM method may not be useful for deorphanization of IRs. Finally, in order to test whether changes in the experimental conditions of the DREAM technique would lead to more reliable results, we varied odorant exposure duration but did not obtain different results.

In summary, while in certain cases, we confirmed that transcription levels of ORs can be significantly affected by prolonged exposure to high concentrations of their (best) ligands, we demonstrate limitations of a universal applicable DREAM method for deorphanization of different types of chemosensory receptors in insects.

MATERIALS AND METHODS

Animals

All flies used in the experiments were WT *D. melanogaster* and belonged to the Canton-S strain (WTcs, stock #1), which was obtained from the Bloomington Drosophila Stock Center[1]. The fly stock was maintained on an artificial diet at 25°C and 70% R.H. with a photoperiod of 12 h:12 h Light:Dark (Stökl et al., 2010). With the exceptions of the pheromone treatment and corresponding control groups, flies were collected 0–3 h after exclusion, pooled to groups of 60 and transferred to a fresh rearing vial. In case of exposure to the pheromone methyl laurate and for the corresponding controls, newly emerged flies were collected 5 days prior to the odorant exposure. On the day of the odorant treatment, the 5-days old flies were transferred to a fresh rearing vial for the exposure. Flies for all odorants were of mixed sex at a ratio of 1:1 and kept together during the length of the odorant exposure.

Chemicals

Odorants used for all experiments were of highest purity commercially available and purchased from Sigma-Aldrich with the exception of methyl butyrate with was purchased from FLUKA. For the DREAM method, general odorants were diluted to 5% vol/vol in dimethyl sulfoxide (Sigma-Aldrich), while methanol (Roth) was used to dilute methyl laurate up to 5% vol/vol. In SSRs, odorant dilutions of 10^{-4} and 10^{-1} were used, dilutions were generated in hexane (Roth) for all general odorants and in methanol (Roth) for methyl laurate.

Odorant Exposure and Tissue Collection

In order to test, whether the DREAM method is suitable for the deorphanization of broadly as well as narrowly tuned ORs and different chemosensory receptor types transcription changes of receptor genes were measured after flies were exposed to high odorant concentrations. Three hours after the beginning of the light phase the odorants or pure solvents were introduced into the rearing vials. To avoid interaction of the chemicals with the artificial diet, 30 µL of the odorants or solvents, respectively, were applied into the well of a detached 2.0 mL reaction tube lid. After an exposure time of 5 h, flies were transferred into new, empty vials, and cooled down for 5 min in a −80°C freezer. The flies were then maintained at −20°C until dissection. For each biological replicate, 50 manually removed fly heads (male–female ratio 1:1) were collected in 2.0-mL microcentrifuge tubes containing mixed zirconium oxide beats of 1.4 and 2.8 mm (CKmix-2 mL, Bertin Instruments) as well as 600 µL TRIzol® (Sigma-Aldrich). For the sample collection of the pheromone treatments and corresponding controls, only the heads of male flies were used. During dissection, samples were stored on ice. After dissection samples were homogenized in a bead mill (TissueLyser LT, Qiagen) for 10 min at 50 Hz. Samples were centrifuged for 1 min at 13,000 *g* and stored at −80°C until RNA extraction.

RNA Extraction and cDNA Synthesis

Total RNA for each replicate and treatment was extracted using an unbiased RNA isolation kit (Direct-zol™ RNA MiniPrep, Zymo Research). The kit included a RNase-free DNase treatment to remove genomic DNA contamination from the samples. RNA concentration was measured with a *NanoDrop*™ spectrophotometer (Thermo Fisher Scientific). First strand cDNA was generated from 1.0 µg of total RNA, using oligo-dT_{20} primers and superscript™ III (Thermo Fisher Scientific). Subsequently, remaining RNA was digested via a RNase H treatment (Thermo Fisher Scientific).

qPCR

Expression levels of target genes were analyzed by reverse transcription-mediated quantitative real-time PCR (qPCR). Following the guidelines proposed to guarantee reproducible and accurate measurements, qPCR reactions were run in a Stratagene Mx3005P qPCR system. Measurements were performed in 96-well plates using the Takyon™ No Rox SYBR® MasterMix dTTP blue (Eurogentec, Belgium) in a total reaction volume of 20 µL. Each reaction was run in triplicate with at least five independent biological replicates for controls and different treatments. Gene-specific primers for Cam, Orco, OR49b, OR67c, as well as OR82a were identical to those used in von der Weid et al. (2015).

[1]https://bdsc.indiana.edu

All other gene-specific primers were designed in Geneious (9.1.5). Primers are listed in **Supplementary Table S1**. The two-step thermal cycling protocol consisted of following steps: initial denaturation (95°C: 3 min), subsequent 40 cycles of denaturation (90°C: 10 s), annealing (60°C: 20 s), elongation (75°C: 30 s), and completed with a final cycle for post-amplification melting-curve analysis. The Cam and Orco genes were used as reference genes. For every primer pair used qPCR efficiency was determined by generating standard curves with mixed cDNA samples. Normalized expression and relative fold change were calculated based on a model by Vandesompele et al. (2002) for normalization against several reference genes when efficiencies of target and reference genes are not similar. Following equation from Vandesompele et al. (2002) was used for the calculations (E: primer efficiency, Cq: threshold cycle, Ref_{xy}: reference gene, Tar: target gene):

$$ratio = \frac{\sqrt[n]{(1+E_{Ref1})^{Cq(Ref1)} \times (1+E_{Ref2})^{Cq(Ref2)} \times \ldots}}{(1+E_{Tar})^{Cq(Tar)}}$$

Single Sensillum Recordings

In order to confirm the published ligand–receptor interactions, SSRs were performed with the same panel of odorants used in the DREAM experiments for all OSN types expressing the chemosensory receptors of interest. Flies of 2- to 7-day-old age were prepared for recordings as described by Clyne et al. (1997) and de Bruyne et al. (2001). With the help of a microscope (10× magnification, 0.30 numerical aperture [NA], Olympus BX51W1) and a micromanipulator (Märzhauser DC-3K) the reference electrode (tungsten wire) was manually inserted into one of the fixated fly's compound eye. Next, changing the magnification to 50× (0.50 [NA]) and using a motorized, piezo-translator-equipped micromanipulator (Märzhauser DC-3K/PM-10), the recoding electrode (tungsten wire) was inserted into the center or shaft of a sensillum. Different OSN types localized inside the sensillum were identified using a set of known, well-established diagnostic odorants (Ebrahim et al., 2015). Spiking frequency of the OSNs expressing chemosensory receptors of interest was recorded for 10 s, starting 3 s before the stimulus (0.5 s stimulus duration), and lasting 7 s after the end of the stimulus. Neuronal signals were converted from a high input resistance to low-output resistance with a pre-amplification step (10×) using a headstage (Syntech Universal AC/DC probe). The pre-amplified signal was then converted (Syntech IDAC-4) and fed into a computer for visualization and analysis via Syntech Autospike v3.2. In order to discriminate between the neural activity of OSNs housed in the same sensillum, spikes were sorted by differences in their amplitude and assigned to distinct OSN types. Spikes with the largest amplitude were considered to belong to the OSN of type A, spikes of the second largest amplitude were assumed to originate from the B OSN and so forth. The amplitude-based spike sorting by Syntech Autospike v3.2 was manually adjusted when amplitudes of co-located OSNs changed after strong odorant stimulation. In cases where amplitudes between OSNs housed in the same sensillum were not distinguishable due to extensive neural activity, the final spike frequency represents the total response of a sensillum. The electrophysiological data was analyzed by subtracting responses to the control solvents from each observed odorant response stimulus (decrease or increase in firing frequency) for each tested chemosensory receptor.

GC–MS Headspace Analysis

As the exposure to the different odorants during the DREAM experiments lasted for several hours, we confirmed by GC–MS analyses that all test compounds were chemically stable and present in high amounts during the whole exposure period. Solvents or diluted odorants were placed into fly vials with artificial diet, simulating the experimental conditions in absence of the actual flies while additionally analyzing the effect of the presence of fly food on the odorant profile. The headspace in the experimental setup was collected for 5 min with a SPME microfiber (StableFlex^{TM}, DVB/CARBOXEN-PDMS, Supelco) after 45 min and 4 h of introducing the solvent or odorant into the system. Headspace samples were manually injected into a GC–MS device (Agilent technologies GC 6896N interfaced with an Agilent technologies 5975B inert XL MSD unit) with an installed HP-5MS UI column (19091S-413U, Agilent technologies). For sample analysis, the temperature of the gas chromatograph oven was held at 40°C for 2 min and then gradually increased by 20°C min^{-1} up to 260°C. Electron impact (EI) was measured at 70 eV and 300 μA in scan mode ranging from 33 to 350 m/z. The temperature of the transfer line was held at 280°C, and the ion source was maintained at 230°C. GC–MS profiles of all headspace samples were interpreted by comparison to a standard library (NIST Mass spectrum library) using MSD ChemStation (F.01.02.2357, Agilent).

Analysis of Transcription Levels

For the analysis of possible regulations in mRNA levels of chemosensory receptors upon prolonged exposure to 5% v/v of odorants, we calculated the significance of relative fold changes in gene mRNA levels that were different from 1 based on One sample *t*-tests (**Figure 1**, *x*-axis). Additionally, for comparison to the original study, we defined an unresponsive zone using the data points of published unresponsive chemosensory receptors to apply a Gaussian distribution to the data set [**Supplementary Figure S1**, gray area (von der Weid et al., 2015)]. Following instructions from von der Weid et al. (2015), the unresponsive zone was defined within 1.4 σ above and below the mean based on the results from the Gaussian fit for all the different odorant treatment series. All data points inside the unresponsive zone were considered as treatment independent variations in mRNA levels and thus not relevant while data points outside were regarded as alterations in mRNA levels caused by the odorant exposure and therefore relevant (**Supplementary Figures S1**, **S2**).

Analysis of our gene mRNA levels in regards to significant fold changes different from 1 as well the definition of an "unresponsive zone" led to similar conclusions. We, hence, focused on analyzing our results looking for fold changes different from 1.

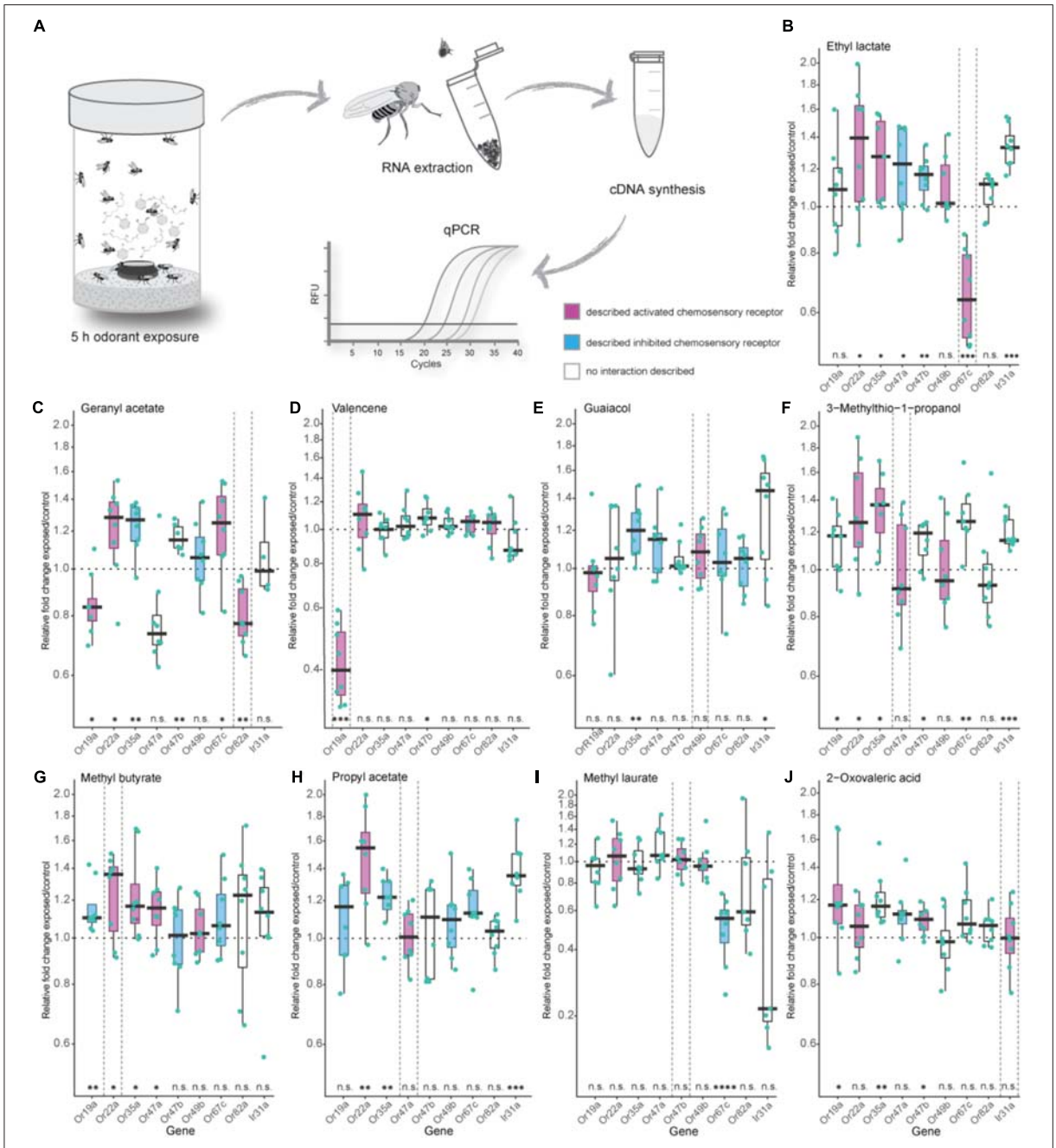

FIGURE 1 | Effects of exposure to high odorant concentration on transcription levels of selected chemosensory receptors. **(A)** Experimental procedure from odorant treatment to a final analysis of gene transcript-levels via quantitative real-time PCR (qPCR). **(B–J)** Evaluation of chemosensory mRNA levels after 5 h exposure to 5 % v/v of depicted odorants using qPCR. Each data point represents a biological replicate with a pool of RNA from 50 fly heads of mixed sex (ratio 1:1), except in **(I)** where heads from males only were used. For every odorant treatment the number of biological replicates was eight with the exception of the Ir31a gene in the geranyl acetate treatment series **(C)** where $n = 4$. Best ligand–receptor pairs are highlighted between dotted vertical lines. Excitatory and inhibitory odorant interactions (doOR database and measured in this study) are indicated in magenta (excitatory) and cyan (inhibitory). Boxplots represent the median (bold horizontal lines) with the interquartile range (whiskers). Results from a One sample *t*-test against 1 are shown on the *x*-axis. Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

RESULTS

Specificity of Chemosensory Receptor mRNA Level Alterations After Odorant Exposure

In *Drosophila melanogaster*, the majority of ORs and IRs have been functionally analyzed, and their ligand spectra have been characterized using electrophysiological approaches like SSR in wildtype flies as well as in mutant flies with different "empty neuron" or "decoder" systems. The doOR (**d**atabase **o**f **o**dorant **r**esponses) online platform provides an extensive database for known ligand–chemosensory receptor pairs[2]. First, we established the DREAM technique in our laboratory by reproducing the results from the original study [**Figures 1B,C** (von der Weid et al., 2015)]. When we exposed 0 to 3 h-old flies to the described best ligand for Or67c and Or82a, that is, ethyl lactate and geranyl acetate, respectively, we observed a significant reduction in the mRNA levels of these genes at the end of the treatment. Long-time exposure to 5% v/v ethyl lactate resulted in a downregulated transcription only of the target receptor Or67c. However, exposure to geranyl acetate did not only downregulate the transcription of Or82a as expected, but also interestingly of Or47a (**Figure 1C**), which is expressed in an OSN that is co-localized in the same sensillum as the OSN expressing Or82a. Moreover, we observed a significant decrease in gene transcription of Or19a after geranyl acetate treatment (**Figure 1C**). While SSR measurements revealed that OSNs expressing Or19a indeed become activated by high amounts of geranyl acetate, Or47a does not seem to have any interaction with this odorant (**Figure 2C**).

Next, we tested the specificity of the DREAM technique with valencene, an odorant previously known to only activate two ORs of the OR repertoire of *Drosophila melanogaster*, that is, Or19a and Or71a (Dweck et al., 2013; Ronderos et al., 2014). Of these two receptors, Or19a shows a substantial activation by valencene, accompanied by a very strong increase in the firing rate (spikes/s) after valencene stimulation of the ai2 sensillum (Dweck et al., 2013). Thus, we chose Or19a as the next target OR to test the specificity of the DREAM technique. Exposure of flies to valencene decreased Or19a mRNA levels substantially and exclusively (**Figure 1D**). None of the other tested ORs showed any reduction in transcription.

We further analyzed the specificity of the DREAM technique by testing a ligand–receptor combination [Or49b and guaiacol (Dweck et al., 2015a)] in which the OR has been identified to possess a narrowly tuned ligand spectrum (de Bruyne et al., 2001; Hallem and Carlson, 2006; Marshall et al., 2010). Surprisingly, after the odorant treatment Or49b mRNA levels did neither show a downregulation nor a significant upregulation (**Figure 1E**). While exposure to guaiacol did not have a measurable influence on the expression levels of the Or49b gene, stimulation with this odorant did lead to an increase in the firing activity of ab6B OSNs in SSRs (**Figure 2C**), confirming the published ligand–receptor interaction of guaiacol and Or49b. We therefore asked whether a lack in downregulation of the receptor's transcription might be due to degradation of guaiacol during the duration of the odorant treatment. However, an analysis of the headspace in the experimental setup confirmed that guaiacol did not break down into other compounds and was present at an abundance comparable to those of ethyl lactate, geranyl acetate, and valencene (**Supplementary Figures S3A–D**).

Subsequently, we wanted to ascertain the effects of prolonged exposure to broadly activating odorants on the transcription of OR genes. High concentrations of odorants, comparable to rates used in the DREAM technique, have been shown to elicit, possibly unspecific, increases in the firing frequency of different broadly tuned OSNs, while lower concentrations of the same compound do not activate these OSNs to a significant degree (Hallem and Carlson, 2006; Kreher et al., 2009). Thus, we were interested in learning if broadly activating odorants used with the DREAM technique cause unspecific up- or downregulation in OR mRNA levels, particularly of those receptors being characterized as broadly tuned. Exposure to neither 3-methylthio-1-propanol, methyl butyrate, nor propyl acetate did coincide with a significant downregulation of any of the tested chemosensory receptor genes (**Figures 1F–H**). Instead, we observed significant increases in transcript levels of those chemosensory receptors, which are described to be either activated or inhibited by the tested odorants (**Figures 1F**–Or22a, Or35a; G–Or19a, Or22a, Or35a, Or47a; H–Or22a, Or35a). Interestingly, odorant treatment with 3-methylthio-1-propanol and propyl acetate did also lead to an upregulation in expression levels of chemosensory receptors electrophysiologically characterized as being unresponsive to these compounds (**Figures 1F**: Or19a, Or47b, Or67c, Ir31a; H: Ir31a).

So far, we had examined the effects of exposure to general, fruit and host odorants on the transcription levels of general ORs, but we were also interested in looking at a possible correlation between exposure to pheromones and changes in regulation patterns of the corresponding PRs. In *D. melanogaster*, OSNs expressing PRs have been shown to exhibit an age-dependent sensitization toward their ligands reaching a maximum after 7 days (Lin et al., 2016). Thus, instead of the previously used 0 to 3 h-old flies we used 5-days-old flies in our pheromone treatment series, in which we exposed the flies to 5% v/v of methyl laurate, a pheromone activating Or47b (Dweck et al., 2015b; Lin et al., 2016). When flies were exposed to 5% v/v of methyl laurate expression levels of the monitored chemosensory receptors, including Or47b, remained unchanged with the exception of Or67c mRNA levels, which were downregulated (**Figure 1I**). Again a screen for methyl laurate in the headspace of the experimental setup validated the presence of the odorant from the beginning to the end of the experiment at an abundance similar to those causing changes in the transcription levels of ORs in prior treatment series (**Supplementary Figure S3G**).

Finally, we were curious to learn if the DREAM technique could be utilized to deorphanize members belonging to the chemosensory receptor type family of IRs. The application of the DREAM technique for odorants activating IRs is limited by the chemical properties of the different odorants. At odorant concentrations used in the experimental setup of the DREAM

[2]neuro.uni-konstanz.de/DoOR/default.html

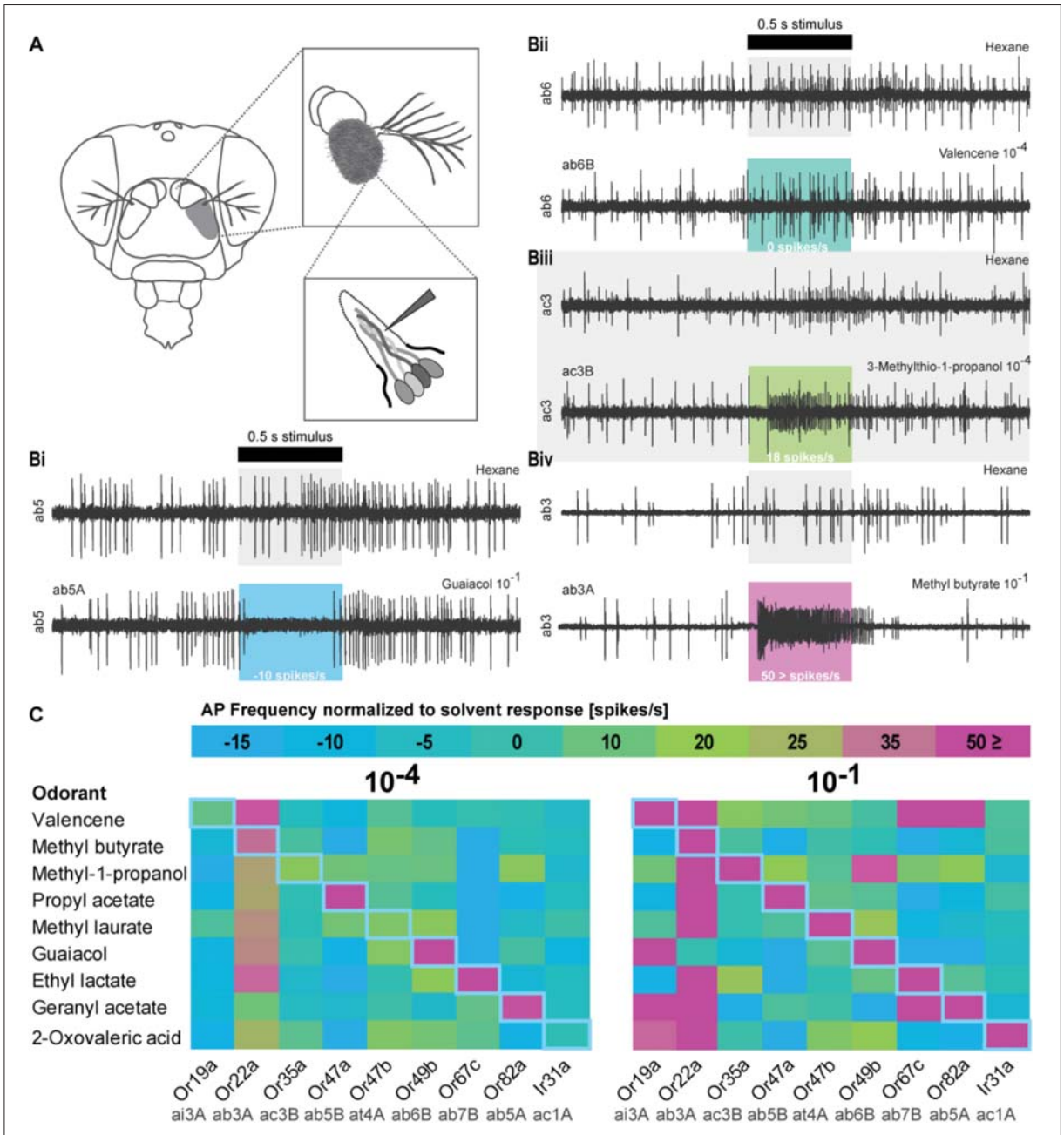

FIGURE 2 | Olfactory sensory neuron (OSN) responses after stimulation with odorants used in the DREAM technique. **(A)** Schematic drawing of Single Sensillum Recording (SSR) procedure. (Bi-Biv) Representative SSR traces of sensilla activity upon presentation of the respective solvents, an inhibitory interaction (guaiacol 10^{-1}, ab5A), no interaction (valencene 10^{-4}, ab6B), an excitatory interaction (3-methylthio-1-propanol 10^{-4}, ac3B), and a highly excitatory interaction (methyl butyrate 10^{-1}, ab3A). The black bar marks stimulus delivery and duration (0.5 s). Colored boxes correspond to heat map in **(C)**. The OSN response after subtraction of possible solvent responses is stated on the bottom of each colored box. **(C)** Color-coded average responses i.e., frequency of action potentials (AP) measured in SSRs to stimulation with odorants at a 10^{-4} and a 10^{-1} dilution ($n \geq 4$). Changes in spontaneous chemosensory receptor activity induced by used solvents have been subtracted from the recorded firing frequency. Numerical values can be found in **Supplementary Table S2**. Blue diagonal line represents expected, ideal results based on original publication (von der Weid et al., 2015).

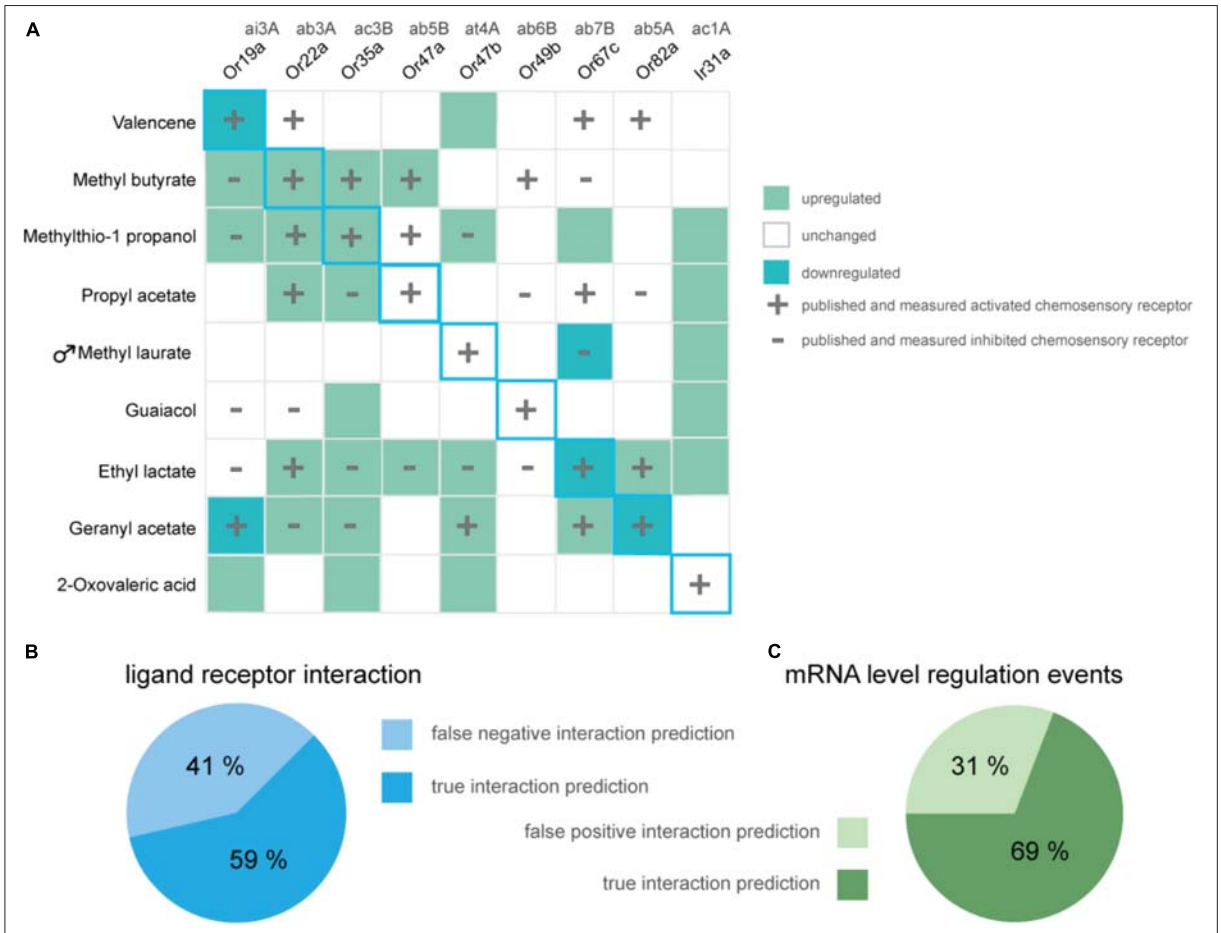

FIGURE 3 | Overview of alterations in chemosensory receptor gene transcript levels after odorant exposure. **(A)** Shown are significant increases (dark green) and decreases (light green) in transcription of chemosensory receptors *in Drosophila melanogaster* flies which were exposed to 5% v/v of displayed odorants for 5 h (One sample *t*-test; $n = 8$, exception Ir31a- geranyl acetate, $n = 4$). Unchanged expression levels are depicted in white. Data for previously characterized interactions of an odorant and chemosensory receptor are indicated with a plus (excitatory interaction) or a minus (inhibitory interaction), respectively. Blue diagonal line represents expected, ideal results based on original publication (von der Weid et al., 2015). **(B)** The pie chart as a whole represents all published and reproduced ligand–receptor interactions for odorants used in the DREAM treatments [plus and minus symbols in **(A)**], visualized are the percentages of overlaps between alterations in mRNA levels and Single Sensillum Recording data ("true interaction prediction") as well as unchanged receptor mRNA levels despite electrophysiologically proven ligand–receptor interactions ("false negative interaction prediction"). **(C)** Visualized are the percentages of all measured changes in receptor mRNA levels which show an overlap with published data for ligand-receptor interactions ("true interaction prediction") or which have occurred without electrophysiological proof for a ligand-receptor interaction ("false positive interaction prediction").

method, the compounds can develop a deleterious influence on the health of the treated flies, possibly leading to unspecific changes in the expression levels of a plethora of genes, including those of chemosensory genes, and/or the death of the tested flies. Hence, we focused on 2-oxo-valeric acid and Ir31a, an IR-ligand combination in which the odorant has no critical impact on the health of flies in the treatment group (**Supplementary Table S4**). Exposure to 2-oxo-valeric acid did not lead to changes in the transcription of the IR31a gene or of the Or22a gene both being chemosensory receptors shown to be activated by stimulation with this odorant [**Figures 1J**, **2C** (Silbering et al., 2011)]. However, we did observe an upregulation in the transcription level of Or19a as an OR being gated by 2-oxo-valeric acid but also an increase in transcription of Or35a and Or47b, both ORs being unresponsive to stimulation with this odorant in SSR (**Figure 2C**).

In summary, from eight tested general odorants, in three cases prolonged exposure to 5% v/v of the described (best) ligand successfully resulted in a decrease in transcription of the corresponding chemosensory receptor (**Figure 3A**, light green squares in diagonal center line). Interestingly, independent of an excitatory or inhibitory ligand–receptor interaction, we did find gene regulation, mostly increases in transcription (**Figure 3A**, dark-green squares) of known

interacting chemosensory receptors in all odorant treatment series (**Figure 3A**, overlap plus and minus symbol with colored squares). However, proven sensitivity to a certain odorant was no predictor for an alteration in mRNA levels of a chemosensory receptor analyzed with the DREAM technique (**Figure 3A**, plus or minus symbol no colored square). Of all described and measured known ligand–receptor interactions 59% of interactions were correctly predicted with the DREAM technique while 41% of interactions were falsely predicted to be negative ("false negative," **Figure 3B**). In some cases, exposure to odorants lead to unspecific changes ("false positive," **Figure 3C**) in transcription of chemosensory receptors prior being identified as unresponsive to those compounds (**Figure 3A**, colored square no plus or minus symbol). Furthermore, application of the DREAM technique to the previously untested chemosensory receptor types (i.e., PRs and IRs) did not result in changes in expression levels of described best ligand–receptor pairs, suggesting the DREAM technique is not applicable to these receptor types for novel deorphanization.

Validation of Ligand–Receptor Pairings With Single Sensillum Recordings

When we found neither up- nor downregulation for some of the described responsive chemosensory receptors after prolonged odorant exposure in the DREAM method, we next performed SSR to confirm the sensitivity of our chemosensory receptors set to all tested odorants (**Figure 2**). Moreover, we wanted to ascertain if we could explain the detection of upregulation events for chemosensory receptors which have been previously described as being unresponsive to the corresponding odorant, with unspecific interactions or artifacts of the high concentrations used in the DREAM technique. We screened the receptor set in SSR with an ecologically relevant odorant dilution of 10^{-4} and an odorant dilution of 10^{-1} (the latter of which was similar to amounts applied within the experimental setup of the DREAM technique) (**Figure 2C**). Recorded spiking frequencies were assigned to the individual OSNs housed in the corresponding sensillum according to differences in spike amplitudes (for details see section "Materials and Methods"). All odorants used in the treatment groups elicited a substantial increase ($\geq$50 spikes/s) in the frequency of OSN firing for the corresponding (best) ligand–receptor combination (**Figure 2C**: 10^{-1}, magenta rectangles). Furthermore, we were not able to attribute all alterations in the transcription of ORs described as unresponsive to unspecific interactions at high odorant concentrations based on our SSR data. We could not elicit changes in the receptor's firing frequency when stimulated with the corresponding odorant at a dilution of 10^{-1} (**Figure 1F**: Or19a, Or67c; C: Or47a, Or47b; J: Or35a). However, in two cases, we measured alterations in the expression levels of ORs that were previously characterized as being unresponsive to the tested odorant and observed discrepancies between published ligand spectra and response profiles based on our recordings at a dilution of 10^{-1} (**Figure 1C**: OR19a; J: Or47b). Finally, comparing the data generated from SSR with the expression levels of Ir31 after exposure to our nine tested odorants, we found that in all four cases of alterations in the IR gene's transcription (**Figures 1A,E,F,H**), stimulation with those odorants did not change the spiking frequency of the OSN in SSR.

Correlation of Alterations in Receptor Transcript-Levels to Either Excitatory or Inhibitory OSN Responses Upon Odorant Stimulation

After completion of our SSR screens, we were curious to learn if there was a correlation between receptor up- and downregulation following an excitatory or following an inhibitory interaction of the odorant used in the DREAM setup with the chemosensory receptor. In other words, does an excitatory ligand–receptor interaction lead to a decrease in mRNA levels of the corresponding receptor and an inhibitory interaction to an increase in transcription? We plotted the odorant treatment induced change in gene transcription against the increase or decrease in spiking frequency observed in SSR (**Supplementary Table S2**). Here, we found significant correlations between these two traits for all genes in which exposure to its best ligand did lead to a decrease in expression levels at odorant dilutions of 10^{-1} (Or19a, Or67c, and Or82a; **Supplementary Figure S4** and **Supplementary Table S3**). However, these correlations depended solely on the data point for the described best ligand interaction. If those data points were excluded from the data pool, the two traits were no longer correlated.

DISCUSSION

The characterization of ligand spectra of chemosensory receptors in *Drosophila melanogaster* has led to a comprehensive database of chemo-signals causing receptor activation or inhibition. Data collection of the ligand spectra for the chemosensory receptor has been a huge undertaking and took tremendous efforts from various laboratories over several decades (Münch and Galizia, 2016). Furthermore, functional characterization of chemosensory receptors has only been possible due to the exceptional availability of numerous genetic tools, like ectopic receptor expression with the Gal4-UAS system (Brand and Perrimon, 1993), and not least due to the ease of accessibility to chemosensory OSNs for electrophysiological recordings. In other non-model insect species, scientists are still struggling to identify ligand–chemosensory receptor combinations as most genetic tools are not yet available. However, a high-throughput characterization of ligand–receptor interactions would highly facilitate the identification of active odorants and their corresponding neuronal circuits involved in mediating ecologically relevant behaviors such as host or mate choice. The recently established DREAM technique (von der Weid et al., 2015) has the potential to be such a tool.

Using published ligand–receptor pairs in *D. melanogaster*, we tested whether DREAM can be used as a reliable tool for the prediction of ligand–receptor interactions of six narrowly and

broadly tuned general ORs, as well as one pheromone OR and one olfactory IR. In an ideal scenario, following the observation from the original study (von der Weid et al., 2015), we would have expected to find a downregulation in transcript levels for all eight of our ligand–OR pairs (**Figure 3**, diagonal blue outline). Moreover, if the decrease in expression levels was a general indicator for an excitatory interaction of odorant and receptor, we expected to measure a downregulation in mRNA levels not only for the interaction of a receptor and its best ligand but for all chemosensory receptors being activated by a corresponding compound (**Figure 3**, plus symbols).

We only found a decrease in gene transcript levels for three out of eight (best) ligand–OR combinations (**Figure 3A**, light green squares with blue outline). At the same time, we measured an increase in expression levels in two of the eight (best) excitatory ligand–OR pairs (**Figure 3A**; methyl butyrate-Or22a, methylthio-1-propanol-Or35a, dark-green squares with blue outline), while the expression levels of the remaining chemosensory receptors remained unchanged after odorant exposure. Taken together, we observed alterations in mRNA levels in five out of eight tested (best) ligand–OR pairings.

Following the conclusions of the original study (von der Weid et al., 2015), we hypothesized that the direction of changes in receptor mRNA levels (up- or downregulation) upon odorant exposure might be correlated to the mode of ligand–receptor interaction (inhibition vs. excitation). A current hypothesis is an adaptive modulating response of the OSN to possible excitatory overstimulation over an extended period of time, which would render the neuron less sensitive to lower odorant concentrations (von der Weid et al., 2015). This was, however, not observed in preliminary SSR experiments, where after 5–6 h of odorant exposure, the measured downregulation in OR gene transcript levels did not translate into changes of the corresponding OR's dose-response curve to the tested odorant (data not shown). The exact duration of conversion of "transcript to protein" is not known for each OR, and it is possible that the protein synthesis occurred in a different time window than what was monitored. Nevertheless, in several cases we also observed increases in the amount of OR mRNA levels after exposure to excitatory odorants; therefore, a simple adaptive, desensitizing response due to overstimulation seems unlikely (e.g., **Figure 3**, methyl butyrate: Or22a, methylthio-1-propanol: Or35a). We conclude that excitation of an OSN with its best ligand does not necessarily result in downregulation of gene transcription of the neuron's corresponding chemosensory receptor. For some ligand–receptor pairs, we found upregulation in gene transcription independent of receptor excitation or inhibition. We thus infer that alterations in chemosensory receptor expression levels following the DREAM technique are not indicative of the nature of the ligand–receptor interaction.

Since the modulation mechanisms induced by prolonged odorant exposure are not known, a correlation of the direction of alterations in receptor mRNA levels to other factors than the mode of ligand–receptor interaction are worth to consider: for instance a correlation to the ligand–receptor binding properties of odorant to the OR followed by possible induced conformational changes in the receptor, leading to differences in the receptor's properties. These induced changes in the receptor's characteristics could then define whether the odorant treatment of the DREAM method leads to an increase or decrease in mRNA levels of the corresponding receptor.

When we compared all characteristics and properties of those ORs where we observed gene transcription alterations upon odorant treatment, we were not able to find a common thread that would connect a successful application of the DREAM method, such as sensillum type, receptor specificity or chemical properties of the used ligands.

For all ligand–receptor pairings that did not show alterations in gene transcript levels (either up- or downregulation), we were still able to confirm active ligand-receptor interactions in SSR measurements. Additionally, we analyzed odorant stability and concentration during the long-term exposure experiments using SPME and we were able to demonstrate odorant integrity as well as presence at high concentrations until the end of the treatment (4 h exposure duration; **Supplementary Figure S3**). Therefore, we can exclude that the lack of alterations in gene transcription was due to inadequate ligand–receptor pairs or deficient odorant stimulation, as each odorant stimulated the receptor of interest and persisted without degradation at high concentration throughout the exposure duration of the DREAM method.

While we were not able to reproduce the trend of correlating an excitatory interaction to a reduction in chemosensory receptor mRNA levels for all ligand–receptor pairs and likewise inhibitory interactions to increases in receptor gene transcription, in 69% of observed regulatory events (**Figure 3C**), we found an overlap between expression alterations and electrophysiologically measurable ligand–receptor interaction.

A possible explanation for "false positives," that is, alterations in mRNA levels although the ligand did not interact with the receptor in SSRs (**Figure 3C** "false positives," e.g., **Figure 1D**: Or47b), could be due to the fact that odorant concentrations in neither our, nor the electrophysiological recordings from available datasets, were as high as those used in the DREAM technique. High concentration stimuli are not occurring in nature and thus outside the typical bounds of receptor function. A critical influence of odorant concentrations on the extent of changes in receptor mRNA levels was already noticed by the authors of the original study (von der Weid et al., 2015). It is thus possible that at concentrations present in the DREAM experimental setup, unspecific odorant and receptor interactions would occur, causing the observed "false positive" alterations in chemosensory receptor mRNA levels.

In cases in which we were not able to find alterations in gene transcript-levels upon odorant exposure despite having evidence for a ligand–receptor interaction ("false negative" predictions, i.e., no alterations in gene mRNA levels although the ligand did interact with the receptor in SSRs), the exposure duration of 5 h may have been too short to induce changes in gene transcription. When we tested this assumption and increased the exposure time to 10 h for guaiacol and its corresponding

receptor Or49b, we did indeed find a tendency toward downregulation of transcription (**Supplementary Figure S5**). Moreover, data from applications of the DREAM method in mice shows that the maximum impact of odorant exposure on the mRNA levels of the corresponding receptor occurs at different hours after the treatment has started, varying between tested ORs (von der Weid et al., 2015). Hence, exposure duration during the DREAM technique appears to be a critical factor that might have to be modified and adjusted for every ligand–receptor interaction, making the technique less applicable.

An additional factor that might hamper the applicability of the DREAM technique for the deorphanization of some olfactory receptors could be differences in transcriptional variability between genes in the olfactory system of *D. melanogaster*. Some ORs might underlie a strict expression and transcript-level control, while other ORs might be less tightly regulated. Transcript-levels of ORs could be regulated differentially between individual OR genes or OR gene groups via distinct post-transcriptional mRNA features, regulating translational repression and mRNA stability (Shum et al., 2015). The relatively small changes in gene mRNA levels following the DREAM treatment would be less prominent on the background of an already high transcriptional variability, making these alterations harder to detect.

There is thus room for customizing the parameters of the DREAM technique to expand its applicability to a broader set of ORs, perhaps even other chemosensory receptor classes like IRs. A starting point for modifications to the parameters of the DREAM method could be the choice of reference genes since we found at least effects of exposure to 3-methylthio-1-propanol on the expression of the reference gene ORCO (**Supplementary Table S5**). Adjusting the DREAM method in regards to odorant concentration or exposure duration in order to find alterations in the corresponding receptor's gene transcription in *D. melanogaster* is only possible due to the availability of extensive databases for ligand–receptors pairs, as it is known exactly which receptors should be affected by which ligand. In most other insect species such databases are of course not available. In the vinegar fly, the identification of new ligand–receptor combinations using the DREAM technique is also hindered by the fact that, according to our findings, there are false positive or non-specific regulatory events that can occur and some ORs seem to be unresponsive to the odorant treatment in regard to differences in expression. In mouse and rat the amount of "false positive" as well as "false negative" ligand–receptor interactions observed after odorant exposure was negligible (0%, von der Weid et al., 2015), and ORs proven to be activated by a ligand *in vivo* also consistently demonstrated alterations in transcript levels (Jiang et al., 2015; von der Weid et al., 2015; Ibarra-Soria et al., 2017). Hence, the DREAM technique seems to be well suited for the deorphanization of ORs in mammals, but less so in insects. When applied to an insect system for identification of possible ligand interaction partners, the relatively high amount of false positive predictions (31%) produced by the DREAM technique is less serious since these predictions when tested in heterologous expression systems or *in vivo* are easy to be characterized as false. However, the even higher amount of false negative predictions (41%) has a more severe impact on the applicability of the technique. The inability of detecting all interaction partners would lead to wrong and/or limited conclusions about ligand–receptor pairings and could prevent the elucidation of important ligand interaction partners.

CONCLUSION

We confirmed the findings from the original study on *D. melanogaster* regarding the down-regulation of Or67c and Or82a upon exposure to their corresponding agonists, ethyl lactate and geranyl acetate, respectively. However, based on our additional results from a broader array of ORs, it seems highly unlikely that the application of the same experimental conditions during the DREAM treatment will work for the deorphanization of a large set of ORs, neither in *D. melanogaster*, nor in other insect species where novel deorphanization is necessary. Further analyses of cases where DREAM does appear successful (such as Or19a-valencene and Or67c-ethyl lactate), may provide more rationale as to where and when this technique can be utilized or as to which parameters have to be modified for a reliable ligand–receptor interaction prediction. Consequently, with the current flaws in the applicability of the DREAM technique there is still no way around time-consuming olfactory receptor deorphanization via the well-established "empty neuron" or "decoder systems" and functional characterization in heterologous expression systems.

AUTHOR CONTRIBUTIONS

BH, MK, and SK conceptualized the study. MK, EG, SK, and LC planned the qPCR experiments. SK carried out all applications of the DREAM technique, the steps leading up to the qPCR experiments, and the qPCR runs themselves. LC and SK analyzed the qPCR data. IK, SK, and MAK performed electrophysiological recordings. SK carried out GC–MS data collection and analysis and performed computational analysis. SK and MK wrote the first draft of the manuscript and all authors reviewed and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00366/full#supplementary-material

FIGURE S1 related to Figure 1 | Comparison of chemosensory receptor mRNA levels after long-time odorant exposure **(A–J).** Analysis of receptor transcript-levels after DREAM treatment implementing two different approaches for the statistical interpretation of observed fold changes. Boxplots show the median (bold horizontal lines) with the interquartile range (whiskers). On the *x*-axis results from a One sample *t*-test against 1 are shown. Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). The gray area represents the "unresponsive zone," which is calculated based on a Gaussian distribution for all data points from described unresponsive chemosensory receptors to stimulation with the applied odorant (for more details see Materials and Methods). Each biological replicate, which includes a pool of RNA from 50 fly heads of mixed sex (ratio 1:1) or heads from males only **(F)**, is represented by one data point. For every odorant treatment the number of biological replicates was eight with the exception of the IR31a gene in the geranyl acetate treatment series **(B)** where $n = 4$. Highlighted between the two dotted lines are the best ligand–receptor pairs for the corresponding odorant treatment. Excitatory and inhibitory odorant interactions (doOR database and measured in this study) are indicated in magenta (excitatory) and cyan (inhibitory).

FIGURE S2 related to Figure 3 | Overview significant fold changes in chemosensory receptor mRNA levels when unresponsive zone is applied. Alterations induced by exposure to 5% v/v of displayed odorants in chemoreceptor gene transcription, which remain significant after application of an "unresponsive zone" (for more details see methods) are indicated in dark green (upregulation) and light green (downregulation). Unchanged expression levels are depicted in white. Data for previously characterized interactions of an odorant and chemosensory receptor are indicated with a plus (excitatory interaction) or a minus (inhibitory interaction), respectively. Blue diagonal line represents expected, ideal results based on original publication (von der Weid et al., 2015).

FIGURE S3 | Odorant integrity remains unchanged and odorant abundance is stable during long-time exposure of DREAM treatment **(A–I)** GC-MS profiles of the headspace in fly rearing vials without fly food and flies after 45 min (white background) and 4 h (light green background). In each panel, the odorant used in the DREAM treatment series is highlighted together with its solvent dimethyl sulfoxide (DMSO), with the exception of methyl laurate in which case the solvent methanol was already completely evaporated before the first headspace was collected **(G)**.

FIGURE S4 | Alterations in chemosensory receptor mRNA levels upon odorant treatment cannot generally be used to predict the nature of ligand-receptor interactions. **(A–J)** Correlation of up- and downregulation events in mRNA levels of Or19a, Or22a, Or49b, Or67c, and Or82a against olfactory sensory neuron spiking frequency upon odorant exposure and odorant stimulation at a dilution of 10^{-4} or 10^{-1}, respectively. Data points represent the individual odorants tested in the DREAM experiments. In each panel, the gray area indicates a confidence interval of 95%.

FIGURE S5 | A 10 h odorant exposure to guaiacol indicates a trend to mRNA level downregulation of the best ligand–receptor pairing which was previously unresponsive after an exposure duration of 5 h. Evaluation of chemosensory receptor mRNA levels after exposure to 5% v/v guaiacol for 10 h with qPCR. Each data point represents one biological replicate and consists of a pool of RNA from 50 *Drosophila melanogaster* heads with a mixed sex ratio of 1:1. The total amount of biological replicates equaled six. On the *x*-axis results from a One sample *t*-test against 1 are shown. Asterisks indicate significant differences (n.s. $P > 0.05$; *$P < 0.05$). Highlighted between the two-dotted lines is the best described chemosensory receptor for guaiacol. A cyan box indicates a described and measured inhibitory ligand–receptor interaction while a magenta box refers to a described and measured excitatory ligand-receptor interaction.

TABLE S1 | Primers used in qPCR experiments. Asterisks indicate primers identical to those from von der Weid et al. (2015).

TABLE S2 related to Figure 2 | Numerical values of Single Sensillum Recording measurements in spikes/s for odorants used in the DREAM treatment at a dilution of 10^{-4} and 10^{-1}.

TABLE S3 | Results of a correlation analysis between up- and downregulation in receptor mRNA levels and spiking frequency of olfactory sensory neurons expressing indicated receptor upon stimulation with odorants tested in the DREAM experiments.

TABLE S4 | Survival rate of 50 *D. melanogaster* flies exposed to dimethyl sulfoxide only (DMSO; control) or 5% v/v of oxovaleric acid in DMSO after 24 h in the DREAM experimental setup.

TABLE S5 | Differences between Cq (threshold cycle) of control and treatment samples for the two reference genes CAM and ORCO.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary information: Evaluation of the DREAM Technique for a High-Throughput Deorphanization of Chemosensory Receptors in *Drosophila*

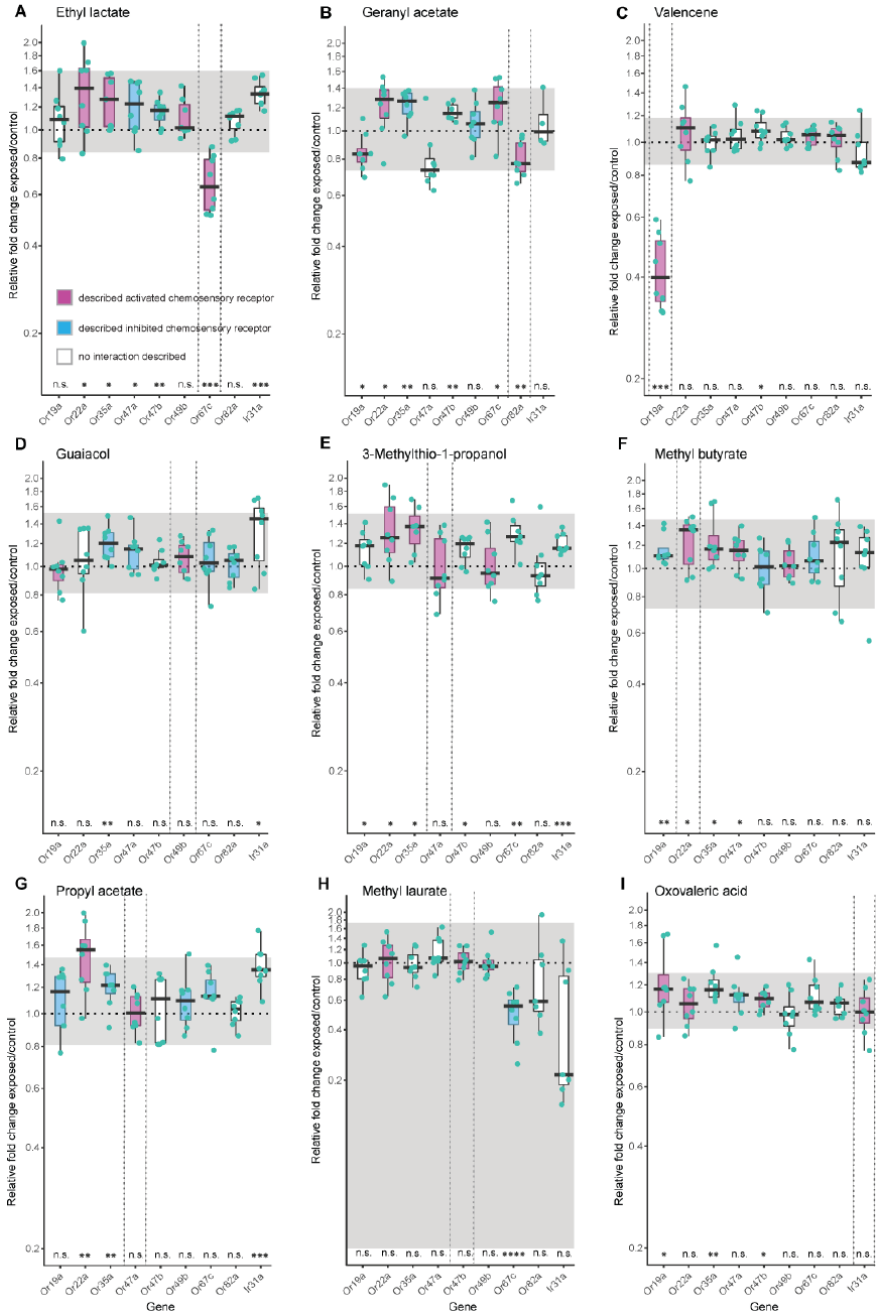

FIGURE S1 related to Figure 1 | Comparison of chemosensory receptor mRNA levels after long-time odorant exposure (A–J). Analysis of receptor transcript-levels after DREAM treatment implementing two different approaches for the statistical interpretation of observed fold changes. Boxplots show the median (bold horizontal lines) with the interquartile range (whiskers). On the *x*-axis results from a One sample *t*-test against 1 are shown. Asterisks indicate significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$). The gray area represents the "unresponsive zone," which is calculated based on a Gaussian distribution for all data points from described unresponsive chemosensory receptors to stimulation with the applied odorant (for more details see Materials and Methods). Each biological replicate, which includes a pool of RNA from 50 fly heads of mixed sex (ratio 1:1) or heads from males only (F), is represented by one data point. For every odorant treatment the number of biological replicates was eight with the exception of the IR31a gene in the geranyl acetate treatment series (B) where $n = 4$. Highlighted between the two dotted lines are the best ligand–receptor pairs for the corresponding odorant treatment. Excitatory and inhibitory odorant interactions (doOR database and measured in this study) are indicated in magenta (excitatory) and cyan (inhibitory).

Supplementary information: Evaluation of the DREAM Technique for a High-Throughput Deorphanization of Chemosensory Receptors in *Drosophila*

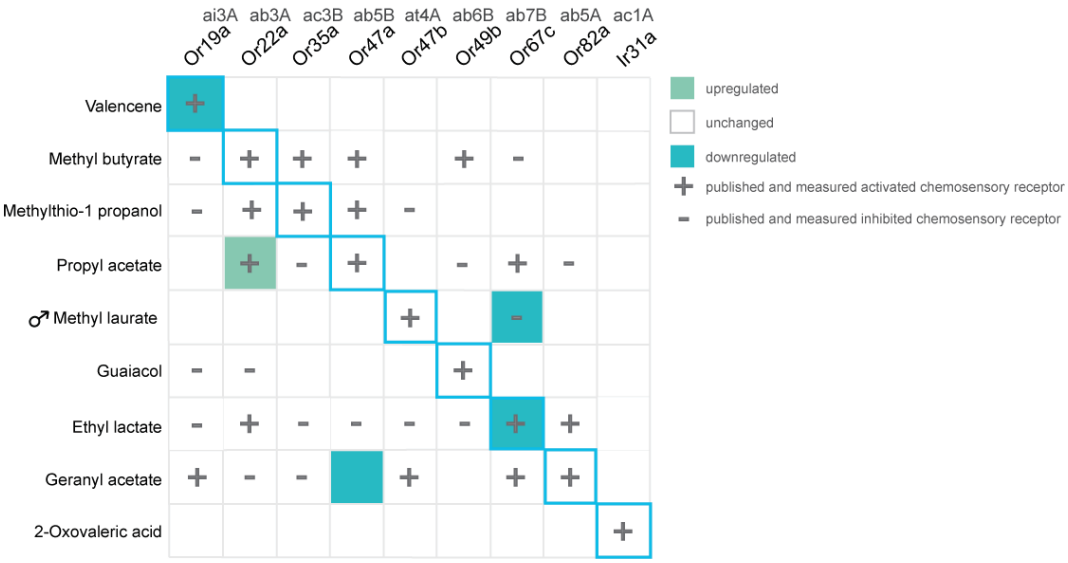

FIGURE S2 related to Figure 3 | Overview significant fold changes in chemosensory receptor mRNA levels when unresponsive zone is applied. Alterations induced by exposure to 5% v/v of displayed odorants in chemoreceptor gene transcription, which remain significant after application of an "unresponsive zone" (for more details see methods) are indicated in dark green (upregulation) and light green (downregulation). Unchanged expression levels are depicted in white. Data for previously characterized interactions of an odorant and chemosensory receptor are indicated with a plus (excitatory interaction) or a minus (inhibitory interaction), respectively. Blue diagonal line represents expected, ideal results based on original publication (von der Weid et al., 2015).

Supplementary information: Evaluation of the DREAM Technique for a High-Throughput Deorphanization of Chemosensory Receptors in *Drosophila*

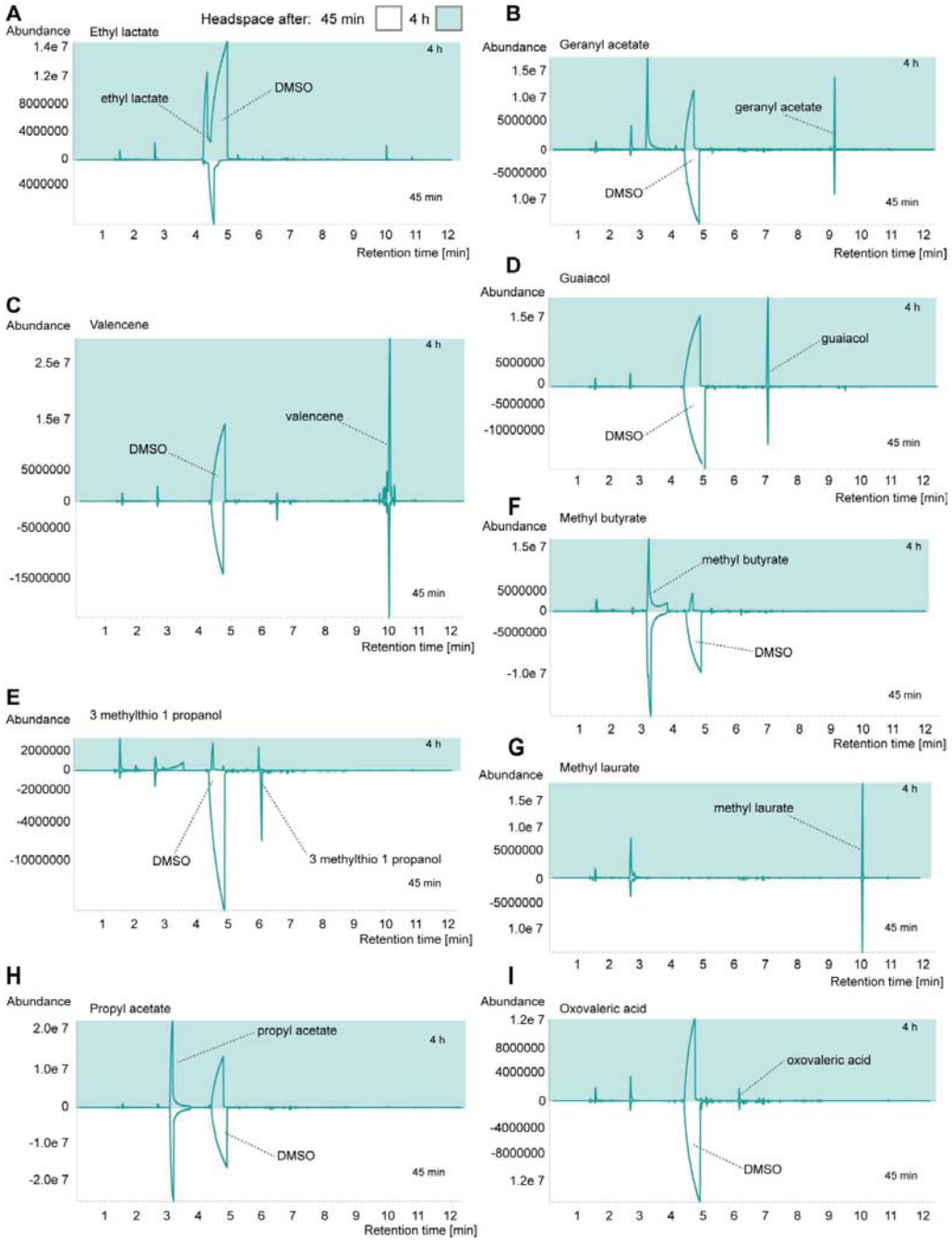

FIGURE S3 | Odorant integrity remains unchanged and odorant abundance is stable during long-time exposure of DREAM treatment (A–I) GC-MS profiles of the headspace in fly rearing vials without fly food and flies after 45 min (white background) and 4 h (light green background). In each panel, the odorant used in the DREAM treatment series is highlighted together with its solvent dimethyl sulfoxide (DMSO), with the exception of methyl laurate in which case the solvent methanol was already completely evaporated before the first headspace was collected (G).

Supplementary information: Evaluation of the DREAM Technique for a High-Throughput Deorphanization of Chemosensory Receptors in *Drosophila*

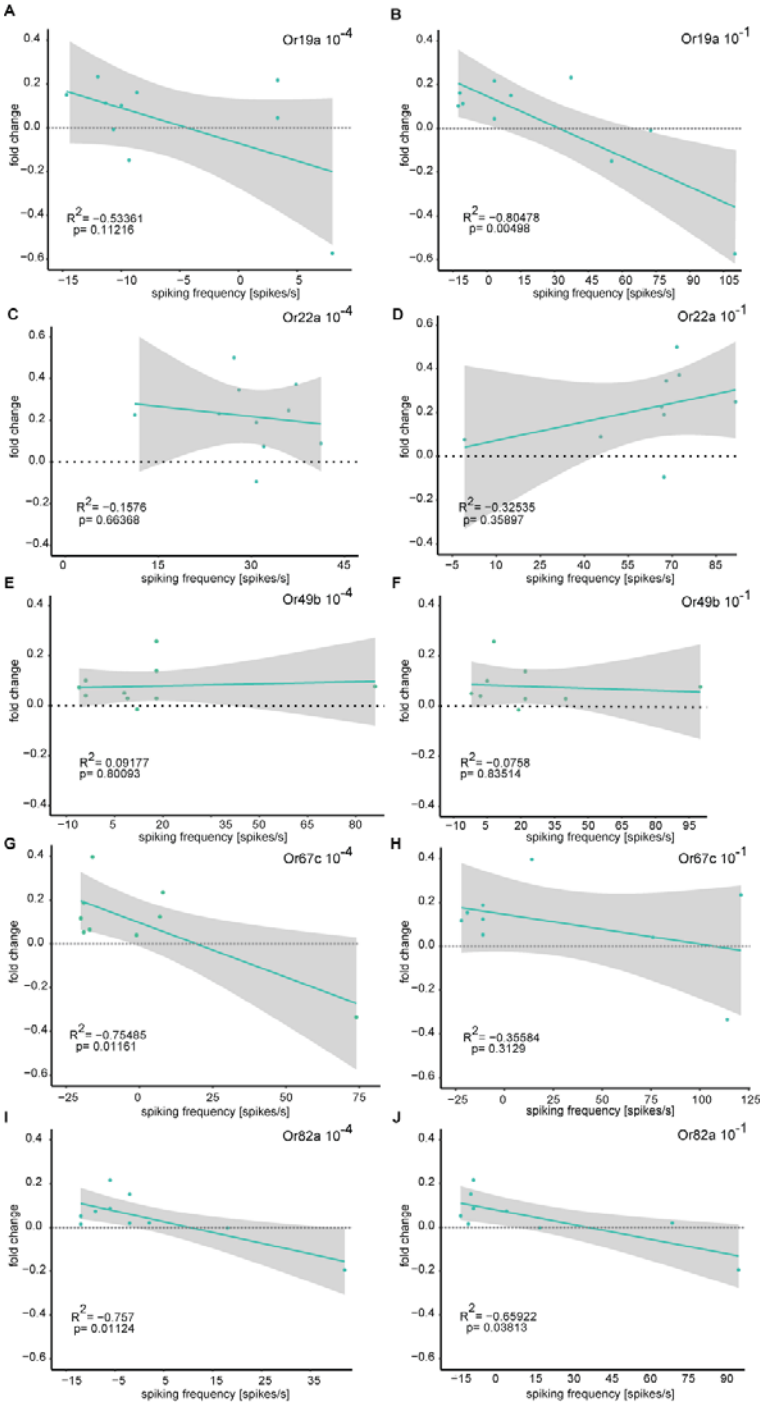

FIGURE S4 | Alterations in chemosensory receptor mRNA levels upon odorant treatment cannot generally be used to predict the nature of ligand-receptor interactions. (A–J) Correlation of up- and downregulation events in mRNA levels of Or19a, Or22a, Or49b, Or67c, and Or82a against olfactory sensory neuron spiking frequency upon odorant exposure and odorant stimulation at a dilution of 10^{-4} or 10^{-1}, respectively. Data points represent the individual odorants tested in the DREAM experiments. In each panel, the gray area indicates a confidence interval of 95 %.

Supplementary information: Evaluation of the DREAM Technique for a High-Throughput Deorphanization of Chemosensory Receptors in *Drosophila*

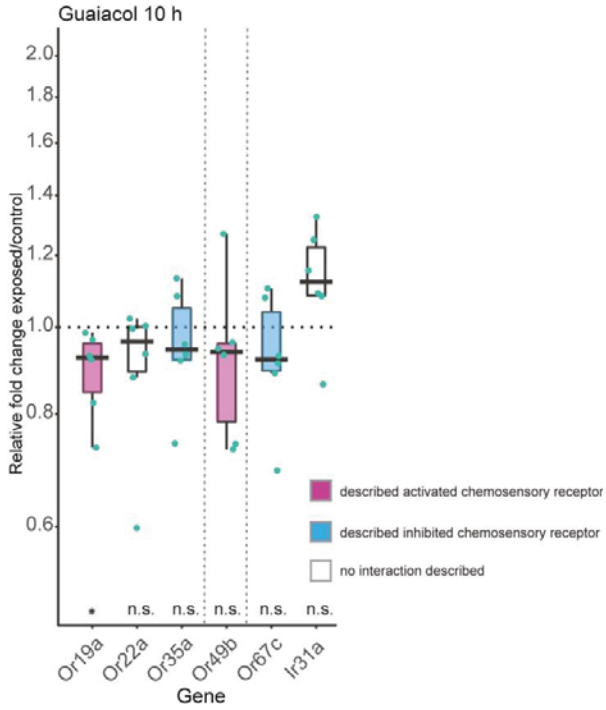

FIGURE S5 | A 10 h odorant exposure to guaiacol indicates a trend to mRNA level downregulation of the best ligand–receptor pairing which was previously unresponsive after an exposure duration of 5 h. Evaluation of chemosensory receptor mRNA levels after exposure to 5% v/v guaiacol for 10 h with qPCR. Each data point represents one biological replicate and consists of a pool of RNA from 50 *Drosophila melanogaster* heads with a mixed sex ratio of 1:1. The total amount of biological replicates equaled six. On the *x*-axis results from a One sample *t*-test against 1 are shown. Asterisks indicate significant differences (n.s. $P > 0.05$; $^{*}P < 0.05$). Highlighted between the two-dotted lines is the best described chemosensory receptor for guaiacol. A cyan box indicates a described and measured inhibitory ligand–receptor interaction while a magenta box refers to a described and measured excitatory ligand-receptor interaction.

Table S2, related to Fig. 2 Numerical values of Single sensillum recording measurements in spikes/s for odorants used in the DREAM treatment at a dilution of 10^{-4} and 10^{-1}

Odorant/chemosensory receptor	Or19a	Or22a	Or35a	Or47a	Or47b	Or49b	Or67c	Or82a	Ir31a
Valencene 10^{-4}	8	41	-4	-10	5	-4	-1	-2	-6
Methyl butyrate 10^{-4}	-9	36	3	-17	13	8	-20	-2	-4
Methyl-1-propanol 10^{-4}	-15	28	18	11	9	9	-16	18	-7
Propyl acetate 10^{-4}	-11	27	-2	56	5	-4	-26	-12	-4
Methyl laurate 10^{-4}	3	31	-3	12	15	18	-19	-6	1
Guaiacol 10^{-4}	-11	32	-1	-12	16	86	-17	2	-10
Ethyl lactate 10^{-4}	-10	37	-7	-19	-1	18	74	-9	-4
Geranyl acetate 10^{-4}	-9	11	-5	-7	1	-6	8	42	-4
2-Oxovaleric acid 10^{-4}	-12	25	6	-17	15	12	7	-12	-2
Valencene 10^{-1}	110	46	17	11	9	2	76	70	3
Methyl butyrate 10^{-1}	-12	92	3	-13	1	-2	-22	-9	3
Methyl-1-propanol 10^{-1}	11	68	109	19	5	40	14	18	-8
Propyl acetate 10^{-1}	-11	72	-1	105	7	5	-19	-10	2
Methyl laurate 10^{-1}	3	67	-1	3	63	22	-11	-8	-4
Guaiacol 10^{-1}	73	-1	-9	-35	5	100	-36	-42	-6
Ethyl lactate 10^{-1}	-13	72	23	-20	-1	8	114	5	-3
Geranyl acetate 10^{-1}	55	66	-14	3	1	-40	121	96	-9
2-Oxovaleric acid 10^{-1}	37	130	1	-16	15	19	-11	-13	53

Table S3 Results of a correlation analysis between up- and downregulation in receptor mRNA levels and spiking frequency of olfactory sensory neurons expressing indicated receptor upon stimulation with odorants tested in the DREAM experiments.

Chemosensory receptor/ SSR data-dilution series	Correlation coefficient	**p-value**	Significance level
Or19a 10^{-4}	-0.53361	0.11216	n.s.
Or19a 10^{-1}	-0.80478	0.00498	**
Or22a 10^{-4}	-0.1576	0.66368	n.s.
Or22a 10^{-1}	0.32535	0.35897	n.s.
Or35a 10^{-4}	0.49894	0.14209	n.s.
Or35a 10^{-1}	0.56733	0.08718	n.s.
Or47a 10^{-4}	-0.2998	0.40002	n.s.
Or47a 10^{-1}	-0.52137	0.12223	n.s.
Or47b 10^{-4}	0.46007	0.02127	n.s.
Or47b 10^{-1}	-0.8589	0.08261	n.s.
OR49b 10^{-4}	0.09177	0.80093	n.s.
Or49b 10^{-1}	-0.08589	0.83514	n.s.
Or67c 10^{-4}	-0.75485	0.01161	*
Or67c 10^{-1}	-0.35584	0.3129	n.s.
Or82a 10^{-4}	-0.7570	0.01124	*
Or82a 10^{-1}	-0.65922	0.03813	*
Ir31a 10^{-4}	-0.33111	0.35003	n.s.
Ir31a 10^{-1}	-0.34603	0.32736	n.s.

Table S4 Survival rate of 50 *D. melanogaster* flies exposed to dimethyl sulfoxide only (DMSO; control) or 5 % v/v of oxovaleric acid in DMSO after 24 h in the DREAM experimental setup.

	Survival after 24 h [%]	
Vial Nr.	**Control**	**Oxovaleric acid**
1	100	100
2	100	100
3	100	100
4	99	100
5	100	100
6	100	100

Table S5 Differences between Cq (threshold cycle) of control and treatment samples for the two reference genes CAM and ORCO.

Odorant		Reference gene ΔCq														
Ethyl lactate	CAM	0.11	0.13	0.42	0.02	0.13	0.92	0.22	0.18	0.17	0.38	0.19	0.07	0.19	0.48	0.25	0.32
	ORCO	0.83	0.86	0.32	0.15	0.92	2.43	0.12	0.22	0.75	0.87	0.20	0.43	1.00	1.90	0.23	0.13
Geranyl acetate	CAM	0.14	0.39	0.55	0.40	-0.68	0.30	0.04	0.36	0.17	0.38	0.19	0.07	0.19	0.48	0.25	0.32
	ORCO	0.64	0.61	0.80	0.74	-0.04	0.72	0.28	0.38	0.75	0.87	0.20	0.43	1.00	1.90	0.23	0.13
Valencene	CAM	0.14	-0.03	-0.08	-0.17	0.28	0.22	0.34	0.31	0.13	0.10	0.14	-0.09	0.01	0.36	0.09	0.60
	ORCO	-0.21	-0.07	0.14	-0.05	0.01	0.18	0.45	0.45	0.02	0.06	0.35	-0.07	0.01	0.08	0.66	0.39
Guaiacol	CAM	0.53	0.22	0.40	0.43	0.41	0.08	-0.11	-0.18	0.37	0.43	0.34	0.50	0.26	0.12	-0.12	0.39
	ORCO	0.51	1.13	0.23	0.78	0.45	0.21	0.29	0.01	0.22	0.76	0.45	0.42	0.30	-0.15	0.29	0.62
3-Methylthio-1-propanol	CAM	0.35	0.14	0.15	0.86	0.72	0.03	0.65	0.32	0.86	0.44	0.35	0.22	0.15	0.37	0.61	0.45
	ORCO	0.53	0.38	0.49	1.21	0.71	0.21	1.37	1.36	0.98	0.59	0.14	0.38	0.50	0.61	1.20	0.79
Methyl butyrate	CAM	0.51	0.51	0.35	0.37	0.12	0.08	0.35	0.37	0.58	0.14	0.52	0.54	0.07	0.19	0.68	0.41
	ORCO	0.21	0.71	0.41	0.73	0.24	0.67	0.41	0.73	0.02	0.54	0.46	0.79	0.26	0.74	0.87	0.79
Methyl laurate	CAM	-0.11	0.36	0.78	-0.04	-0.18	2.03	-0.84	-0.05	-0.37	0.17	0.85	0.12	-0.13	2.20	0.90	0.38
	ORCO	-0.02	0.15	1.34	0.66	-0.18	2.12	-0.42	0.01	1.33	0.00	-0.13	2.09	-0.56	0.15	0.21	0.16
Oxovaleric acid	CAM	-0.19	0.09	0.41	0.24	0.69	-0.04	0.34	0.28	-0.25	-0.13	0.51	0.63	0.52	0.10	0.33	0.58
	ORCO	-0.19	0.37	0.79	0.62	0.99	0.09	0.77	0.82	-0.14	0.57	0.62	0.81	0.69	0.18	0.60	0.94

Manuscript IV

Inverse resource allocation between vision and olfaction across the genus *Drosophila*

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Inverse resource allocation between vision and olfaction across the genus *Drosophila*

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Divergent populations across different environments are exposed to critical sensory information related to locating a host or mate, as well as avoiding predators and pathogens. These sensory signals generate evolutionary changes in neuroanatomy and behavior; however, few studies have investigated patterns of neural architecture that occur between sensory systems, or that occur within large groups of closely-related organisms. Here we examine 62 species within the genus *Drosophila* and describe an inverse resource allocation between vision and olfaction, which we consistently observe at the periphery, within the brain, as well as during larval development. This sensory variation was noted across the entire genus and appears to represent repeated, independent evolutionary events, where one sensory modality is consistently selected for at the expense of the other. Moreover, we provide evidence of a developmental genetic constraint through the sharing of a single larval structure, the eye-antennal imaginal disc. In addition, we examine the ecological implications of visual or olfactory bias, including the potential impact on host-navigation and courtship.

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A pivotal question in neuroscience focuses on how the morphology and structure of the brain relates to its function and thereby its behavioral relevance. Neuroscience in general utilizes a wide array of techniques, including both genetics and neuroanatomical imaging, in order to unravel neural mechanisms underlying animal behavior and to understand how these circuits translate into the natural behaviors that are associated with an animal's specific ecological niche, for example, in regard to decisions concerning host navigation or mate selection[1].

One of the ultimate goals of neuroethology is to understand the principles organizing and defining these complex neural circuits, both from an ecological as well as an evolutionary perspective, and to decipher how the brain processes information while guiding behavioral responses toward naturally occurring stimuli. Previous research has supported the notion that structural size in a sensory phenotype correlates with its functional significance, for example, the reduction of sight in cave fish[2,3], the enlarged ears of echolocating bats[4–6], or the enlarged eyes of predatory birds[7]. Moreover, neuroanatomical studies have also shown that the size of each brain region corresponds to the organism's morphological specialization, thus for example, the smaller the eyes, the less importance of visual stimuli, and the smaller the brain region dedicated toward vision[2,3]. Other studies have also sought to associate sensory size with behavioral or ecological importance, such as the enlarged male-specific macroglomerular complex (MGC) in the Lepidoptera[8,9], the enlarged DM2 glomerulus in *Drosophila sechellia*[10], or an enlarged glomerulus based on the number of OSNs or synapses[11,12]. In each of these cases, the enlarged structure is indicative of the importance of a particular ecological stimulus, and moreover, that the relative morphological size of a sensory structure relates to its importance. However, just as studying a single neuron will not be sufficient to understand the function of the whole brain, the study of a single animal species will not be sufficient to address overarching ecological and evolutionary questions. Consequently, as the field of neuroethology moves in the direction of understanding and incorporating the roles of multimodal signals for behavioral decision-making (i.e., visual, olfactory, gustatory, mechanosensory, and auditory cues), similarly, neuroethology is also beginning to examine a multitude of closely related animal species for evolutionary comparisons of morphology, behavior, and adaptation[13–15], which can help identify the selective pressures that drive these changes in sensory systems and neural development or neural plasticity.

One of the original genetic model organisms, the vinegar fly, *Drosophila melanogaster*, has been a workhorse of advanced genetics for the last several decades. The advantage of this invertebrate model is attributed to its short generation time, ease of colony establishment in the laboratory, the huge diversity of available molecular and genetic tools, as well as the immense efforts toward the complete mapping of neural circuits for both the adult and the larvae of this one species[16–18]. However, the genus *Drosophila* also provides between 1200 and 1500 individual species, with an ecology spanning nearly every imaginable environment and host choice, from deserts to forests, from islands to mountains, and across incredibly unique or specialized food resources, such as the gills of land crabs, protein sources within bat guano, or otherwise toxic fruits;[10,15,19–21] therefore, the potential to transform an already powerful model organism from a singular species into an entire genus is now possible due to the recent advances in cellular and genetic tools for examining the complex neurological mechanisms of natural behavior in novel, non-model species. Moreover, the expansion from a single species into an entire genus affords scientists the opportunity to address larger ecological, developmental, and evolutionary questions using the full gamut of molecular and genetic tools that have already been generated for *D. melanogaster*. Research into non-*melanogaster* species is already well underway, with researchers beginning to highlight individual species, often selecting those based on economic impact or behavioral specialization[22–27], with studies now also including CRISPR-cas9, the powerful gene editing tool, such as the studies in *D. suzukii*, *D. subobscura*, *D. simulans*, and *D. pseudoobscura*[28–31].

An emerging integrative field of the biological study, called ecological evolutionary developmental biology, or more commonly known as eco-evo-devo, focuses on the underlying interactions between an organism's environment, its genes, as well as its development in regard to how these three factors shape evolutionary trends and help create a map or framework for better understanding and predicting speciation[32–35]. The field of eco-evo-devo is built on the premise that evolution is animal development controlled by ecological and environmental forces. Thus with the above-mentioned factors in mind, one of the goals of the present study is to encourage the expansion of the *D. melanogaster* model to become the *Drosophila* system, and thereby encompass a broader array of species within this genus for comparative, ecological research into what drives the evolution of the nervous system.

Based on the many examples from the animal kingdom as well as our previous observations from a number of Drosophilid species[27,36], we set out to test the hypothesis that sensory systems occupy a restricted niche in the nervous system of these flies, where relative size and energy allocation prevents one sense from expanding without having an effect on another. Also, as an entry to creating a larger ecological and evolutionary framework for this genus of flies, our study samples a wide, phylogenetic array of 62 different species within the genus *Drosophila*, and begins to analyze both host navigation and mate selection or courtship with regard specifically toward visual and olfactory sensory modalities. This study includes investigation at the periphery, such as morphometrics of the antenna and compound eye, as well as measurements within the antennal lobe (AL), optic lobe (OL), and the central brain for each selected species. This phylogenetic comparative approach allows for a more precise study of adaptation, and making these interspecific comparisons allows us to assess the general rules governing evolutionary phenomena via observations of repeated, independent evolutionary events within a group of organisms.

In our study, we identify a consistent, inverse resource allocation between vision and olfaction across these 62 species, and we use a combination of phylogenetic, phenotypic as well as developmental data in order to examine the evolutionary pressures and constraints underlying this potential tradeoff between two critically important sensory structures in regard to both host navigation and mate selection.

Results

Phylogeny, species selection, and general morphometrics. An array of 62 species within the Dipteran family Drosophilidae were selected to span the diversity contained within the genus *Drosophila* (Fig. 1a, b). This genus of flies covers a multitude of hosts and host ranges, including examples such as rotten fruits, cacti, flowers, tree sap, and mushrooms. Each species was measured for a number of physical metrics, including body size, head size, eye surface area, and the surface area of the third antennal segment (the funiculus) (Supplementary Figure 1A). In general, there was a huge variety of physical sizes noted within this single genus of flies, providing much more variability in absolute or overall size between species than we initially anticipated. Not surprisingly, as fly species increased in either body or head size, eye surface area and funiculus surface area

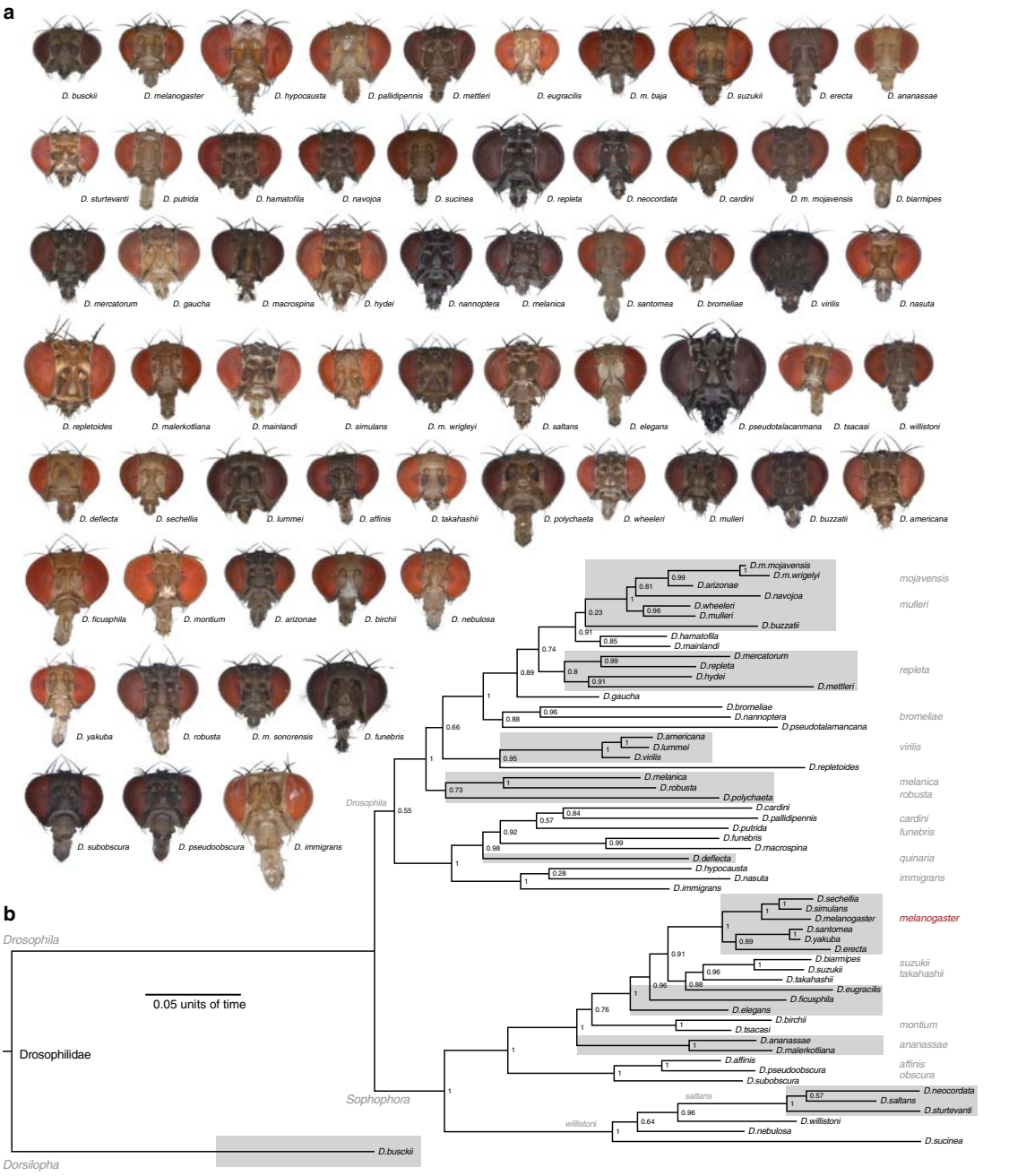
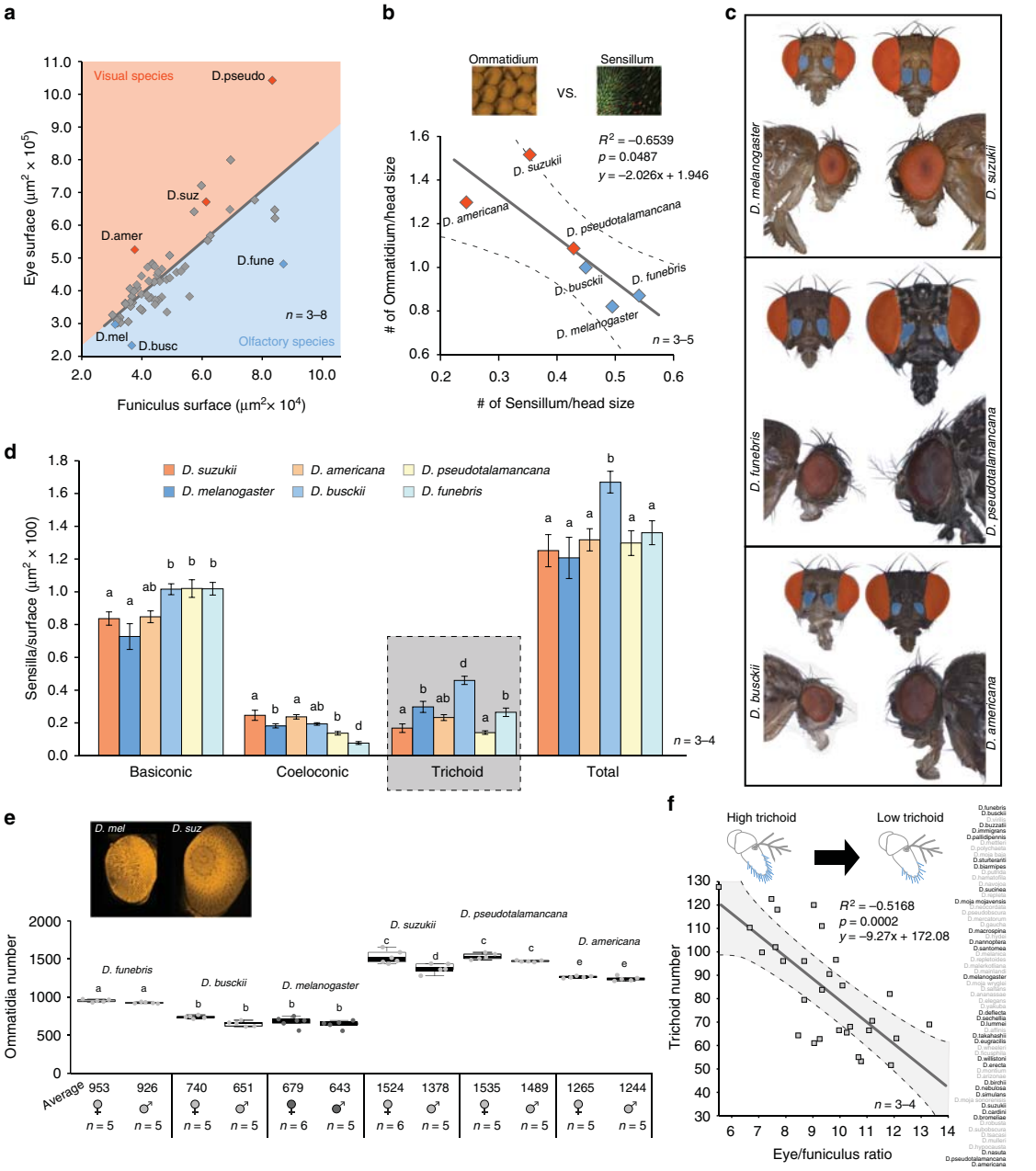


Fig. 1 Frontal head images of all tested *Drosophila* species and their associated phylogeny. **a** Frontal view of the head of all 62 species, illustrating the diversity in overall size, as well as in the variance of the visual and olfactory sensory systems across this genus. Also worth noting is the disparity in pigmentation that extends across the whole head, including the antenna and the compound eye. **b** Phylogeny of 59 species of *Drosophila* where genetic material was available for use in this study (*D. montium* and two subspecies of *D. mojavensis* are missing). Species were selected to span the width of subgroups and represent the genetic diversity within this genus of insect. Some species are denoted with gray boxes to provide more visual separation between subgroups. (Data are provided at https://doi.org/10.17617/3.1D)



both increased as well, with head size always having a tighter positive correlation than body size for both eye and antennal metrics (Supplementary Figure 1). However, there was also quite a bit of variability in these sensory structures, both among similar body sizes and between flies with similar eye or funiculus sizes (Supplementary Figure 1). Here, we found that the eye and funiculus surface area scale isometrically with respect to both the body and head measurements (Supplementary Figure 1H); moreover, that the variance in these two sensory systems could not be explained by the absolute size of a species.

Ommatidium and sensillum comparisons among main species. For more in-depth comparison, we next sought to compare the sensory regions associated with visual and olfactory stimuli (Fig. 2a), and while again there was a general trend across the 62 species that larger insects had both larger eye surface area and larger funiculus surface area, there was still significant variability between these two sensory systems that was not explained by body or head size alone (Supplementary Figure 1H, I). From our robust array of species, we selected six Drosophilids for a more in-depth analysis of their sensory structures (Fig. 2a). These six

Fig. 2 External comparison of visual and olfactory system. Red color signifies vision or predicted visual bias, while blue indicates olfaction or potential olfactory bias. **a** All 62 species measured for eye and funiculus surface area, where six species were selected for additional measurements. These flies were selected to compare species with similar antennal surface area but contrasting eye sizes (e.g., *D. pseudotalamancana* and *D. funebris*, or *D. americana* and *D. busckii*) or species with similar eye size but contrasting antennal sizes (e.g., *D. americana* and *D. funebris*). We also selected two well-established species, *D. melanogaster* and *D. suzukii*, for an additional comparison and points of reference. **b** Inverse correlation between ommatidium number and sensillum number when corrected for head size from six species of *Drosophila*, suggesting a possible tradeoff between these sensory systems at the periphery. **c** All species were photographed for more detailed measurements of eye and antennal features across several frontal and lateral views. Highlighted in blue are the antennal surface area, and in red, the eye surface area. **d** Shown are the sensillum density metrics taken from stacked lambda mode scans (maximum intensity projections) of the anterior portion of the antenna for all six species examined, identifying strong differences for example in trichoid sensillum density, where potentially olfactory biased species (in blue) showed the significantly larger trichoid densities. Error bars represent standard deviation. **e** Ommatidium counts from each species, which illustrates the large differences in visual capabilities across this genus of fly, with some species having 2–3 times larger eyes. Boxplots represent the median (bold black line), quartiles (boxes), as well as the confidence intervals (whiskers). **d**, **e** Means with the same letter are not significantly different from each other (ANOVA with Tukey–Kramer multiple comparison test). **f** Expanded study to include additional species (that were selected using stratified random sampling), where we show that trichoids are consistently and inversely correlated with increasing eye-to funiculus ratio across the entire genus. (Data are provided at https://doi.org/10.17617/3.1D)

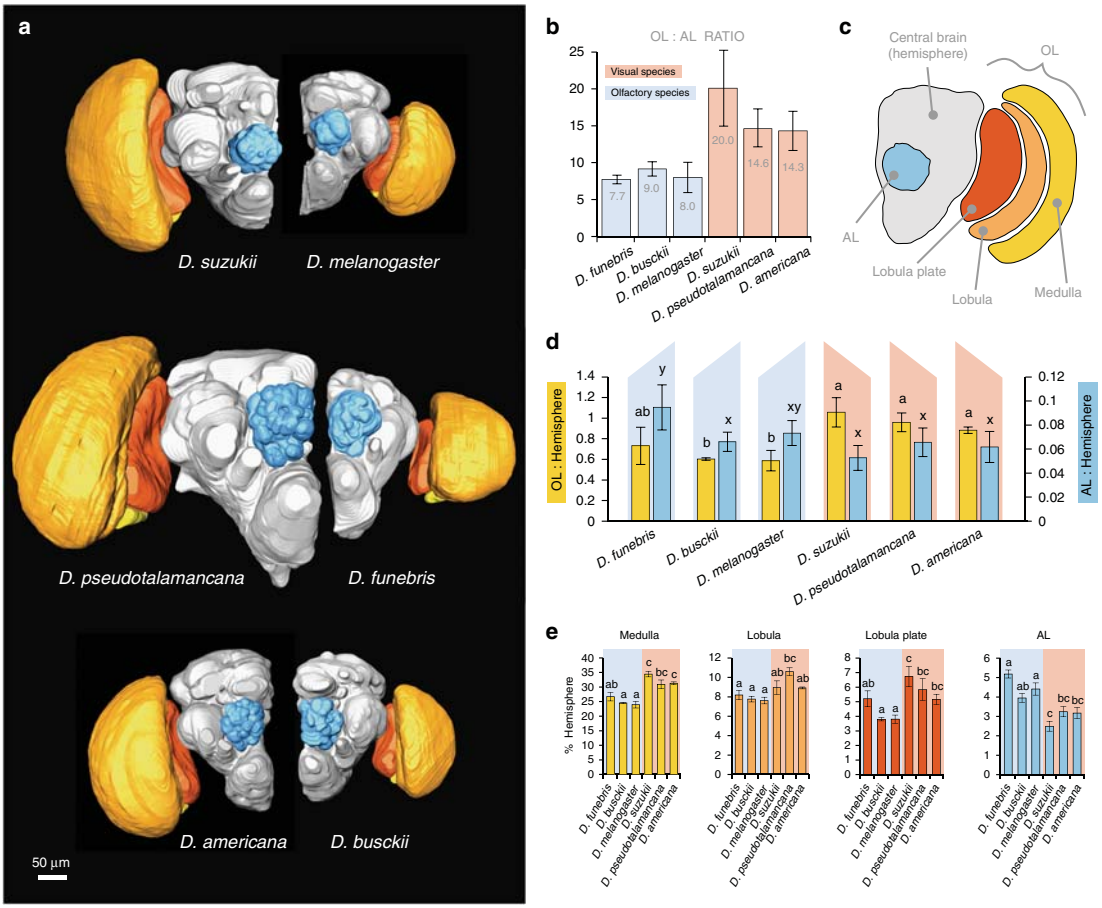
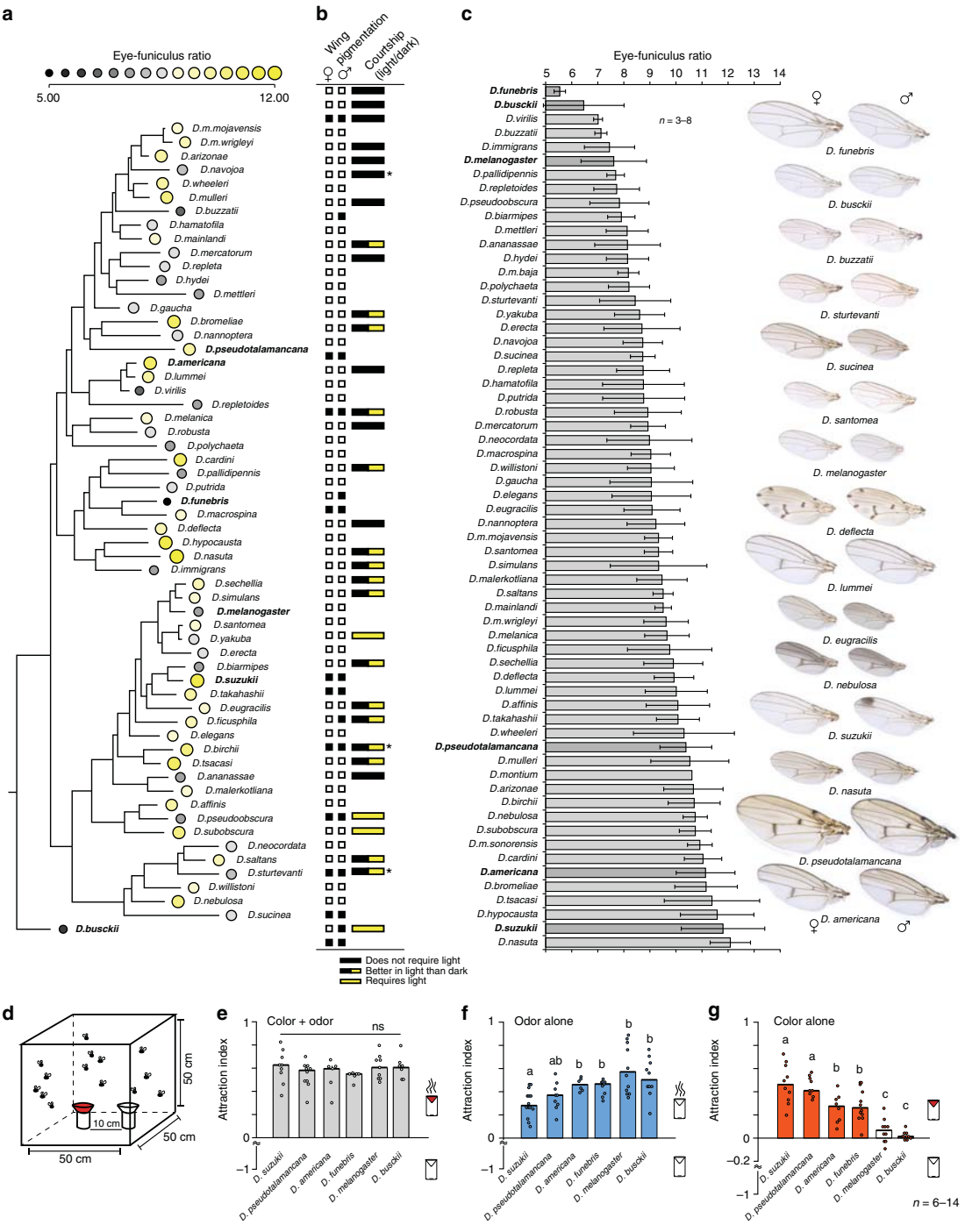


Fig. 3 Three-dimensional reconstructions of the visual and olfactory neuropils in six *Drosophila*. Red to yellow (warm) color signifies vision or visual bias, while blue indicates olfaction or olfactory species. **a** Whole brain reconstructions, highlighting visual (yellow to red) and olfactory (blue) regions, with central brain in gray. **b** The optic lobe (OL) to antennal lobe (AL) ratio for each species, showing the division between olfactory and visual bias among species. **c** Diagram of all measured volumes for comparison between species. **d** Relative sizes of OL (yellow) and AL (blue) as compared to the central brain, where the data show an inverse correlation between visual or olfactory investment. **e** Separate regions of OL and AL that were measured as a percentage of the central brain to provide a comparable value between insects of differing absolute size, again highlighting that brain regions mirror external measurements of visual or olfactory size bias. **d**, **e** Means with the same letter are not significantly different from each other (ANOVA with Tukey–Kramer multiple comparison test). Error bars represent standard deviation. (Data are provided at https://doi.org/10.17617/3.1D)



species were selected as either having similar funiculus size, but disparate eye size (i.e., *D. americana* and *D. busckii*; *D. pseudotalamancana*, and *D. funebris*), or vice versa (e.g., *D. americana* and *D. funebris*) (Fig. 2a). We also included *D. melanogaster*, given its prevalence in this genus as a model organism, and we included *D. suzukii*, as it has risen to become both an important invasive species for agricultural research as well as an important model for evolutionary neuroethology.

We were interested in documenting any drastic differences in sensory structures beyond surface area (Fig. 2a, c), and we next

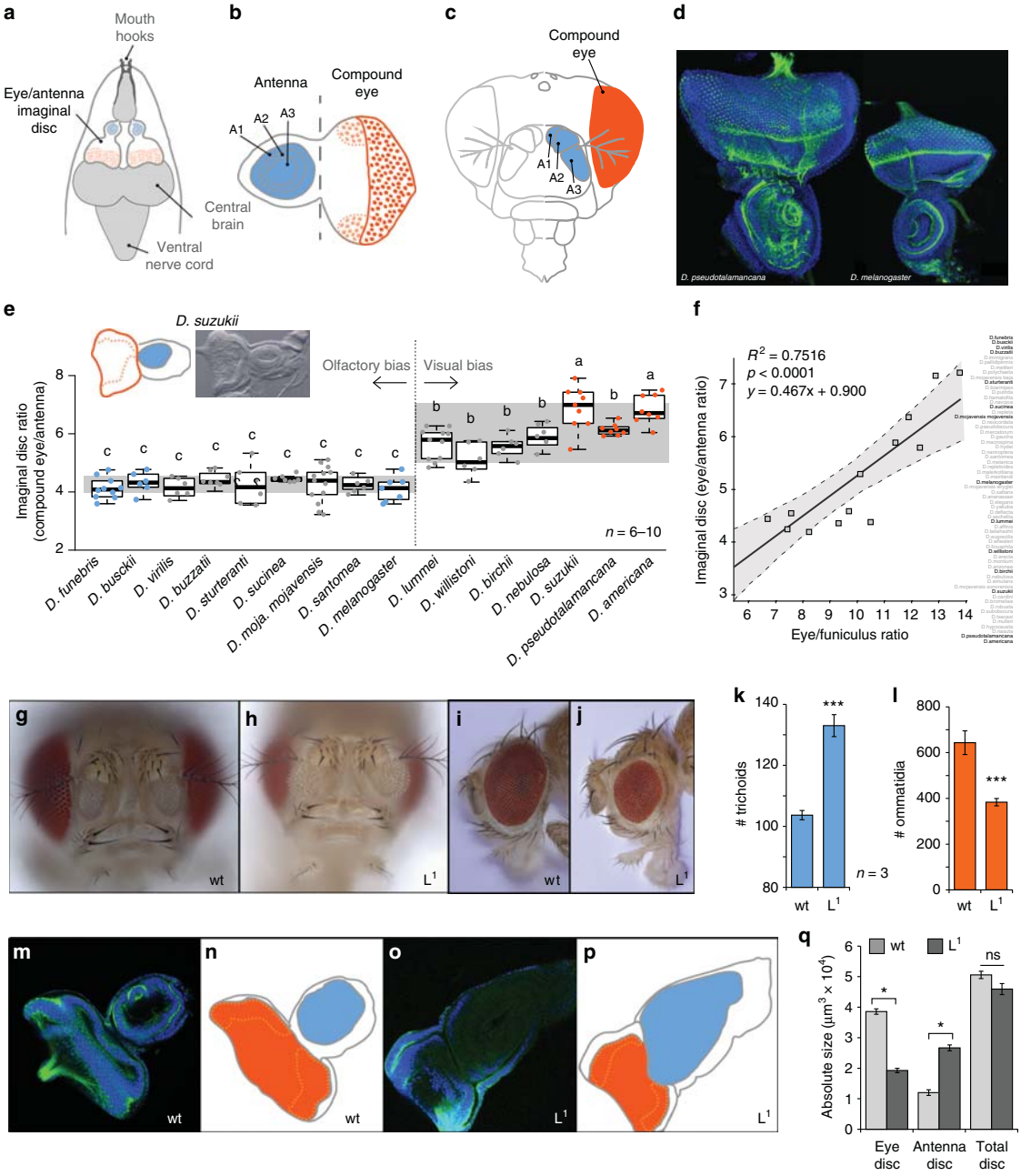
Fig. 4 Host navigation and courtship differences across *Drosophila*. **a** Molecular phylogeny for 59 species that includes the eye-to-funiculus trait (EF ratio), which is visualized by both dot size and color. Two statistical tests (Blomberg K and Pagel's lambda) reveal that this sensory trait is not strongly supported by the phylogeny (K = 0.478, $p = 0.041$; $\lambda = 7.102e^{-05}$, $p = 1$). We note large variance within subgroups, and across habitat or ecological niche. **b** There was a significant correlation between both male/female wing pigmentation and EF ratio after phylogenetic correction ($p = 0.043$ and $p = 0.026$, respectively), suggesting that larger eyes correlate with pigmentation, which is not explained by phylogeny. Also shown are courtship values for mating pairs within light/dark environments, where light-based courtship is strongly correlated with larger EF ratio after phylogenetic correction ($p = 2.406e-07$), suggesting larger eye ratios correlate with visual mating. Asterisk indicates new data from this study. All other data from refs. [81–92]. **c** All 62 species arranged according to EF ratio, with wing pigmentation examples (standard deviation shown). **d** Diagram of behavioral assay used to test navigation of each species towards visual and olfactory objects. **e**–**g** Attraction indices for each species when stimuli were presented **e** together, **f** with odor alone, or **g** with visual target alone. While all species perform equally well when both odor and visual object are presented together, we observe a trend in behavioral preference where larger-eyed species perform more poorly in navigation towards odor objects when presented alone, but better towards visual objects, and vice versa for relative antennal size. (Data are provided at https://doi.org/10.17617/3.1D)

pursued additional metrics for visual and olfactory signal reception by quantifying sensillum and ommatidium number. Interestingly, the trend between visual and olfactory sensory structures was inversed among these six flies when we corrected for absolute head size (Fig. 2b), where large ommatidium counts in a fly species seemed to correspond with reduced sensillum counts, and vice versa. We also examined whether antennal surface area alone was a predictor of specific sensillum types, but surface area did not always predict the number of sensilla (Supplementary Figure 2G). In regard to olfaction, while these six species differed greatly in their absolute size, we discovered striking similarities in the density of sensilla found on either the anterior surface or the whole antennae (Fig. 2d; Supplementary Figure 2E, F). While both basiconic and coeloconic counts were roughly similar in their density, the largest difference between the species was in the number of trichoid sensilla (which have been shown to house sensory neurons detecting pheromone compounds[26,37,38]) (Fig. 2d). These trichoid differences were also apparent when we compared the absolute sensillum counts between species (Supplementary Figure 2D–F). Trichoids also varied in length and curvature. In addition to olfaction, we examined visual capabilities of each of these six species by counting the visual receptors or ommatidia (Fig. 2e; Supplementary Figure 2A–C, H), and again we noted large differences between these selected species, where ommatidia number was proportional to our previous measures of eye surface area. In order to further test the hypothesis that a tradeoff occurs between visual and olfactory sensory systems, we expanded our evolutionary comparison beyond these six examples to include additional species across the phylogeny (which were selected using stratified random sampling in order to represent as many subgroups as possible). Here, as before, we observed a significant inverse correlation between trichoid number and the eye-to-funiculus ratio (EF ratio) (Fig. 2f), where again, trichoid numbers were not correlated with antennal surface area or antennal size (Supplementary Figure 2G).

Neuroanatomy of visual and olfactory sensory circuitry. Given the disparity in external sensory morphology between our six species, we next sought to compare neuroanatomical metrics for the primary visual and olfactory processing centers within the brain (Fig. 3; Supplementary Figure 3). The species with the enlarged compound eyes also had a much larger OL relative to the AL, while the species with enlarged antenna had a relatively smaller OL (Fig. 3a, b). This matched our metrics related to external anatomy, suggesting as we predicted for example, that larger eyes correlates with larger OL volume. In order to account for differences in absolute size between each species, we used the central brain as a means to generate a weighed value for both OL and AL comparison (Fig. 3c–e). While it was not surprising that larger eyes or larger antennae matched with a larger brain region associated with these sensory structures, we started to see a pattern where an increase or an exaggeration of one sensory structure correlated with a relative reduction in the other. For example, that while *D. suzukii* has a much larger (OL:AL) ratio or (OL: central brain) ratio when compared with *D. melanogaster* (Fig. 3b, d), at the same time *D. suzukii* also had a significantly smaller (AL:central brain) ratio by comparison (Fig. 3d). This trend is true for each of the other reconstructions and species comparisons. We also assessed the selected six *Drosophila* species in regard to subunits of the OL, including the medulla, lobula, and lobula plate, where again we saw a similar pattern of a significant increase in size for each subunit of the OL in larger-eyed species; moreover, that the medulla represented the largest increase relative to central brain volume (Fig. 3e; Supplementary Figure 3G). Here, we also documented again that the AL of the larger-eyed species was relatively smaller when compared with larger antennal species, as expressed by a ratio to central brain volume (Fig. 3e). While these six species varied in their absolute sizes (Supplementary Figure 3A–G), we noted that the central brain relative to the whole brain was consistent in size across all tested species (Supplementary Figure 3E), thus a relative comparison of OL or AL to the central brain within each species gave a consistent measure or weighted value for comparison.

Phylogenetic correction of traits of interest. To examine whether the phylogeny of our species could account for the variations, that we measured in the eye and antenna, we compared the EF ratio trait to all relatives within the genus (Fig. 4a). Here, we utilized two independent statistical tests of phylogenetic signal, including the Blomberg K value and Pagel's lambda (K = 0.478; $p = 0.041$; $\lambda = 7.102e^{-05}$; $p = 1$), where we assess phylogenetic signal to indicate the tendency for closely related species to resemble each other more than a random species selected from the tree. Here, we found that both statistical measures agree that this phenotypic trait (EF ratio) is not strongly supported by the phylogeny, where a K value less than one indicates that variation is larger within subgroups than between subgroups (Fig. 4a). Thus, while we considered phylogenetic associations as a driver of trait variation, we did not find a relationship between phylogeny and trait variation. In addition, we noted that eye and antennal size diverge repeatedly throughout the genus and were not predicted by known ecology or shared habitats (e.g., EF ratio was not correlated with cactus-feeding or desert-living species; Fig. 4a); however, more ecological data are still needed for a multitude of species to discern the role ecology plays in the observed sensory variation.

Behavioral effects of sensory bias between species. Given the trends and correlations we observed in our in-depth analyses of six species, and in order to assess potential behavioral courtship



implications from the size variance of visual and olfactory sensory systems, we wanted to expand our comparative model to include all 62 species in our study (Fig. 4b, c). Here, we arranged all 62 species in regard to their EF ratio, as provided by measures of the surface area of each sensory structure, with smaller values indicating relatively large antennae, and bigger EF ratio values indicating a larger compound eye relative to the antenna (Fig. 4c). Photographs of wings from males and females were taken and used to provide information about wing spots or pigmentation for each species that was tested (Fig. 4b, c), and we also used previous literature to assess whether each species is influenced by light (lux intensity) during courtship or whether light is required for successful mating to occur (Fig. 4b). There was a significant correlation between female wing pigmentation and EF ratio after phylogenetic correction ($p = 0.0429$) (Supplementary Figure 3H, I). In addition, there was a significant correlation between male pigmentation and EF ratio after phylogenetic correction ($p = 0.0256$); therefore, because there was a correlation between wing

Fig. 5 Tradeoffs and developmental constraints. Red color signifies vision or visual bias, while blue indicates olfaction or olfactory species. **a**–**c** Diagrams of a single imaginal disc from larval development that gives rise to two separate adult structures, namely the eye and the antenna. **d** Two part staining (Hoechst & Phalloidin) of *Drosophila* species to visualize differences in absolute size of imaginal discs, highlighting the need for a ratio of eye to antenna for comparisons between species. **e** Imaginal disc ratios (eye to antenna) across each tested species where two groups were noted, olfactory biased and visually biased. Means with the same letter are not significantly different from each other (ANOVA with Tukey–Kramer multiple comparison test). Boxplots represent the median (bold black line), quartiles (boxes), as well as the confidence intervals (whiskers). **f** The significant correlation between larval imaginal disc measurements per species and the EF ratio from adult flies. **g**–**j** Eye and antennal mutants were compared to wild-type flies for both ommatidium and trichoid numbers. **k**, **l** From the mutants we screened, a single mutant, Lobe[1], displays increased trichoids and decreased ommatidia compared the the wild-type. An asterisk denotes statistical significance between two groups (*$p \leq 0.05$, ***$p \leq 0.001$; T test). **m**–**p** Eye–antennal imaginal disc comparisons between wild-type and Lobe[1] mutant, visualizing the tradeoff between visual (red) and olfactory (blue) development. **q** Measurements show that while the total size of the imaginal disc is the same between wild-type and mutant, that the proportion of eye and antenna are inversely correlated, suggesting a developmental constraint between these two sensory systems. (*$p \leq 0.05$, ***$p \leq 0.001$; *T* test) (Data are provided at https://doi.org/10.17617/3.1D)

pigmentation and EF ratio when we include the phylogenetic correction, the correlation between these two traits has no phylogenetic signal (i.e., the covariance of the residuals for the EF ratio and wing pigmentation regression do not follow phylogenetic signal). From the analyses of the light/dark courtship data in regard to EF ratio, we found these traits were strongly correlated both before phylogenetic correction ($p < 0.0001$) as well as after the correction based on relatedness of the species ($p = 2.406e\text{-}07$) (Supplementary Figure 3H, I). Thus in summary, it appears that proportionally larger eye size provides a potential visual bias in courtship that is associated with light-enhanced mating success. Moreover, we show that species with larger EF ratios (and thus those species with relatively larger eye size) were significantly more likely to possess wing pigmentation, and have significantly more successful copulation in light conditions (or display light-dependent courtship), perhaps as part of a successful visual display. However, due to the paucity of natural history for most species, additional work is needed to address all species-specific mating behaviors within this genus, including for example, pheromone-related courtship (or pheromone-related olfaction) in larger antennal species that display light-independent courtship.

As we had established a consistent difference between the visual and olfactory senses of the six species in regard to external and internal neuroanatomy as well as courtship, we wanted to next test if there was also any behavioral relevance to these sensory structure differences in regard to host navigation (Fig. 4d–g; Supplementary Figure 4A–D). When we combined visual and olfactory stimuli, all six species performed equally well in trap assays, including tests with several different olfactory cues, such as vinegar, blueberry, and strawberry (Fig. 4e; Supplementary Figure 4A). However, when we tested the olfactory stimuli alone, without any visual target, we observed a biased trend in that larger-eyed species navigated more poorly than larger-antennal flies (Fig. 4f), suggesting an olfactory advantage to large antennal species toward the odor object alone. The opposite phenomenon occurred when we tested visual stimuli in the absence of an odor source, where larger-eyed species performed significantly better than those species with enlarged antennae (Fig. 4g); moreover, we caught almost no flies from the larger antennal species using color alone. We also tested for species differences in their preference toward specific colors, with red and black being the most consistently attractive to all species, regardless of behavioral assay, but with *D. suzukii* also being attracted to green (Supplementary Figure 4A, B). However, this may be in part due to differences in contrast detection. Interestingly, *D. suzukii* was also more attracted to the combination of blue when presented with odor from blueberry, which may be linked to this species being reared for dozens of generations on this food source in our laboratory, and additional work will be required to test this combinatorial bias (Supplementary Figure 4A). In order to compare visible qualities of each color used, we generated a diffuse reflection gradient for each visual stimulus, to confirm the primary visible wavelength associated with each color we used in this study (Supplementary Figure 4C). We also confirmed the reliance on visual stimulus for host navigation by repeating a trial in either full light and complete darkness (Supplementary Figure 4D). Here, for example, *D. melanogaster*, a large antenna, olfactory-driven species, navigated equally well toward an odor source regardless of light conditions (Supplementary Figure 4D). However, in the same experimental design, *D. suzukii*, a large eye, potentially more visual species, performed as well as *D. melanogaster* toward an odor source in the dark, but roughly split capture with the visual stimulus and the odor source when in light conditions. In this case, as all species were still able to locate a host source successfully using a single-stimulus type (i.e., odor object in the dark), it would appear that the difference in size of a sensory structure indicates an innate preference or behavioral bias for certain navigational cues, but that both sensory systems still work well. Although again, visual and olfactory stimuli worked optimally in tandem, or when the two stimuli were in agreement in regard to the location of the host (Fig. 4e). Future work should examine the behavioral response of each species when the visual and olfactory objects are not in spatial congruence in regard to the location of the host or food source.

Evolutionary development of visual and olfactory structures. Although insect development is a complicated and delicate process under strict genetic control, the process by which *D. melanogaster* undergoes development has been relatively well elucidated. In general, there are 19 imaginal discs from the *Drosophila* larvae, each of which gives rise to a different adult structure (Supplementary Figure 6A); however, there is only one disc that gives rise to several separate adult structures, namely the eye–antennal imaginal disc (Fig. 5a–d). Here, a single larval developmental structure generates primarily both the eye and the antenna for the adult fly (Fig. 5b, c). With this in mind, we next examined the relative ratio of the two sides of this imaginal disc, including both the eye and antennal portions across a multitude of species (Fig. 5e). Although species varied in egg to pupal developmental time, by dissecting the tissues from late third instar larvae (wandering phase; Supplementary Figure 7), we could generate consistent ratios for each species during the same time window of development (Supplementary Figure 6B, C). To confirm these measurements, we used two stains (Hoechst & Phalloidin) in order to more closely monitor areas separating these two portions of the same developmental disc in each new non-*melanogaster* species (Fig. 5d). By using a ratio between the two parts of the same imaginal disc, we could account for any issues during the comparison of species that differed drastically in absolute size, for example between *D. pseudotalamancana* and *D.*

melanogaster (Fig. 5d). Using the data taken from a multitude of *Drosophila* species, we could identify essentially two main groups or two common ratios, either antennal biased or visually biased (Fig. 5e). This developmental data matched very well with the previously established external metrics taken from the compound eye and antennal surface areas, and thus further support the theory that there is a tight link between the imaginal disc size for the eye and antenna in comparison with the corresponding adult structures (Fig. 5f). This data again provide evidence for an inverse resource allocation between the eye and the antenna during development, as these two sensory structures would essentially be competing for the same resources within a single disc (Supplementary Figure 6D).

Genetic constraints on vision and olfaction. While we could not further examine the role development plays in non-*melanogaster* species of Drosophilidae, we could in fact, examine established genetic lines within *D. melanogaster* for either eye or antennal mutations (Fig. 5g–q). In these experiments, we used previously identified mutations for either eye or antennal development in *D. melanogaster*, and analyzed both of these adult sensory structures in order to test our hypothesis that there is a tradeoff or inverse resource allocation (Fig. 5g–q; Supplementary Figure 6E–G). Here, we counted trichoid sensilla and individual ommatidia from each mutant line in order to assess any potential candidate genes that match the phenotype we observed in the wild-type species (Fig. 5g–l; Supplementary Figure 6E–G)). Although some fly mutants have been previously published for either visual or olfactory abnormalities, most lines have not to our knowledge ever been examined for both sensory structures within a single mutant. While not an exhaustive screen of all possible gene candidates in *Drosophila* development, we did uncover a single-mutant allele in our screen that appeared to have a similar tradeoff between visual and olfactory sensory structures to that observed across the genus, more specifically, Lobe[1] (L^1), which has a significant reduction in the number of ommatidia while possessing a significant increase in the number of trichoid sensilla present on the funiculus (Fig. 5k, l), something that was consistent with the observations from wild types. This mutant has a reduced eye size, which has been previously published;[39–41] however, the alteration leading to increased antennal size (enlargement of all three segments) and the increase in trichoid sensillum number has not been previously described for this mutant (Fig. 5g–l).

In order to further test our hypothesis that the imaginal disc provides the framework for an inverse resource allocation based on the sharing of a single disc for two adult sensory structures, we next sought to examine the imaginal disc of this L^1 mutant in regard to eye and antennal ratio (Fig. 5m–p). Here, we observed that the Lobe[1] mutant has a marked reduction in the portion of this developmental disc that gives rise to the compound eye (Fig. 5o, p), while also showing a marked increase in the portion that gives rise to the antennal segments. When we measured the two portions of the developmental disc for both wild-type and mutant, we discovered that there was no significant difference in the total size of these imaginal discs (Fig. 5q), but rather that the proportion of the disc dedicated to each sensory structure had shifted in the mutant from the eye to the antenna (Fig. 5q). Thus, this new data lends additional support to our previous observation that a tradeoff might occur between visual and olfactory sensory systems, in this case during development, and that this inverse resource allocation is perhaps necessitated by the sharing of a single larval structure. Thus, for example, in order for the antennal region to increase in Lobe[1], there is necessarily a decrease in eye size to compensate. Recently, a preprint[31] has addressed this same developmental mechanism, and has proposed a similar tradeoff hypothesis by comparing two *Drosophila* species using CRISPR mutants, where they conclude that a single amino-acid shift can alter the functional timing of a gene, and explain the natural variation between eye and antenna during larval development. However, more research is needed to address whether this same developmental constraint can dictate the inverse correlation between visual and olfactory sensory systems that we have observed in all tested *Drosophila* species.

Discussion

In this study, we provide large-scale evidence for an inverse relationship between visual and olfactory anatomical investment across this genus of Drosophilid flies. The potential tradeoff seems to stem from a theoretically restricted resource allocation between the eye and antenna during larval development, which is linked to a single shared structure giving rise to both adult sensory systems (Fig. 5d–i). It remains to be seen whether this push–pull between the eye and antennal region of the imaginal disc is under similar genetic control in all non-*melanogaster* species; however, our study and a recent preprint[31] provide evidence that a simple mutation can mirror inverse variation in ommatidia and sensilla numbers for *D. melanogaster*, something which is consistent with our observations of repeated, independent evolutionary events across this genus of fly in regard to visual and olfactory divergence.

Investment in an exaggerated sensory structure might be costly[42], thus prominent structures often result in a tradeoff with another trait to minimize energetic costs[43–47]. Tradeoffs can occur across populations or between species within a single subfamily or genus, and each different sensory structure often has differing ecological and environmental pressures acting upon it[48,49]. An example from vertebrates of a similar tradeoff hypothesis examines trichromatic color vision in primates[50], where researchers found that primates with heightened color vision also had a higher number of olfactory pseudogenes or non-functional gene mutations. In order to test this pseudogene argument, we also examined the olfactory genes from many *Drosophila* species using previously published data on OR, GR, IR genes, and their associated pseudogenes across 14 members of Drosophilidae (Supplementary Figure 1J)[51], but we did not find any meaningful correlation between olfactory pseudogenes and eye size or visual enhancement. However, it is possible that gene expression levels differ between *Drosophila* species, either across rhodopsin types or other visual pigmentation genes, or perhaps across olfactory-related genes. For example, while the most-studied *Drosophila* species have roughly the same diversity of chemosensory genes and ommatidium types[51,52], different olfactory receptor ratios exist across basiconic or trichoid sensillum types, where variation in olfactory receptor expression is often associated with specialization[10,25,26]. This was the case in *D. sechellia*, where this species has similar olfactory gene diversity (or number of chemosensory genes) when compared with *D. melanogaster*, but vastly different expression levels of a few specific receptors. Additional research is required to assess this type of expression-level comparison for visual and olfactory genes between a wider array of *Drosophila* species, as it is not clear if fly species with increases in ommatidia or sensilla numbers represent a uniform increase across receptor types. It is also important to mention that there are some limitations in our extrapolation to true wild-type insects due to the usage of stock center or laboratory flies, but we anticipate that our findings will extend to natural populations as well.

From an ecological point of view, we considered mate-finding and host navigation when examining sensory systems in *Drosophila*. Both of these behaviors have been shown to rely heavily on visual and olfactory inputs in several species that have previously been investigated. For example, wing pigmentation has been extensively studied in *Drosophila*[53–56], although never before in correlation with olfactory function such as pheromone detection (Fig. 4b, c). The removal of pigmentation heavily influences sexual selection and courtship, thus further confirming the importance of visual cues during courtship in spotted wing *Drosophila* as well as in the visual courtship of other animals[57,58]. In addition, it was recently shown that *D. subobscura*, which requires light for courtship success[59,60], has enhanced fruitless-labeled gene expression and circuitry that maps to the OL, unlike *D. melanogaster*, where courtship is light-independent[29]. Moreover, that study also highlighted fruitless-labeled visual enhancement into the lobula and lobula plate of *D. subobscura*, a specific increase in brain volume which we also show in all three of our visually biased species examples (Fig. 3e). Another well-studied example of courtship and incipient speciation is the diverging populations of *D. mojavensis*[22–24], where our data again show that the largest divergence is found between the closest relatives and geographically overlapping subspecies, suggesting character displacement as an additional driving force for the observed differences in visual and olfactory investment (Fig. 4a, c). In fact, the vast majority of *Drosophila* species we tested show the largest differences within a species clade or subgroup (e.g., *D. virilis* vs. *D. americana*; *D. biarmpies* vs. *D. suzukii*; *D. pseudoobscura* vs. *D. subobscura*), where courtship, mate selection, and host competition pressures are potentially highest, and perhaps driving repeated speciation events that favor either visual or olfactory bias to differentiate the species' niche (Fig. 4a, c). Although recent work has examined differences in the visual and olfactory systems of *D. melanogaster* and *D. pseudoobscura*[31], we do not feel this is a good direct comparison, given the poor phylogenetic connection between these more distantly related species (17–30 million years apart), and that other pairings would perhaps better tackle the genetic, ecological, and evolutionary pressures that underpin this sensory tradeoff (e.g., that *D. subobscura* or *D. affinis* would be a better comparison for *D. pseudoobscura*, while *D. simulans* or *D. sechellia* would be a better comparison for *D. melanogaster*). Thus, we conclude that the correlations and model provided by our study, including eye size and wing pigmentation as well as light-dependent courtship, match with previous publications from the *Drosophila* genus and our study provides a large dataset for further testing. In addition, our data continue to strongly support the theory that visual investment and OL increases mirror the behavioral priority of vision for courtship and/or host navigation in those species with larger EF ratios and wing pigmentation (Fig. 4b, c; Supplementary Figure 3H, I).

Although additional work is required to confirm any differences in pheromone production or increased olfactory courtship reliance in species with larger antennal ratios, our data already support the inverse investment between the eye and antenna in regard to copulation based on the number of trichoid sensilla versus ommatidia (Fig. 2b, d, f; Supplementary Figure 2 E–G). Moreover, within the suzukii subgroup, it has been well established that *D. suzukii* produces very low amounts of the male pheromone known as cis-vaccenyl acetate (cVA; detected by trichoid at1, and Or67d) and that this species has a greatly reduced glomerular volume within the AL for this odor[26]. The previous research matches our findings here that *D. suzukii* flies have a reduced total number of trichoids, and in addition, that these flies instead possess an enlarged compound eye that is 2.5 times larger than in *D. melanogaster*. Similarly, *D. biarmipes*, the closest relative of *D. suzukii*, has also been previously studied and shown to have a large amount of cVA production, which is opposite to *D. suzukii*[36]. In the present study, we also found a correspondingly higher number of trichoid sensilla for *D. biarmipes* when compared with *D. suzukii*, even given the smaller overall size of *D. biarmipes*, matching a potential tradeoff between olfactory and visual investment between close relatives for courtship, again suggesting character displacement as a potential means of speciation or divergence (Fig. 4a, c).

Resource allocations have been well documented within other insects, such as in courting scarab beetles, where there is an inverse correlation of investment between physical horn size for fighting and sperm production for increasing the likelihood of paternity[61]. Examples of visual and olfactory variation have also been recently documented in other insects, such as in Lepidoptera, where nocturnal and diurnal species within the Sphingidae family of hawk moths vary widely in morphological investment toward either eye or antennal structures, as well as in their relative OL and AL sizes;[62] however, while a tradeoff between these sensory systems has not been previously proposed, these studies have shown by comparing two hawk moth species that relative brain structure increases match behavioral preferences, with diurnal species having enlarged visual centers and visual preferences, and nocturnal species having enlarged olfactory centers with olfactory behavioral preferences. Moreover, that these sensory brain measurements can be used to explain and predict differences in the importance or priority of these two senses (vision and olfaction) for host navigation. In these studies of Lepidopteran neuropils, it can be inferred from the data that investment in vision is perhaps associated with a relative decrease in olfactory processing centers, and vice versa, both for host-finding and migration, suggesting that perhaps an insect species cannot increase both sensory systems[62–64]. It has also been shown recently that a potential tradeoff might also occur between diurnal and nocturnal dung beetle species[65], where there was a difference across the two examined species between visual and olfactory brain regions based on circadian rhythm or daily activity patterns. Here, the diurnal species have a larger OL and are more visual, while the nocturnal species relies more on olfaction as well as possessing an enlarged AL. Another insect example of visual variation exists across Formicidae, where different ant species, or even different castes members within a species, have differing investment in vision depending on their ecological roles within the colony or depending on the amount of time they spend underground[66,67]. In addition, more distant insect relatives have been compared across visual brain structures[68], where the visual centers from Mantodea, Blattodea and Orthoptera were addressed for their anatomical similarities and differences. Although some of these latter studies did not address olfactory centers for relative comparison between both vision and olfaction, each example lends support to the hypothesis that all insects potentially demonstrate a tradeoff in sensory systems. However, additional work is still required in more orders of insects to assess this tradeoff hypothesis and the evolutionary pressures that lead to these potential compromises between sensory structures.

In many insect examples, the differential investment in OL or AL was linked to differences in activity (diurnal and nocturnal). These differences in circadian rhythm are not as well studied in all non-*melanogaster* species, and the timing of both courtship and host-seeking behaviors are not known for all species. However, in the *Drosophila* species that have been examined, they all share a similar crepuscular activity cycle, thus it is unlikely that differences in visual and olfactory sensory systems in *Drosophila* arise from nocturnal versus diurnal activity[60,69]. Additionally, tradeoffs between visual and olfactory signaling have been long

recognized in plant species, especially between odorous nectar or visual floral displays that are used in order to attract insect pollinators[70]. The difference in plants is evident where you have a visually large and distinct floral petal arrangement, but with reduced smell or reward. In contrast, other plants have little in the way of visual attraction, but utilize sweet nectar rewards or strong, pungent odor plumes to draw in olfactory-driven pollinators[71–73]. These plants examples again highlight potential differences across insect pollinators, such as hymenopterans and dipterans, where the plant takes advantage of insects that favor either visual or olfactory stimuli for host navigation, but perhaps not both sensory modalities[73]. It is possible in these cases that vision could assist some Drosophild species in finding their preferred plant hosts (i.e., flowers, or fruit ripening within leaves or tree canopies), although the paucity of ecological information for most species within this genus has made this impossible to examine so far.

In summary, our assessment of the genus *Drosophila* supports the hypothesis that the visual sensory system expands consistently at the expense of structures related to olfaction, and vice versa. In addition, we provide robust evidence that the inverse correlation observed between visual and olfactory sensory systems occurs repeatedly within the family Drosophilidae, and we conclude that our theory of a tradeoff is consistent with all observed patterns, and perhaps is necessitated by a developmental constraint. Moreover, while additional research is required to address the specific molecular genetic mechanism(s) that control this observed phenomenon across the entire genus, the data provided herein generate a solid foundation to continue to test this sensory tradeoff hypothesis in the future. By using a large subset of close relatives within one genus of Dipterans and creating an extensive overview of their visual and olfactory systems, including a robust molecular phylogeny, we were able to generate a finely tuned evolutionary framework, and we provide the first step in establishing a larger model system to encompass dozens of *Drosophila* species for additional study beyond *D. melanogaster* and its subgroup. In the end, we have also started to build evidence about the pressures and general rules governing developmental, ecological, and evolutionary phenomena related to differences in neuroanatomy and behavior across all insects, where the data provided support previous research as well as encourages new ideas and new avenues for the study of speciation, specialization, and the evolution of the nervous system.

Methods

Fly stocks. All wild-type species, stock numbers, and rearing diets are in Supplementary Table 1. Unless otherwise noted, all fly stocks were maintained on standard diet (normal food) at 25 °C with a 12 h light/dark cycle in 70% humidity. Stock population density was controlled by using 20–25 females per vial. Mutants lines included oc^1 (ocelliless; Bloomington #2291), ar^1 (arista-less; Bloomington #210), Antp (antennapedia; Bloomington #2235), Dll (distal-less; Bloomington #3306), $Diap^1$ (thread; Bloomington #618), L^1 (lobe; Bloomington #318), gl^1 (glass; Bloomington #506), and gla^1 (glazed; Bloomington #1951). Stocks were maintained according to previous publications[74], and for all behavioral experiments we used 2–7 -day-old flies of both sexes.

External morphometrics from head and body. For each fly species or mutant line, 3–8 females were photographed using a Zeiss AXIO microscope, including lateral, dorsal, and frontal views. Flies of the 62 wild-types were dispatched using pure ethyl acetate (MERCK, Germany, Darmstadt). Lateral body (40×), dissected frontal head (128×), and dissected antenna views (180×) were acquired as focal stacks on an AXIO Zoom V.16 (ZEISS, Germany, Oberkochen) with a 0.5x PlanApo Z objective (ZEISS, Germany, Oberkochen). The resulting stacks were compiled to extended focus images in Helicon Focus 6 (Helicon Soft, Dominica) using the pyramid method. Based on the extended focus images, we measured body length (abdominal tip to antennal tip), head width (between eye margins), eye width, and eye height, as well as funiculus width and length, all measurements are in μm (Supplementary Figure 1A). Assuming the eye as a full ellipsoid, we calculated the 3D surface based on the average eye width and half eye height as the ellipsoid radius (r), and used the formula $[4 \times (\pi) \times r^2]$ for the area of a sphere, then dividing the result by 2 to generate the eye surface area as a half-ellipsoid for each species. Calculations for the funiculus surface used its half-length and half-width as radius for the 3D ellipsoid surface area. Accounting for the proximal connection between funiculus and pedicel, we subtracted the circular base area, and then calculated with the funiculus width. In addition, we compared these calculations with previous publications for available species[52,75] in order to confirm that our metrics were similar, and while some of our estimates were low relative to other publications, they were consistent across replicates within each species. All raw measurements are available with the online library, as are the stock photos for all replicates (https://doi.org/10.17617/3.1D; 01 Species Images; Excel tables).

In order to test the validity of the usage of ratios for our comparisons made between visual and olfaction sensory systems, we have provided a statistical assessment of allometry (including a multiple regression analysis). First, we found that the eye and funiculus surface area measurements scale isometrically with respect to the measurements taken from the body and the head. Thus, we feel it continues to make sense to use the EF ratio as our primary trait, given that there is no real allometry in our data. Moreover, we show that neither body size ($p = 0.294$) nor head size ($p = 0.590$) significantly correlate with this EF ratio trait (Supplementary Figure 1H), and we have plotted the analyses of the residual variance (Supplementary Figure 1H). Last, we have also conducted a multiple regression analysis (using the EF ratio, eye, funiculus, body, and head measurements from all 62 species), and indeed again, the EF ratio does not correlate with body or head size in this multiple regression ($p = 0.354$ and $p = 0.295$, respectively). Overall, we continue to feel that we can safely maintain the usage of ratios, as the EF trait does not simply scale allometrically with body or head size, and these statistical tests again strengthen and further support our interpretations of the data that an inverse correlation exists between these sensory modalities that is not reflective of absolute body size. In addition, an online copy of the curated R scripts is available, including all measurements used to test allometry and to perform the multiple regressions (https://doi.org/10.17617/3.1D; 12Allometry).

Ommatidium measurements. In order to count ommatidia, the compound eye of each species was dissected and mounted on slides in water using a coverslip, and then photographed using a confocal microscope (Fig. 2e). A total of 5–6 individuals per species were used, and counts were done manually using ImageJ (Fiji) software tools (Supplementary Figure 2A). Diameters of single ommatidia were also assessed (Supplementary Figure 2B, C), with most species having roughly similar size.

Sensillum counts. Three different individuals from each species were anesthetized with CO_2, and their antennae were dissected. After removal, antennae were dipped into phosphate buffer (0.1 M pH, 7.3) with 5% Triton-X (Sigma-Aldrich) and they were washed in phosphate buffer and embedded in VectaShield (Vector Laboratories) between two cover slips[11]. To visualize the anterior surface of the antennae, lambda scans were obtained via confocal laser scanning microscopy (Zeiss LSM 880; Carl Zeiss) using a 40x water immersion objective (W Plan-Apochromat 40×/1.0 DIC M27; Carl Zeiss) in combination with the internal Argon 488 -nm laser (LASOS) and the 405 -nm Laser diode (Carl Zeiss). The broad emission spectrum of the samples auto-fluorescence was detected with the quasar detector (Carl Zeiss). Thereby images with 32 separate channels (each with a range of 9.7 nm) are generated simultaneously (Supplementary Figure 2D). To visually support the following sensilla quantification, lambda scans were post processed using the linear un-mixing technique (Carl Zeiss; http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/introduction.html). This technique enables the determination and separation of spectral profiles for every pixel and assigns each pixel, according to its spectral profile, to a manually defined spectral group. Three spectral groups were defined by selecting reference points in each stack (diameter 5 pixels) using the ZEN software (Carl Zeiss). This technique enables reassignment of one color for each group to a region (or group of pixels) that would otherwise appear as mixed color, and therefore supports visual separation of olfactory sensilla from other structures as well as the characterization of different sensillum types, due to structural differences (e.g., between trichoid, coeloconic, and basiconic shapes) that cause distinct emission spectra in their auto-fluorescence.

The sensillum quantification was done with the cell counting plugin (https://imagej.nih.gov/ij/plugins/cell-counter.html) in ImageJ (Fiji). Linear unmixed lambda stacks were visualized as a composite of all three channels and sensilla were manually counted by going through the stack. Each sensillum was assigned to one group (trichoid, basiconic, and coeloconic) and marked separately, and then each group was summed in the end.

Sensilla density of each anterior surface side was calculated as follows:

$$\text{Sensilla density} = \frac{\text{Sensilla number}}{\frac{1}{2}\text{funiculus surface}(\mu m^2)} \quad (1)$$

For trichoid sensillum counts of the other 24 species, counts were done manually for either the anterior or posterior or for both sides of the antennal surface. Counts were conducted with images from a Zeiss AXIO microscope under bright-field light, using arista up single sensillum recording preparations for each

insect that was examined (Supplementary Figure 5A, B), as this was the best preparation for viewing and counting trichoid sensilla[37]. A total of 3–6 individuals were counted per species, and where possible, these totals were compared with previous scanning electron microscopy (SEM) images, or lambda scans, or the previously published counts from the available species.

Phylogeny of *Drosophila* species. Species were initially selected, ordered, and arranged to include close relatives in pairs or triplicates for each major subgroup within the genus. Our initial molecular phylogeny search consisted of 16 mitochondrial and nuclear genes that were identified and used previously for studies of Drosophilidae[76,77]. However, many of these sequences were partial, or from older literature, while in addition, some genes had representation in only a few species. Therefore, we replaced much of the previously published data with the newer sequences that are currently available in public sources such as GenBank and Flybase repositories, with new sequences being either complete or longer in length than those that were previously published. In particular, no segments of the same gene in a species have been combined, as had been done in previous publications. We retrieved only the nucleotide coding sequence (CDS) regions of protein-coding genes, as well as the nucleotides for non-coding ribosomal RNA genes. In cases where mitochondrion genomes were available (bold after species names), then all the target mitochondrion genes sequences were retrieved from the same genome data. Moreover, in cases where the sourced data contained multiple genes, the specific region of the target gene sequence is given. After we assessed each individual gene, we generated trees for each gene individually, and ultimately narrowed our list from 16 down to 5 genes for concatenation (ADH-1, Amyrel, NADH-2, NADH4, and NADH4L). Raw molecular data, including sequences and accession numbers, are available at https://doi.org/10.17617/3.1D; 02 Molecular Phylogeny and in Supplementary Data 1.

For phylogenetic tree construction, we used available sequences from 59 Drosophila species drawn from the *Sophophora* and *Drosophila* clades, including *D. busckii* as an out group in the *Dorsilopha* clade of this genus. We assessed the dataset for each of the 16 gene families for quality in terms of representation or coverage across the sampled species, completeness of sequence length, the nucleotide multiple sequence alignment conservation, as well as the ability of each gene to reconstruct the phylogeny of the species represented (for individual phylogenetic trees see https://doi.org/10.17617/3.1D; 02 Molecular Phylogeny). This assessment enabled us to also determine the sequential order for concatenating the genes. Our final concatenated dataset were comprising two nuclear protein coding genes, amylase related (AmyRel) and alcohol dehydrogenase subunit 1 (ADH-1), as well as three mitochondrion genes, NADH: ubiquinone oxireductase subunit 2, −4, and −4L (NADH-2, NADH-4, and NADH-4L). We excluded non-coding mitochondrion genes for the reason that they individually failed to reconstruct the phylogenetic tree, as the sequences were often partial, had biased representation across the species, or failed to reproduce a consistent phylogeny, though we still include them for future reference in the online library (https://doi.org/10.17617/3.1D; 02 Molecular Phylogeny). The final dataset consisted of 229519 bp data points, in 59 concatenated sequences. The sequences were multiply aligned using a MAFFT tool with L-INS-I parameters, with 10000 bootstrap (Kato & Toh, 2008) and the final tree was reconstructed using maximum-likelihood approach with GTR+G+I model of nucleotide substitution and 1000 non-parametric bootstrapping, re-sampling of 10 initial random trees in Fasttree program. We did not partition the concatenated gene sets in this analysis. All emanating trees were visualized, and rendered using Figtree v.1.4.2.

Using this newly created phylogeny, we analyzed in two different ways the phylogenetic relationship for the eye–funiculus trait that we had generated for each species. First, we tested the Blomberg K value ($K = 0.478$; $p = 0.041$), where the K value being less than one suggests a lower phylogenetic signal than expected from Brownian motion; moreover, this low K value indicates that the variance is mostly within a given subgroup, and not between subgroup clades. Here, we determine phylogenetic signal to indicate the tendency for closely related species to resemble each other more than a random species selected from the tree. Second, we tested the Pagel's lambda value ($\lambda = 7.102e^{-05}$; $p = 1$), where again, a λ value that is not significantly different from zero indicates very little phylogenetic signal in this trait. Thus, given the consistency of these two different statistical measures, we determined that the eye–funiculus ratio is not strongly supported by the phylogenetic relationship of the species that we tested.

3D reconstructions and neuropil measurements. In order to assess neuroanatomy, the dissection of fly brains was carried out according to established practices[78]. The confocal scans were obtained using multiple photon confocal laser scanning microscopy (MPCLSM) (Zeiss laser scanning microscopy [LSM] 710 NLO confocal microscope; Carl Zeiss) using a 403 water immersion objective (W Plan-Apochromat 40×/1.0 DIC M27; Carl Zeiss) in combination with the internal Argon 488 (LASOS) and Helium-Neon 543 (Carl Zeiss) laser lines. Reconstruction of whole OLs and ALs was done using the segmentation software AMIRA version 5.5.0 (FEI Visualization Sciences Group). We analyzed scans of at least three specimens for each and reconstructed them in using the segmentation software AMIRA 5.5.0 (FEI Visualization Sciences Group). Using information on the voxel size from the laser scanning microscopy scans as well as the number of voxels labeled for each neuropil in AMIRA, we calculated the volume of the whole AL as well as the individual sections of the OL and the central brain (where central brain values exclude the AL volume).

Behavioral assays for visual and olfactory stimuli. Trap experiments were performed as previously described for individual odors[27,36], but using white or colored paper cones as an entrance to the trap (as non-*melanogaster* adults were too large to enter pipette tips). We also used an additional 200 µl of light mineral oil (Sigma-Aldrich, 330779-1L) that was added to capture and drown flies upon entering to the paper cone trap, and to ensure they did not escape over the 24 h testing window. Trials were conducted with 30 adult flies (15 males, 15 female), and each species was run separately. All behavioral cone traps consisted of 60 -ml plastic containers (Rotilabo sterile screw cap, Carl Roth GmbH, EA77.1), with one trap used as a white control and the other containing a colored cone entrance (red) (Fig. 4a–d, Supplementary Figure 4A, D). In experiments with whole fruit, each fruit was placed individually into traps that were presented simultaneously, where the sides of the container were opaque to avoid any extra visual stimuli, and as before, a large arena was used (BugDorm-44545 F) (Fig. 4a; Supplementary Figure 4A, D). For Petri dish behavioral traps (Supplementary Figure 4B), color paper circles were cut out and placed onto standard 10 -cm Petri dishes, either with or without an odor source, where mineral oil was again used to capture flies that landed on the paper disks. A total of 60 adults (30 males, 30 females) were used per trial, with a 16 L:8D photoperiod during testing. All odor dilutions were prepared in hexane or water, and all behavioral trials were conducted with odors diluted to 10^{-3} unless otherwise noted. Statistics were performed using GraphPad InStat version 3.10 at both $\alpha = 0.05$ and $\alpha = 0.01$ levels. No differences were noted between the sexes in regard to behavior, and thus, the data were pooled.

Color and wavelength measurements. The measurement of the backward light scattering with directed reflection took place using a Lambda 950 spectrometer (Perkin Elmer). This device is suitable for measurements in the UV/VIS/NIR range from about 200 nm to 2500 nm. The measurement of each colored paper was conducted at discrete wavelengths in this range with a distance of 1 nm (Supplementary Figure 4C), which allows for the more discrete characterization of each color used (i.e., green reflected light between 480 and 580 nm, and was well within the expected range for this color).

Wing pigmentation and light/dark courtship. The wings from male and female adults from each species were dissected and mounted with a slide and coverslip, with images generated using a Zeiss AXIO microscope under bright field and transmitted light (Fig. 4e, f). Wing pigmentation was noted for males and females from all species (https://doi.org/10.17617/3.1D; 08 Wings), with examples shown for most wings with any spots or pattern, where there was a significant trend of wing pigmentation being correlated with larger eye species relative to antennal size (Fig. 4c; Supplementary Figure 3H). Previously published data for courtship that required light, or where courtship was better under light conditions (yellow bars in Fig. 4e) or where courtship was possible in the absence of light (black bars in Fig. 4e) are shown (Supplementary References), with new data denoted by an asterisk. Light-dependent courtship, as well as mating better in light conditions, was also correlated with larger eye size relative to the antenna, suggesting a connection between vision and visually-mediated courtship signals such as wing pigmentation (Supplementary Figure 3 I). For statistical measurements, we used the package caper (Comparative Analyses of Phylogenetics and Evolution in R)[79] as well as the packages ape (Analyses of Phylogenetics and Evolution) and phytools (Phylogenetic Tools for Comparative Biology) to perform phylogenetic generalized least squares (pgls) and employed Pagel's lambda, Blomberg K, and the Brownian model of phylogenetic relatedness, with the R-script available online. We chose the caper package as we were most comfortable with the way it handles missing data, for example during the analyses of light/dark courtship, where published behavioral data are missing for several species. For all three phenotypes (female wing pigmentation, male wing pigmentation and courtship in light-dark), the estimates of Pagel's lambda for the branch length transformation significantly deviate from a strict Brownian motion process model of phylogenetic relatedness (i.e., deviate from lambda = 1; for more details, please see R-script at doi.org/10.17617/3.1D; 02 Molecular Phylogeny).

Staining of imaginal discs. Fly species were selected using stratified random sampling in order to represent as many subgroups as possible. Third instar larva were allowed to self-clean for several minutes in 1 M phosphate-buffered saline (PBS) and then dissected in fresh PBS. In a first dissection step, the imaginal discs were kept attached to mouth hooks and central brain to add structural stability. This coarse dissection product was transferred into 0.5 -mL reaction tubes with fresh, cold 300 µL of 1 M PBS. The PBS was exchanged against cold 400 µL of fixative, and the tissue was incubated in the paraformaldehyde solution on ice for 35 min. Next, tissue samples were washed in cold 400 µL of 1 M PBS five times for 5 min each. After removal of the PBS, the dissection products were incubated in the blocking solution on ice for 45 min. Then the blocking solution (1 M PBS plus 7% normal goat serum) was replaced with the staining solution (blocking solution with 0.07% Hoechst and 1% Phalloidin 488) and samples were incubated on a rotator at 4 °C for 2 h. Subsequently, the tissue was washed again in cold 400 µL of 1 M PBS

five times for 5 min each. In a fine dissection step, the imaginal discs were then freed from all other connected tissues, and then mounted on object slides using a drop of Entellan® (Merck, Darmstadt, Germany). Sections of the imaginal disc were measured in Fiji software, and ratios were generated of surface areas for the eye divided by the corresponding antennal surface area (Fig. 5h; Supplementary Figure 6C), with 6–14 replicates per species, always taken from third instar wandering phase larvae just prior to pupation (Supplementary Figure 7).

Statistics and figure preparation. Statistical analyses were conducted using GraphPad InStat 3 (https://www.graphpad.com/scientific-software/instat/) and R Project (https://www.r-project.org/), while figures were organized and prepared using R Studio, Microsoft Excel, and Adobe Illustrator CS5. Additional details concerning tests of allometry, multiple regression, and phylogenetic correction are contained within the publically available R scripts that are described below in the Code availability section.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Code availability. All scripts for R, including curation of what tests were conducted, as well as the raw data files used for each statistical analysis are available at DOI: 10.17617/3.1D [10.17617/3.1D] (see 02 Molecular Phylogeny; 12 Allometry)[80].

Data availability

All data supporting the findings of this study, including methodology examples, raw images and z-stack scans, molecular sequences, accession numbers, statistical assessments as well as species information are all available through Edmond, the Open Access Data Repository of the Max Planck Society, https://doi.org/10.17617/3.1D [10.17617/3.1D][80].

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Author contributions

This study was built on an idea conceived by I.W.K., while V.G., B.S.H., and M.K. all contributed to the design of this study. V.G. and I.W.K. completed the images and measurements associated with body morphometrics and ommatidium metrics. V.G. handled all neuroanatomy measures as well as the 3D reconstructions. L.G. and I.W.K. worked on the sensillum counts, while L.G. completed the lambda scans for antennal descriptions. I.W.K., G.B., and B.A.B. conducted the behavioral trials. I.W.K. and S.K. performed the imaginal disc experiments and metrics, including labeling, staining as well as confocal scans, with S.L.L. and J.R. providing their expertise. I.W.K. and D.R.V. worked on the courtship and wing images, as well as the data analyses. G.F.O., I.W.K., and M.A.K. assessed and built the molecular phylogeny, where D.R.V. and G.K. completed the statistical analyses for phylogenetic correction. M.A.K. and I.W.K. selected, ordered, and maintained fly species. I.W.K. prepared the original paper and all figures, while I.W.K., B.S.H., and M.K. all contributed to the final manuscript and subsequent revisions.

Additional information

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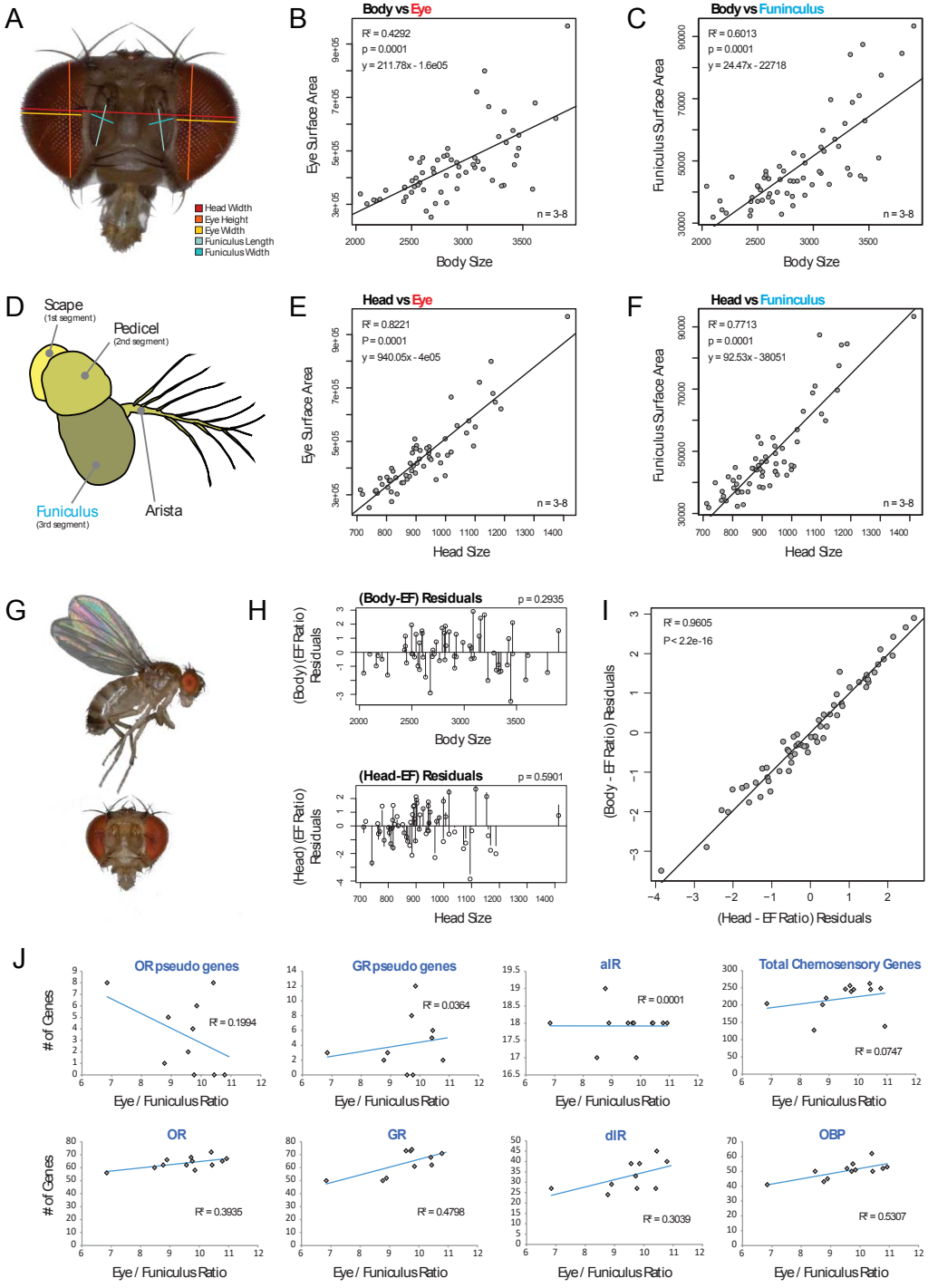
Supplementary Information

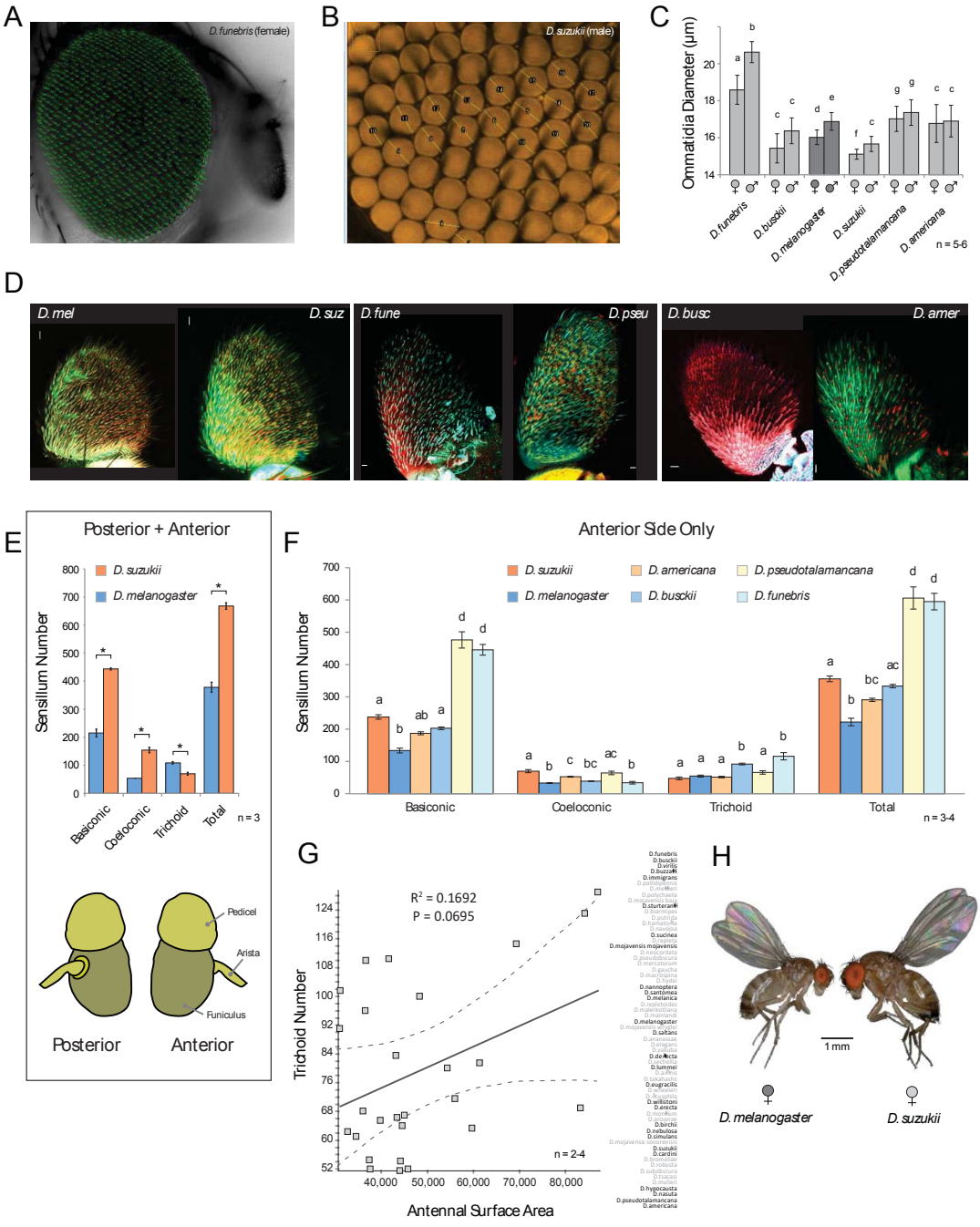
Inverse resource allocation between vision and olfaction across the genus *Drosophila*

Keesey et al.



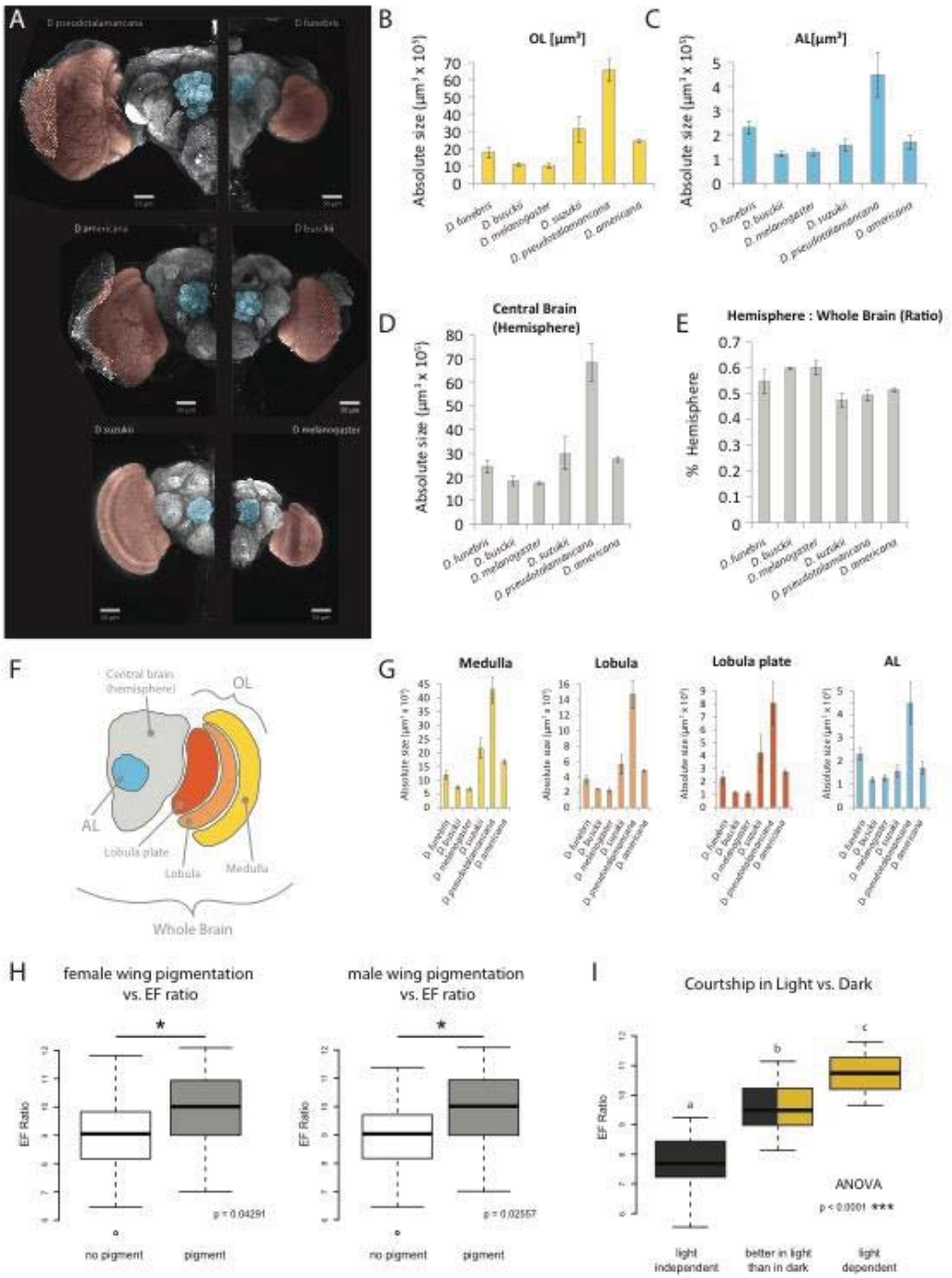
Supplementary Figure 1: External morphometrics from 62 species and functional chemoreceptor genes. (A) Example of measurements taken to calculate eye and funiculus surface area for each species. (B,C) Eye and funiculus surface area (μm^2) as compared to body size for each species. (D) Diagram of the *Drosophila* antenna, highlighting the 3rd antennal segment, also known as the funiculus (where the majority of chemosensory sensilla are located). (E,F) Eye and funiculus

surface area (μm^2) as compared to head size for each species. (G) Example of lateral and frontal views (*Drosophila melanogaster*), which were used to measure the body, head, eye and funiculus. (H) Plotting of the residuals, where neither body nor head size significantly correlate with the EF ratio trait, suggesting that this trait does not simply scale allometrically with respect to body and head size. (I) Residuals of head and body have highly similar deviations from EF-ratio, supporting that body and head size are highly correlated across all species. (J) Different chemosensory genes from 12-14 *Drosophila* species genomes and their correlation to the EF ratio [1], where number of olfactory pseudogenes, for example, does not suggest a sensory tradeoff. (Data are provided at doi.org/10.17617/3.1D).



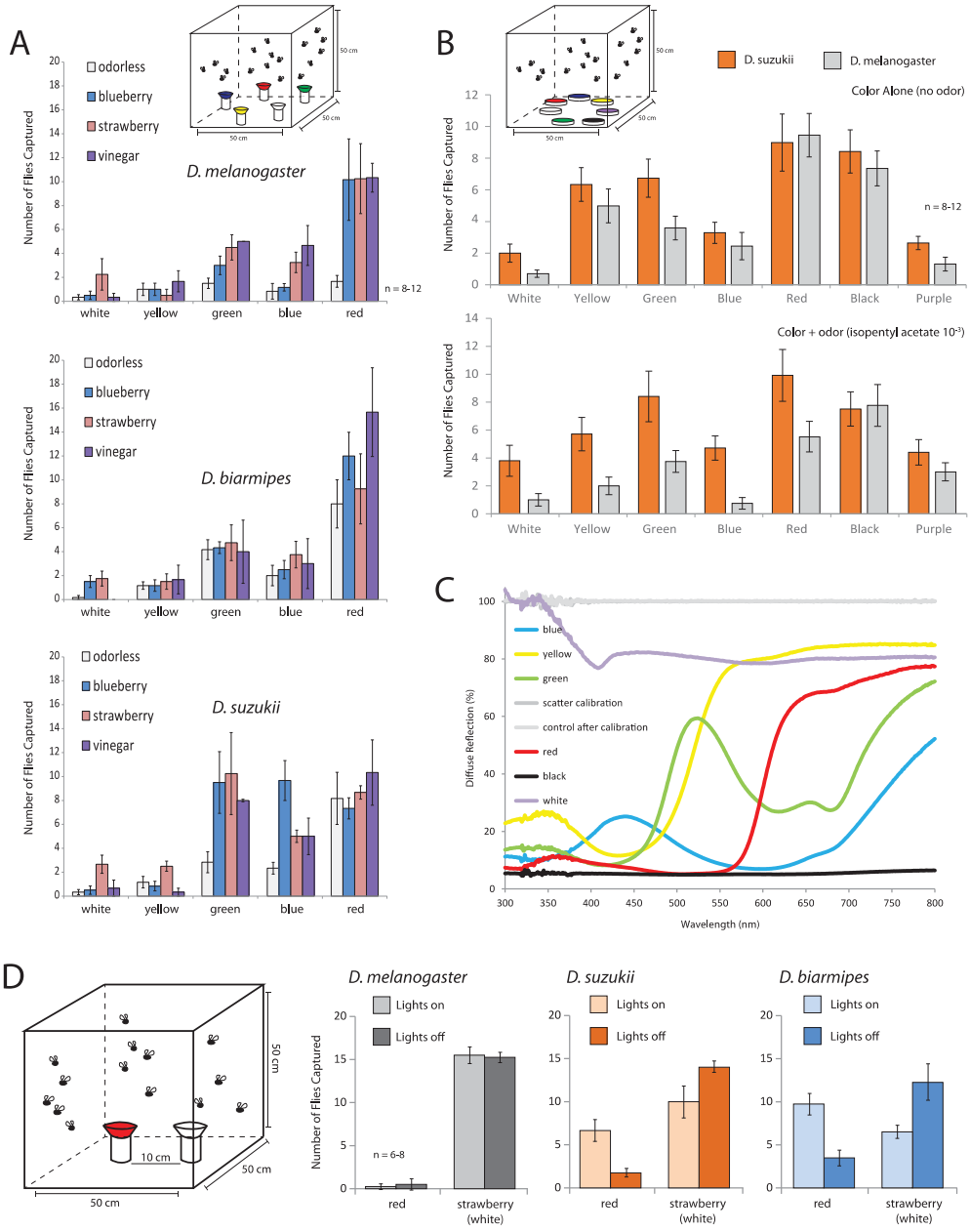
Supplementary Figure 2: Visual and olfactory sensory receptor measurements. (A) Example of ommatidium counts from photomontage of lateral view of *D. funebris* female head. (B) Examples of measurements taken to compare ommatidium diameters between species. (C) Ommatidia diameters. Means with the same letter are not significantly different from each other (ANOVA with Tukey-Kramer multiple comparison test). Error bars represent standard deviation. (D) Shown are examples of the images used for sensillum counts that were taken from stacked lambda mode scans (maximum intensity projections) of the anterior portion of the antenna for all 6 species examined. (E) Absolute sensillum counts from both sides of the antenna, as well as a diagram of anterior and posterior sides. Red to yellow color

signifies vision or visual bias, while blue indicates olfaction or olfactory species. An asterisk denotes statistical significance between two groups (*P ≤ 0.05, ***P ≤ 0.001; T-test). (F) Sensillum counts from lambda scans from only the anterior side of the antenna and the comparisons between all six species. Means with the same letter are not significantly different from each other (ANOVA with Tukey-Kramer multiple comparison test). Error bars represent standard deviation. (G) There is no correlation between trichoid number and antennal surface area, arguing against the idea that larger species necessarily have more trichoids. (H) Absolute size comparisons between two species, illustrating the differences in body, head, and eye morphology, where the body of the *D. suzukii* female is 1.5 times larger, but possesses a 2.5 times larger eye than the *D. melanogaster* female. (Data are provided at doi.org/10.17617/3.1D).



Supplementary Figure 3: Optic and antennal lobe measurements from 6 species. Red to yellow color signifies vision or visual bias, while blue indicates olfaction or olfactory species. (A) Confocal scans of each *Drosophila* species, with colored highlights for optic lobe (OL; red) and antennal lobe (AL; blue). Shown are the absolute measures of optic lobe (B), antennal lobe (C), and central brain volume (D), for each target species. (E) Although each species differed in absolute size, the ratio of central brain to total or whole brain (OL, AL, and central brain) for each species was roughly the same.

(F) Schematic of measurements taken from different species. (G) Absolute size of components of the OL and the AL from each species. (H) Female and male wing pigmentation plotted against EF ratio, where there is a correlation between relatively larger eyes and wing pigment across both sexes. An asterisk denotes statistical significance between two groups (*P ≤ 0.05, ***P ≤ 0.001; T-test). (I) Data from courtship in light or dark conditions as tested against EF ratio, where there is a highly significant difference in EF ratio across the three groups of courtship. Here again, relatively larger eyes correlate with better performance in light conditions, or with complete light-dependence for courtship. Means with the same letter are not significantly different from each other (ANOVA with Tukey-Kramer multiple comparison test). (Data are provided at doi.org/10.17617/3.1D).

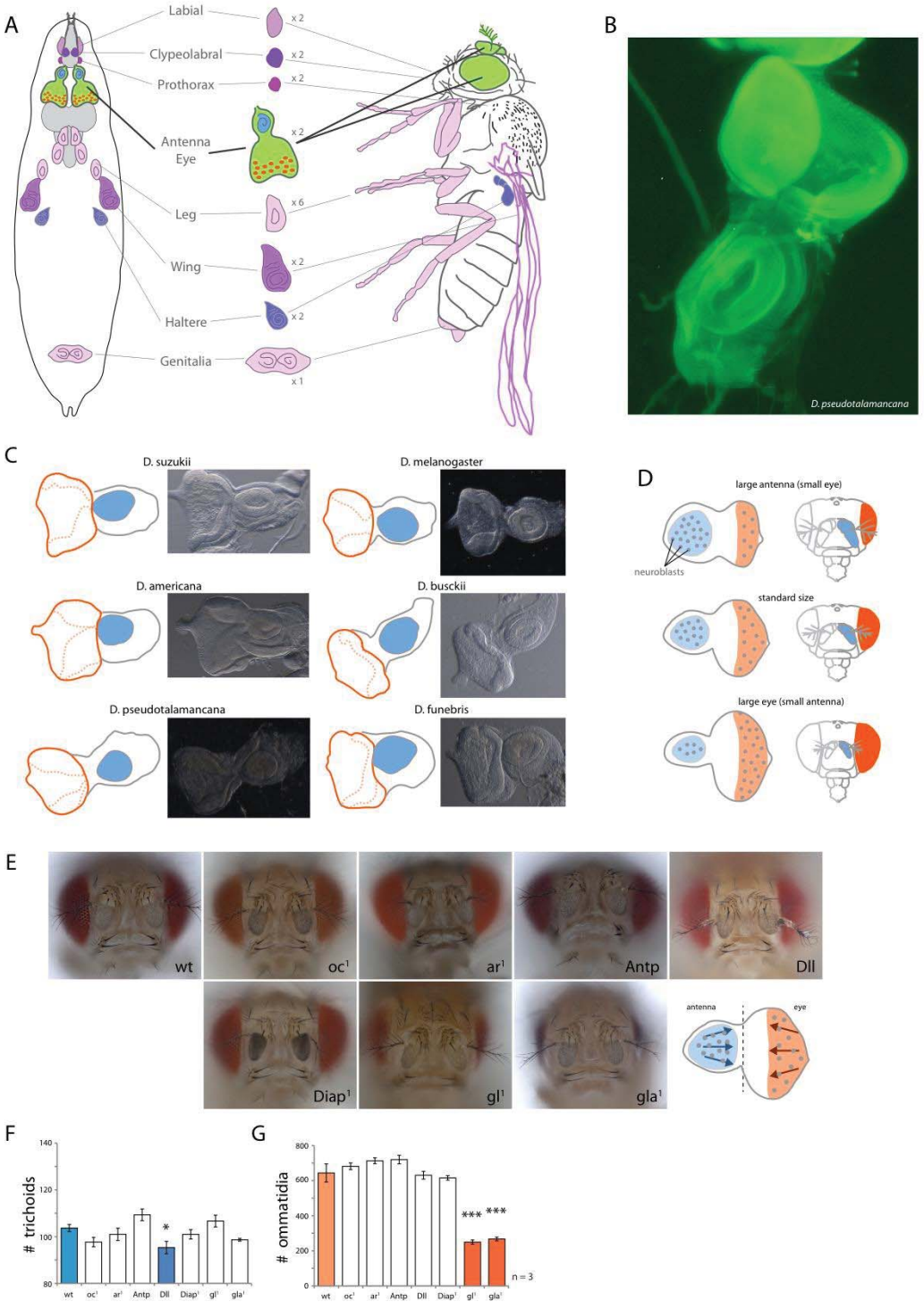


Supplementary Figure 4: Behavioral assays for visual and olfactory host navigation. (A) Design of trap assays using several visual and olfactory objects in testing attractive stimuli for each species. Red was the most attractive against the white background for all species regardless of the odor type, and even without odor, red was sufficient to capture spotted wing species. There was no significant difference in attraction to red when in combination with the three tested odors. The only color difference between species was noted to be an attraction to green for *D. suzukii*, as well as blue when in combination with blueberries, which they were reared upon. (B) Petri dish behavioral assay comparing *D. melanogaster* and *D. suzukii*, where both species showed similar color preference when presented without odor, although when with an odor, *D. suzukii* had a higher tendency towards white, yellow, green, blue and red than the other species. (C) Reflection index and wavelength for each color used in the behavioral assays. (D) Two-choice trap assay,

conducted in either full light, or full darkness. With lights off, all tested species were able to successfully navigate to the odor source; however, with lights on, the spotted wing species often mistakenly selected the visual object and not the odor object containing the fruit or food source, suggesting perhaps a visual bias or preference. In contrast, *D. melanogaster* always navigated to the odor source regardless of light condition or visual object, suggesting an olfactory bias or priority for this sensory cue. (Data are provided at doi.org/10.17617/3.1D).

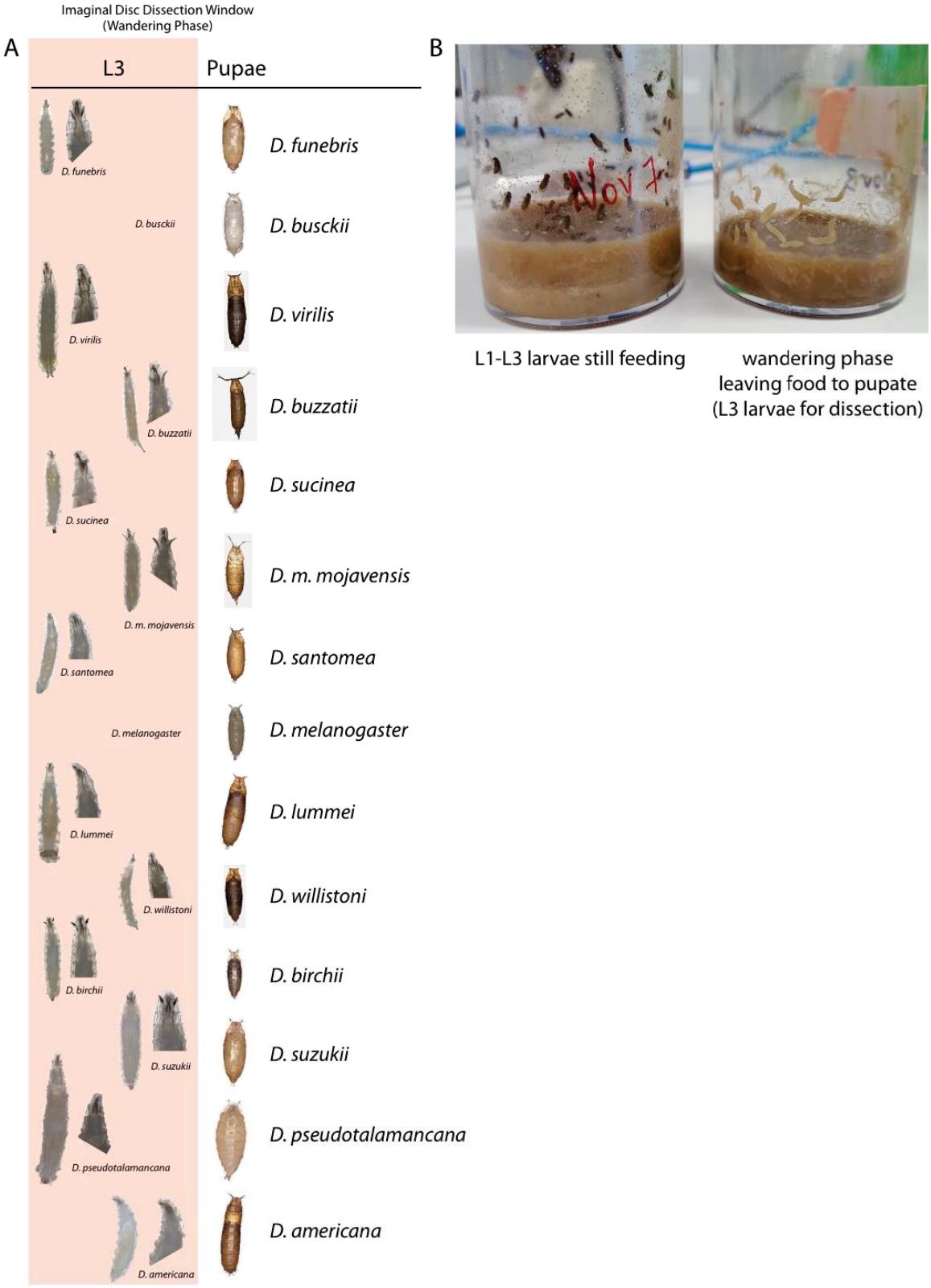

A
D.funebris
D.immigrans
D.hypocausta
D.santomea
D.busckii
D.melanogaster
D.buzzatii
D.erecta
D.simulans
D.moja moja
D.suzukii
D.sucinea
D.virilis
D.pseudotalamancana
D.eugracilis
D.lummei
D.cardini
D.deflecta
D.sturtevanti
D.nasuta
D.neocordata
D.nannoptera
D.willistoni
D.birchii
D.nebulosa
D.americana
D.melanica
D.saltans
B
D.biarmipes
D.takahashii
D.suzukii

Supplementary Figure 5: Antennal preparations and trichoid counts from selected species. (A) Each *Drosophila* species was mounted using single-sensillum recording (SSR) preparation techniques, and a series of images was taken to generate a z-stack photomontage. Trichoid sensilla were counted from male individuals over the same region of the funiculus for each *Drosophila* species. Images were taken with the arista mounted upward for consistency and for the best viewing angle as previously described for this sensillum type [2]. (B) Example of *Drosophila* species from a single phylogenetic clade that show a decreasing number of trichoid sensillum (left to right), and differences in surface area containing these sensilla, as well as differing sensillum length.



Supplementary Figure 6: The eye-antennal imaginal disc. (A) Diagram of the 19 total imaginal discs from *Drosophila* larvae and their corresponding location on the adult, highlighting that only one disc gives rise to two separate adult structures, namely the eye-antennal disc. (B) GFP labeling of *D. pseudotalamancana* imaginal disc, used to visualize the three-dimensional folding of the eye portion, as well as the shape and border of the antennal portion within the disc. (C)

Outlines and relative size measurements for eye and antenna from the imaginal discs of all 6 main species. Red color signifies vision or the visual system, while blue indicates olfaction. (D) Illustration of evo-devo theory of inverse resource allocation within one disc in order to generate a negative correlation between two adult sensory systems, the eye and antenna. (E) Wildtype and *melanogaster* mutants screened for either eye or antenna development, focusing on the ommatidium and trichoid numbers. (F) Trichoid number for each tested mutant, where only one was significantly different, Dll, which has an enlargement of the arista, and a decrease in each antennal segment size. Asterisk denotes significant difference from wildtype flies (T-test). (G) Ommatidium numbers from each mutant compared to the wildtype, where two lines showed marked reduction in ommatidia development. Asterisk denotes significant difference from wildtype flies (T-test). (Data are provided at doi.org/10.17617/3.1D).



Supplementary Figure 7: Pupae and 3rd instar wandering phase larvae. (A) Given that each species had a different

developmental duration from egg to adult, we selected larvae for imaginal disc dissection during the same developmental window of time, namely the 3rd instar wandering phase larvae, which occurs just prior to the onset of pupation. (B) Example of 3rd instar larvae feeding on top layer of food (left) and 3rd instar wandering phase larvae (right) that have stopped feeding and are in search of a suitable pupation site. The latter of which were selected from each species for consistent dissection of the imaginal disc. (Data are provided at http://doi.org/10.17617/3.1D)

Supplementary Table 1: All scientific names, rearing media and stock numbers. (A) *Drosophila* species in alphabetical order, in conjunction with media used for rearing, as well as stock center identity. More information about each species is available through these stock numbers (e.g. site of insect collection, collection date, and reference specimens) (B-C) Recipe for diets used in this study. Green and blue colored diets were supplemented with either *Opuntia* cactus powder or fresh blueberries to enhance oviposition. Flies were maintained in a density-controlled manner, with 20-25 females per vial.

A

	Species Name	Diet/Media	UCSD/Cornell Stock #
1	*Drosophila affinis*	banana food	14012-0141.00
2	*Drosophila americana*	banana food	15010-0951.00
3	*Drosophila ananassae*	normal food	14024-0371.12
4	*Drosophila arizonae*	banana food	15081-1271.33
5	*Drosophila biarmipes*	normal food	14023-0361.10
6	*Drosophila birchii*	normal food	14028-0521.00
7	*Drosophila bromeliae*	banana food	15085-1682.00
8	*Drosophila busckii*	banana food	13000-0081.00
9	*Drosophila buzzatii*	normal food	15081-1291.02
10	*Drosophila cardini*	banana food	15181-2181.03
11	*Drosophila deflecta*	banana food	15130-2018.00
12	*Drosophila elegans*	normal food	14027-0461.00
13	*Drosophila erecta*	normal food	14021-0224.01
14	*Drosophila eugracilis*	normal food	14026-0451.02
15	*Drosophila ficusphila*	banana food	14025-0441.01
16	*Drosophila funebris*	normal food	15120-1911.05
17	*Drosophila gaucha*	banana food	15070-1231.03
18	*Drosophila hamatofila*	banana food	15081-1301.05
19	*Drosophila hydei*	normal food	15085-1641.03
20	*Drosophila hypocausta*	normal food	15115-1871.04
21	*Drosophila immigrans*	normal food	15111-1731.00
22	*Drosophila lummei*	wheat food	15010-1011.01
23	*Drosophila macrospina*	wheat food	15120-1931.00
24	*Drosophila mainlandi*	banana food	15081-1315.02
25	*Drosophila malerkotliana*	banana food	14024-0391.00
26	*Drosophila melanica*	normal food + blueberry	15030-1141.03
27	*Drosophila melanogaster Canton S*	normal food	Hansson Lab Strain
28	*Drosophila mercatorum*	normal food	15082-1521.00
29	*Drosophila mettleri*	banana food	15081-1502.11
30	*Drosophila mojavensis baja*	Banana-Opuntia	15081-1351.30
31	*Drosophila mojavensis mojavensis*	Banana-Opuntia	15081-1352.10
32	*Drosophila mojavensis sonorensis*	Banana-Opuntia	15081-1352.32
33	*Drosophila mojavensis wrigleyi*	Banana-Opuntia	15081-1352.30
34	*Drosophila montium*	banana food	14028-0701.00
35	*Drosophila mulleri*	Banana-Opuntia	15081-1371.01
36	*Drosophila nannoptera*	banana food	15090-1692.00
37	*Drosophila nasuta*	normal food	15112-1781.01
38	*Drosophila navojoa*	Banana-Opuntia	15081-1374.12
39	*Drosophila nebulosa*	normal food	14030-0761.00
40	*Drosophila neocordata*	banana food	14041-0831.00
41	*Drosophila pallidipennis*	banana food	15210-2331.01
42	*Drosophila polychaeta*	normal food	15100-1711.01
43	*Drosophila pseudoobscura*	banana food	14011-0121.00
44	*Drosophila pseudotalamancana*	normal food	15040-1191.00
45	*Drosophila putrida*	banana food	15150-2101.00
46	*Drosophila repleta*	banana food	15084-16611.02
47	*Drosophila repletoides*	banana food	15250-2451.01
48	*Drosophila robusta*	banana food	15020-1111.01
49	*Drosophila saltans*	banana food	14045-0911.00
50	*Drosophila santomea*	banana food	14021-0271.01
51	*Drosophila sechellia*	normal food + blueberry	14021-0248.07
52	*Drosophila simulans*	normal food	14021-0251.01
53	*Drosophila sturtevanti*	normal food	14043-0871.01
54	*Drosophila subobscura*	banana food	14011-0131.04
55	*Drosophila sucinea*	normal food	14030-0791.00
56	*Drosophila suzukii*	normal food + blueberry	14023-0311.01
57	*Drosophila takahashii*	normal food + blueberry	14022-0311.00
58	*Drosophila tsacasi*	banana food	14028-0701.00
59	*Drosophila virils*	normal food	15010-1051.00
60	*Drosophila wheeleri*	banana food	15081-1501.04
61	*Drosophila willistoni*	normal food	14030-0811.24
62	*Drosophila yakuba*	normal food	14021-0261.38

B

Normal Food

		500ml
treacle	g	59
brewer`s yeast	g	5.4
hot water	ml	101
agar	g	2.1
cold water	ml	135
Polenta	g	47
fill up with hot water	ml	135
flush out with hot water	ml	34
cold water	ml	54
propionic acid	ml	1.2
Nipagin 30%	ml	1.65

C

Banana Food

agar	g	85
yeast	g	165
methylparaben	g	13.4
blended bananas	g	825
Karo syrup	g	570
liquid malt extract	g	180
100% ethanol	ml	134
water	L	6

D

Wheat Food

		1L
semolina (corn based)	g	50
wheatgerm	g	50
sugar	g	50
dry yeast	g	40
agarose	g	8
water	ml	1000
propionic acid	ml	5
methylparaben	ml	3.3

Supplementary References

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2. Lin, C. & Potter, C. J. Re-classification of *Drosophila melanogaster* trichoid and intermediate sensilla using fluorescence-guided single sensillum recording. *PLoS One* **10** e0139675 (2015). doi:10.1371/journal.pone.0139675

Manuscript V

Variable dependency on associated yeast communities influences host range in *Drosophila* species

ARTICLES PREPRINT

Variable dependency on associated yeast communities influences host range in *Drosophila* species

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The tight association between yeast metabolites and the attraction of fly species provides key evolutionary innovations that generate immense diversity within the genus *Drosophila*. Why and how changes in *Drosophila* niche preference occur, and what role yeasts play in local adaptation, is still largely unknown. Here, we analyze adult preference and larval development across three species of *Drosophila* as well as niche modifications through joint efforts by both insect and yeast. In general, we found that restrictions in host use are more a result of larval constraints than adult oviposition choice. We also determined that different life stages of fly development vary in their respective yeast preference, which may reduce cross-generational competition for resources. In this way, natural selection pressures may act quite differently on adult or larval performance, where adaptation events in larval stages likely being the stronger driver of niche evolution. Furthermore, we provide evidence that *Drosophila* and yeast together process host material to promote mutual benefits.

INTRODUCTION

What drives host preference across the genus *Drosophila*? Does the association of *Drosophila* species with certain habitats arise from benefits in fitness for the flies themselves and how does the nature of their associated microorganisms determine the host preference of each fly? Studying the principles and mechanisms underlying the odor-mediated interaction of *Drosophila* flies with yeast species that share the same habitat and ecological niche allows for insights into the evolution of mutualism and local adaptation. It is believed that metabolic activities of a sessile organism (e.g. yeast), together with physical modifications of its habitat, have the potential to create an ecological niche (also known as niche construction), which in return affects other organisms living in the same environment (Laland, Odling-Smee and Feldman, 1999; Odling-Smee, Laland and Felman, 2003; Goddard, 2008; Odling-Smee *et al.*, 2013; Buser *et al.*, 2014). Moreover, organisms populating the same habitat as a niche constructor such as yeast may experience benefits to their reproductive success through these habitat modifications (Odling-Smee *et al.*, 2013). In this case, chemical and visual cues that originate from the niche constructor can become mediators of an interaction between constructor and beneficiary. Thus, what may have started as a one-way interaction between beneficiary (insect) and niche constructor (yeast) can instead evolve into an interspecies mutualism if the niche constructor in return also profits from an association with the beneficiary (Doebeli and Knowlton, 1998; Herre *et al.*, 1999; Odling-Smee *et al.*, 2013; Buser *et al.*, 2014). A prominent example for niche construction is the release of ethanol, heat and CO_2 into the surrounding environment by the fruit-associated yeast *Saccharomyces cerevisiae* as part of the yeast's energy production via fermentation of sugars found in its host material (Goddard, 2008). The combination of these niche modifications can suppress the growth of other microorganisms and secure essential resources for *S. cerevisiae* by creating a competitive advantage (Goddard, 2008). In much the same way, the presence of *S. cerevisiae* at breeding sites of *Drosophila* species could prevent microorganisms that are harmful for adult and larval insect stages to establish in the flies' food source (e.g. pathogens and fungal molds).

The interactions between *Drosophila* flies and yeasts is hasbeen shown to be mediated by odors, as is the interaction of many other insects with yeasts (Davis *et al.*, 2013; Andreadis, Witzgall and Becher, 2015; Madden *et al.*, 2018). This phenomenon traces back to a co-evolution of chemosignal production in yeasts along with the development of corresponding detecting units for these odorants by insects (Engel and Grimaldi, 2004; Dujon, 2012; Davis *et al.*, 2013; Nel *et al.*, 2013; Becher *et al.*, 2018). Yeasts have been found to dedicate entire metabolic pathways towards the production of volatile chemosignals that are attractive to *Drosophilidae* and other insect species (Christiaens et al., 2014; Becher et al., 2018). For example, the production of acetyl esters by *S. cerevisiae*, such as ethyl acetate which is a highly attractive odorant for *Drosophila* flies, is mediated by an alcohol acetyl transferase encoded by the yeast's ATF1 gene (Christiaens *et al.*, 2014). Beyond the production of these attractive chemosignals, it is not known whether yeasts invest additional resources into the maintenance of an association with *Drosophila* flies, such as the generation of further compounds that are not directly necessary for the yeasts' own survival.

In the case of *D. melanogaster*, emission of volatiles by associated yeasts has been proven to be a key factor in the flies' attraction and preference towards a substrate as a food source or oviposition site, while odorants released by the host material itself play only a secondary role in this behavior (Becher *et al.*, 2012; Scheidler *et al.*, 2015). Furthermore, recent findings show that the presence of viable yeast spores in fly diet causes changes in larval development and survivorship as well as changes in adult phenotypic traits (e.g. fecundity and cuticular hormone production), adult behavior (e.g. food choice) and longevity (Anagnostou, Dorsch and Rohlfs, 2010; Fischer *et al.*, 2017; Bellutti *et al.*, 2018; Grangeteau *et al.*, 2018; Murgier *et al.*, 2019). As such both *Drosophila* species and yeasts may mutually benefit from a lifestyle in close association with each

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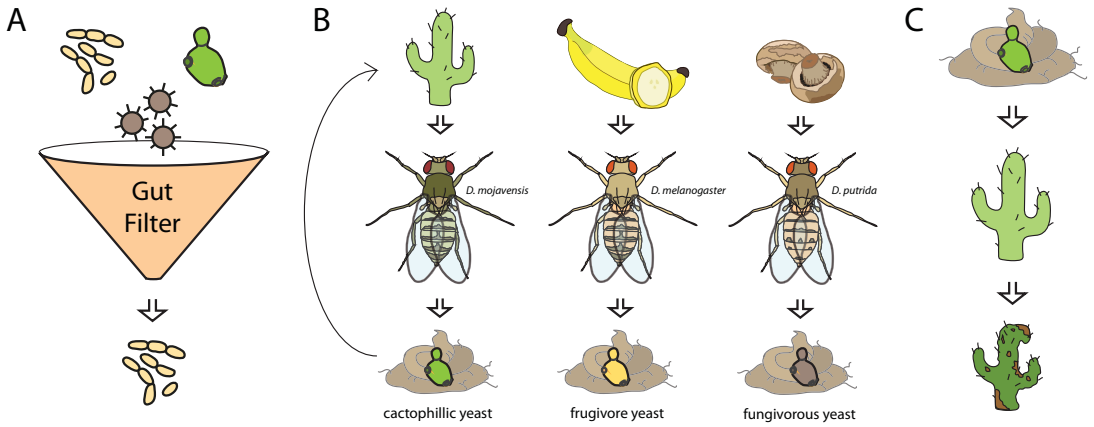


Figure 1 | Hypothesized interplay of insect, yeast and their hosts. (A) A fly species-specific lifestyle and gut physiology allow only a certain set of specific microorganisms to survive and thrive in the insect's alimentary canal. Thus, the fly's gut could act as a filter selecting the microorganisms it excretes. (B) *Drosophila* species may display adaptive behaviors towards not just their host, but also towards yeast species associated with that ecological niche. The interaction of insect and yeast species could be mutually beneficial providing the flies with nutrition and enabling dissemination of the yeast. (C) Example of an interplay described in (A) and (B): Cactophilic *Drosophila* species are attracted to cacti and cacti-associated yeast. Yeast spores are ingested by the flies and vectored via an oral-fecal route onto new hosts. The cactophilic yeast efficiently breaks down the host material, releasing nutritional factors. We hypothesize that growth of both the insect and the yeast will be optimal when placed on cactus material, and that this will be additionally measurable by the observed rate of host decay.

other. In many cases, yeasts enrich the flies' host substrate with nutritional factors such as amino acids, antioxidants, fatty acids, sterols and vitamin B's, which are all essential dietary components that the host alone cannot provide (Loeb and Northrop, 1916; Tatum, 1939; Becher *et al.*, 2012; Dweck *et al.*, 2015). These yeast-produced nutrients might in turn affect the ability of *Drosophila* flies to resist pathogens and parasitoids (Vass and Nappi, 1998; Rivera *et al.*, 2003; Li *et al.*, 2007; Lee, Simpson and Wilson, 2008; Anagnostou, LeGrand and Rohlfs, 2010).

In exchange for nutrients supplied by yeasts, *Drosophila* flies act as a vector for the dispersal of microbial species, carrying yeast spores on their body or transferring them to new substrates through the oral-fecal route (Ganter, 1988; Starmer, Peris and Fontdevila, 1988; Reuter, Bell and Greig, 2007; Coluccio *et al.*, 2008). In addition, passage through the alimentary canal of *Drosophila* flies has been shown to release yeast spores from their tetrads and enable the yeasts to sexually reproduce, which in return promotes genetic mixing (Reuter, Bell and Greig, 2007). In this case, sexual reproduction then allows the yeasts to adapt faster to changes in their environment through outbreeding (Reuter, Bell and Greig, 2007), which is similar to the advantages afforded to flowering plants via insect-driven pollination. Besides the function of dispersal and outbreeding through *Drosophila* species, previous data suggest that the presence of *D. melanogaster* larvae in host material additionally affects yeast cell numbers and composition of yeast communities to the benefit of a few selected yeast species (Stamps *et al.*, 2012). Flies achieve this through the deposition of frass, which contains nutritional factors and preselected microbes as well as through physical modifications of the substrate structure (Stamps *et al.*, 2012). It is known that the alimentary canal of insects and vertebrates can act as a filtering unit for the cultivation of a selected community of microbes, including yeasts (**Figure 1 A**, (Stefanini, 2018)). *D. melanogaster* and *Drosophila* flies in general need to actively promote the growth of their specific beneficial yeast species at new breeding sites since these sites already possess an established microbial community before flies arrive, feed, and lay their eggs. Growth promotion in return helps fly-associated yeasts in their efforts of niche construction, which further aids in the establishment of a mutualistic relationship. However, in *Drosophila* species it is not yet known whether flies actively seek out and select certain yeasts in order to acquire a species-specific microbiome that is optimized for their reproductive success. Are *Drosophila* flies farming species-specific yeast communities, transporting the yeasts to suitable substrates, promoting the yeasts' growth and suppressing the colonization of harmful or non-beneficial microorganisms? This would be comparable to other well-studied insect-microbe partnerships such as leafcutter ants with *Lepiotaceae* fungi or the association of the European beewolf with actinobacterium '*Candidatus* Streptomyces philanthi' (Chapela *et al.*, 1994; Kaltenpoth *et al.*, 2005; Mueller *et al.*, 2005; Kroiss *et al.*, 2010). In each case the microbe and insect act in a partnership with mutual benefits.

In the present study, we test the hypothesis that *Drosophila* flies will favor an association with yeast species from a shared habitat over those yeast species from unfamiliar ecological niches (**Figure 1 B**). We predict that *Drosophila* species can discriminate between yeasts based on species-specific chemosignals emitted by the yeasts and that flies will be most attracted to yeast species from a common host substrate to

ensure mutual benefits. Presumably, attractiveness of a yeast from a familiar environment would be linked to an increase in larval and adult performance. Similarly, we suspect that yeasts will also preferably attract *Drosophila* species from a shared habitat to ensure their transfer to suitable substrates for growth (**Figure 1 C**). In agreement with previous studies we find that *Drosophila* flies can distinguish between different yeasts based on chemosensory cues. However, we observed that *Drosophila* species may favor yeasts from new environments over yeast species from a familiar ecological niche. Thus, we propose that the association of *Drosophila* with yeast species from new habitats can lead to local adaptation processes and may be a driving force behind new speciation events and the establishment of novel insect niches. We also found that larval and adult performance is differently affected by yeast species caused by differences in nutritional needs in these two stages, and furthermore, that the yeast preference of the female fly at oviposition sites does not necessarily correlate with optimized larval performance. Lastly, we provide evidence that *Drosophila* flies and yeasts from a shared habitat together participate in activities of niche construction that enhance both insect and yeast performance on a given substrate and thus both partners mutually benefit from specific associations (**Figure 1 C**).

RESULTS

Attraction of flies towards yeast cultures

For our study, we chose to work with the cosmopolitan *Drosophila* species *D. melanogaster* and two species with a specialized lifestyle, *D. mojavensis mojavensis* and *D. putrida*, which are found on cacti and mushrooms, respectively. For each *Drosophila* species and host combination we then

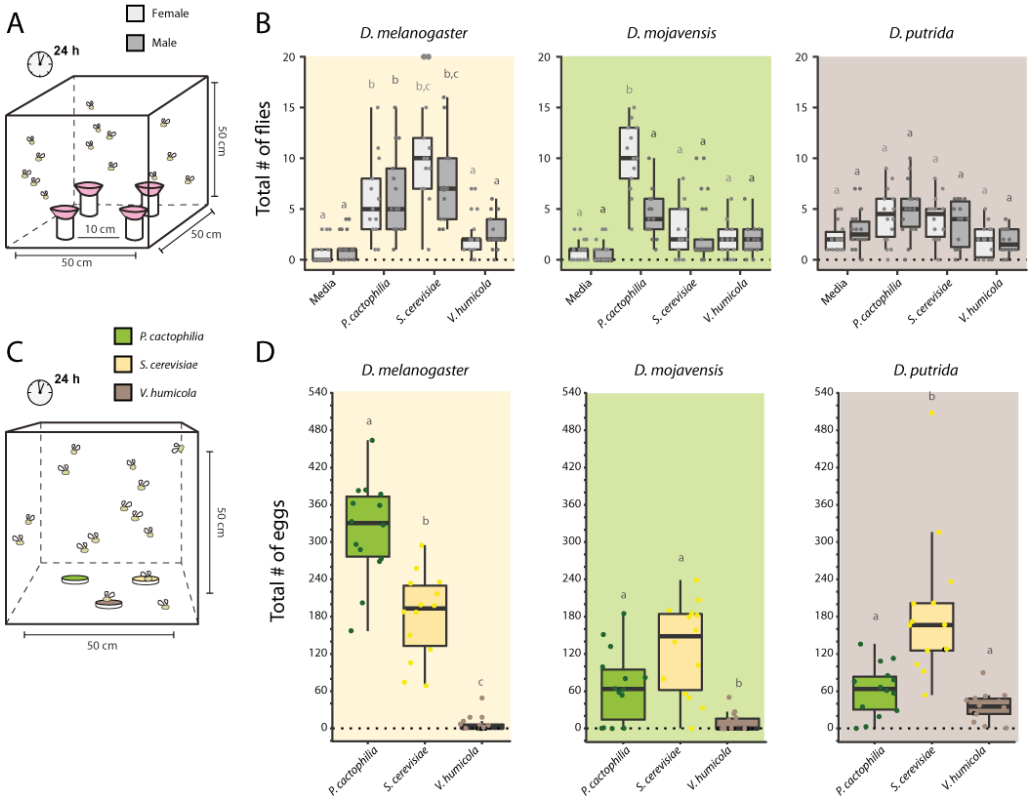


Figure 2 | Behavioral preferences of *Drosophila* species towards different yeast species without the background of host material. (A) Trap assays in which the flies were simultaneously presented with three different yeast species, as well as the growth medium (YM) as a control. Colored cones served as trap entrances. (B) Total number of flies caught in the different traps of the attraction assay separated by sex (n=14 replicates with each 40 individuals at a 1:1 sex ratio). Significant differences are indicated with letters above the box plots (Friedman test with Dunn's post test). Boxplots represent the median (bold horizontal lines) with the interquartile range (whiskers). (C) Design of oviposition assays, where gravid females could choose between three different oviposition plates, which consisted of modified standard diet inoculated with one of the tested yeast species. (D) Total numbers of eggs found on each oviposition plate for the three target *Drosophila* species in presence of the different yeast species. (n=14 replicates with each 20 female flies). Letters above the box plots indicate significant differences between the numbers of eggs found on the oviposition plates (Friedman test followed by Dunn's Multiple Comparison post test). Box plots show the median (bold horizontal lines) and whiskers the interquartile range.

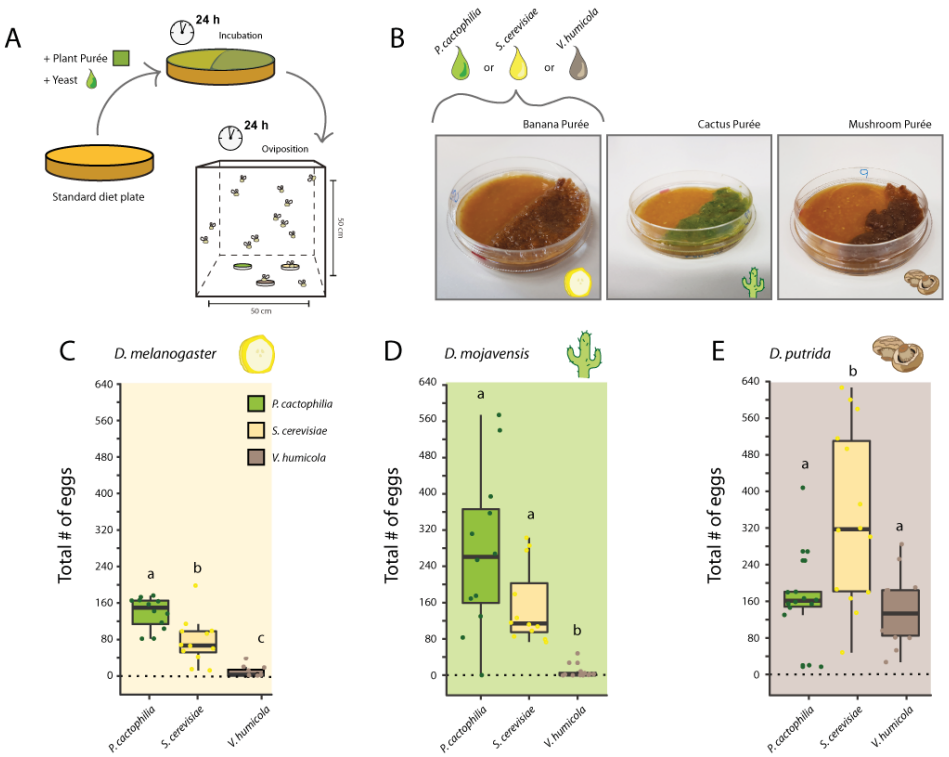


Figure 3| *Drosophila* oviposition preference on host material inoculated with different yeast species. (A) Oviposition assays on host material inoculated with target yeast species. (B) Examples of oviposition plates with the three different host materials. (C-E) Total number of eggs laid by females of *D. melanogaster* (C), *D. mojavensis* (D), and *D. putrida* (E) on the different host material. Letters above the box plots indicate significant differences between the numbers of eggs found on the oviposition plates (Friedman test followed by Dunn's Multiple Comparison post test; n=12-14 replicates with each 20 female flies). Boxplots represent the median (bold horizontal lines) with the interquartile range (whiskers).

selected one yeast species that has been isolated from the flies' host or has also been found in association with the respective *Drosophila* species (**Table S2**). Our first interest was to examine whether the target *Drosophila* species can distinguish between different yeast cultures and whether they show species-specific preferences in their attraction towards the provided yeast. For this, we simultaneously presented the flies with four options: the yeast malt (YM) growth medium alone or YM inoculated with one of the three different yeast species and allowed each *Drosophila* species to display their preference (**Figure 2 A, B**). For *D. melanogaster* adults, we observed an attraction towards *P. cactophilia* as well as *S. cerevisiae* cultures. Traps with *V. humicola* or YM medium contained significantly less flies (**Figure 2 B**).

In the case of *D. mojavensis* females, we observed a strong preference in attraction for *P. cactophilia* while none of the other yeast species produced significant attraction different from the control. Although little information is available about the yeast associated with *D. putrida*, this fly species is known to be mycophagous (Grimaldi, 1985). In our attraction assays with *D. putrida* we did not find significant differences in preference towards our target yeast species or the growth medium when we differentiated between fly sex. However, if we pooled the data, total fly numbers of *D. putrida* caught in traps with *P. cactophilia* culture were significantly higher than in traps with the other two yeast species or the YM control.

Oviposition assays with yeast on artificial diet

After ascertaining whether the target *Drosophila* species can differentiate between selected yeast species and whether the flies show species-specific attraction, we next pursued choice-tests of egg-laying behaviors using artificial diet containing the three yeast species (**Figure 2 C, D**). Flies were simultaneously provided with three oviposition plates that only varied in the supplemented yeast species, allowing each *Drosophila* species to display any preference for oviposition. *D. melanogaster* females showed a preference to oviposit on plates inoculated with *P. cactophilia* or *S. cerevisiae*, preferring *P. cactophilia* over *S. cerevisiae*. Plates with either of these yeast species were significantly different compared to *V. humicola* plates, where few if any eggs were deposited (**Figure 2 D**). In oviposition assays with *D. mojavensis* we found again a preference for both *P. cactophilia* as well as *S. cerevisiae*, in this case with a trend for oviposition in presence of *S. cerevisiae*. Similarly, *D. putrida* also preferred oviposition plates inoculated with S. *cerevisiae*. Interestingly, we observed that *D. putrida* laid a higher average

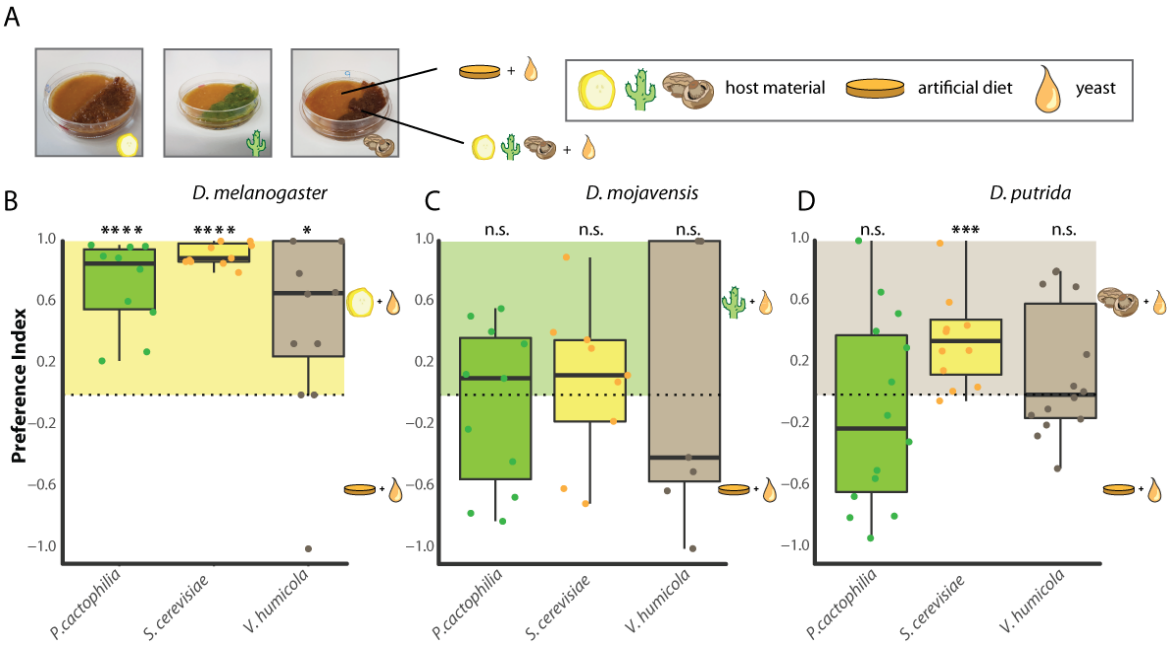
number of eggs on plates containing *V. humicola* than either of the other two *Drosophila* species (**Figure 2 D**, *D. melanogaster* 7 ± 13, *D. mojavensis* 7 ± 15, *D. putrida* 35 ± 23). Thus, despite its preference for *S. cerevesiae*, *D. putrida* appeared to be more willing to accept mycophagous yeast and to lay eggs in the presence of a yeast species that was seemingly less desirable for the other two *Drosophila* species that we examined.

Oviposition assays with yeast and host material

In order to examine the role that plant and yeast play in *Drosophila* oviposition preference, we next performed oviposition assays using host material in addition to our target yeast species (**Figure 3**). The base of the oviposition plate consisted of modified artificial diet. We covered half of the diet with homogenized host material (e.g. banana, cactus or mushroom) and then inoculated the whole plate with a pre-culture of one of the three target yeast species. Here, the flies were simultaneously presented with all three yeast species across a single host material (**Figure 3 A, B**). For *D. melanogaster*, we observed a nearly identical trend to that observed when we presented the yeast alone without host material (**Figure 3 C-E**). Moreover, the preference for *P. cactophilia* was even more pronounced when combined with banana purée, though we did not observe changes in the oviposition preference of *D. melanogaster* females towards *S. cerevisiae* or *V. humicola* in the presence of the host material (**Figure 3 C**). Again, there were only a few eggs laid on plates containing *V. humicola*, even in combination with banana purée. When we had a closer look at which half of the oviposition plates the *D. melanogaster* females favored, we found a clear preference of the females for the half containing host material (**Supp. Figure S1**). For *D. mojavensis*, we observed that when host and yeast were combined, females now showed a tendency to lay eggs preferably on substrate inoculated with *P. cactophilia* (**Figure 3 D**). Interestingly, we also found a drastic increase in total egg numbers laid by *D. mojavensis* when we combined yeast with their natural host material (**Supp. Figure S2**) as compared to the trials with yeast alone; however, we again documented hardly any oviposition on plates inoculated with *V. humicola*. Females of *D. mojavensis* did not demonstrate a preference whether to lay eggs on the host material or the artificial diet sides of the plates (**Supp. Figure S1**). In regard to *D. putrida*, this fly species significantly preferred to oviposit on *S. cerevisiae* but again laid also many more eggs than the other two *Drosophila* species on *V. humicola* (**Figure 3 E**). Additionally, on oviposition plates inoculated with *S. cerevisiae*, *D. putrida* favored the host material side of the plate (mushroom) as a substrate for oviposition (**Supp. Figure S1**). Similar to *D. mojavensis* overall oviposition numbers were greatly increased in *D. putrida* when both yeast and host material were combined (**Supp. Figure S2**).

Larval performance in the presence of different yeast species

As we had now demonstrated adult preferences for both attraction and oviposition, we next wanted to examine whether there was a correlation of this adult preference in regards to subsequent larval development (**Figure 4**). For all



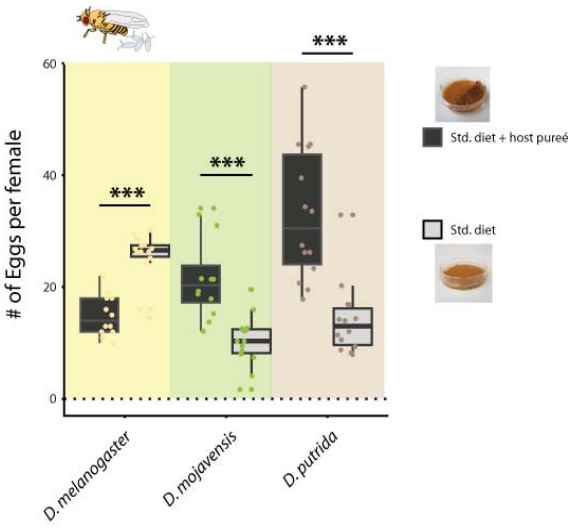
Supp. Figure S1 | Preference of *Drosophila* species to oviposit on artificial diet or host material inoculated with different yeast species. Distribution of eggs on oviposition plates from oviposition assays with host material vs artificial diet (Preference Index=(Number of eggs on host material-number of eggs on artificial diet)/number of eggs total)). For each *Drosophila* species the PI of six to 14 oviposition plates per yeast species was calculated. Filled box plots indicate a significant difference from zero, transparent box plots show no significant (ns) difference from zero (two tailed One-sample t-test; ns P> 0.05). Box plots show the median (bold horizontal lines) and whiskers the interquartile range.

target *Drosophila* species, we monitored larval development, survivorship and the time point of adult eclosion using cohorts of 15 individual first instar larvae that were transferred onto modified standard diet, which was mixed with one of the three yeast species. On average, *D. melanogaster* pupae first appeared on day four, with no trends or significant difference in timing or numbers for each of the different yeast treatments (**Figure 4 A**). Larvae of *D. mojavensis* took longer than *D. melanogaster* larvae to reach pupation, with an average of six or seven days for most replicates. We noted that *D. mojavensis* larvae growing up on *P. cactophilia* developed on average one to two days faster than those which had been reared on the other two yeast species. Moreover, we also documented that more *D. mojavensis* larvae reached pupation when reared on diet supplemented with *P. cactophilia*. In assays with *D. putrida* differences in larval development and survivorship were more drastic where few if any larvae survived when reared on artificial diet supplemented with *S. cerevisiae* or *P. cactophilia*. Strikingly, only in the presence of *V. humicola* did we observe considerable numbers of *D. putrida* larvae to survive until pupation.

Similar to our examination of larval developmental rate, we also tracked adult eclosion for each *Drosophila* species in association with different inoculated yeast species (**Figure 4 B**). Here overall numbers of *D. melanogaster* adults that hatched were comparable for all yeast treatments. However, adult eclosion was on average one day delayed when *D. melanogaster* larvae developed on artificial diet containing V. *humicola*. In assays with *D. mojavensis*, adults hatched on average fastest on the yeast from their natural habitat, *P. cactophilia*. If larvae had been reared in association with yeast that are less prevalent in their ecological niche, *S. cerevisiae* or *V. humicola*, we observed fewer overall adult *D. mojavensis* surviving to eclosion. In the case of *D. putrida* the few larvae which made it until pupation in the presence of *P. cactophilia* also successfully eclosed, while larvae on a diet containing *S. cerevisiae* never reached further developmental stages beyond first or second larval instar. Only food supplemented with *V. humicola* led to *D. putrida* adult survivorship numbers higher than 6 %, where survivorship was on average 39 % in the presence of this mycophagous yeast species. Our data suggest that while the generalist, *D. melanogaster*, can perform equally well on a wide range of different yeast species, that both of the other *Drosophila* specialists display larger variation in developmental time as well as survivorship when ingesting and exposed to different yeast species. This may be related to evolutionary restrictions on larval development due to specific nutritional needs.

Adult phenotypes from individuals grown on different yeasts

We further analyzed the ramifications of insect rearing in association with the target yeast species by addressing fly performance and reproductive development. Here, we analyzed these changes by collecting data on ovary size and adult weight as indicators for fecundity and overall fitness after being reared on different yeasts. We collected females from each *Drosophila* species across each of the three yeast treatments, where we first weighed them, and then dissected female ovaries (**Figure 5**). In agreement with the adult



Supp. Figure S2 | Comparison of egg numbers on either artificial diet or host material both inoculated with yeast. For each *Drosophila* species the data from the oviposition assays in figure 2 and 3 was compared for the numbers of eggs per female. Box plots show the median (bold horizontal lines) and whiskers the interquartile range. Asterisks above the box plots indicate statistically significant differences (*D. melanogaster* & *D. mojavensis*: two-tailed Mann-Whitney test, *D. putrida*: unpaired t-test with Welch correction; n=12-14).

preference for *P. cactophilia* (**Figure 2 D and 3 C**), ovary size and adult weight of *D. melanogaster* females grown on diets containing *P. cactophilia* were both significantly increased compared to these metrics from females reared on the other two yeast species. Development of *D. melanogaster* females was poorest in association with *V. humicola*, where ovaries generally appeared underdeveloped (**Figure 5 A**). In assays with *D. mojavensis* females, ovaries were significantly larger from individuals reared on diet containing *S. cerevisiae*; however, in contrast to assays with *D. melanogaster*, larger ovaries did not correlate with generally heavier females. Highest weight measurements were documented for *D. mojavensis* females that emerged from larvae fed on diets supplemented with *P. cactophilia* (**Figure 5 B**). Overall, *D. mojavensis* females preferred to lay eggs on diet inoculated with *S. cerevisae* (artificial diet plus yeast) or *P. cactophilia* (host material plus yeast), which are the yeast species that produced progeny with the biggest ovaries with numerous ovarioles and heavy, well-nourished females (**Figure 2 D, Figure 3 D**). As larvae of *D. putrida* did not survive on diet containing *S. cerevisiae,* and as the few adults emerging from diet inoculated with *P. cactophilia* were all males, we could only assess measurements from females reared on *V. humicola* (**Figure 5**).

Odor profile of target yeast species

After finding evidence that our target *Drosophila* species had a species-specific preference towards the different yeast species and thus were able to differentiate between these yeast species based on volatile odors, we next wanted to analyze the

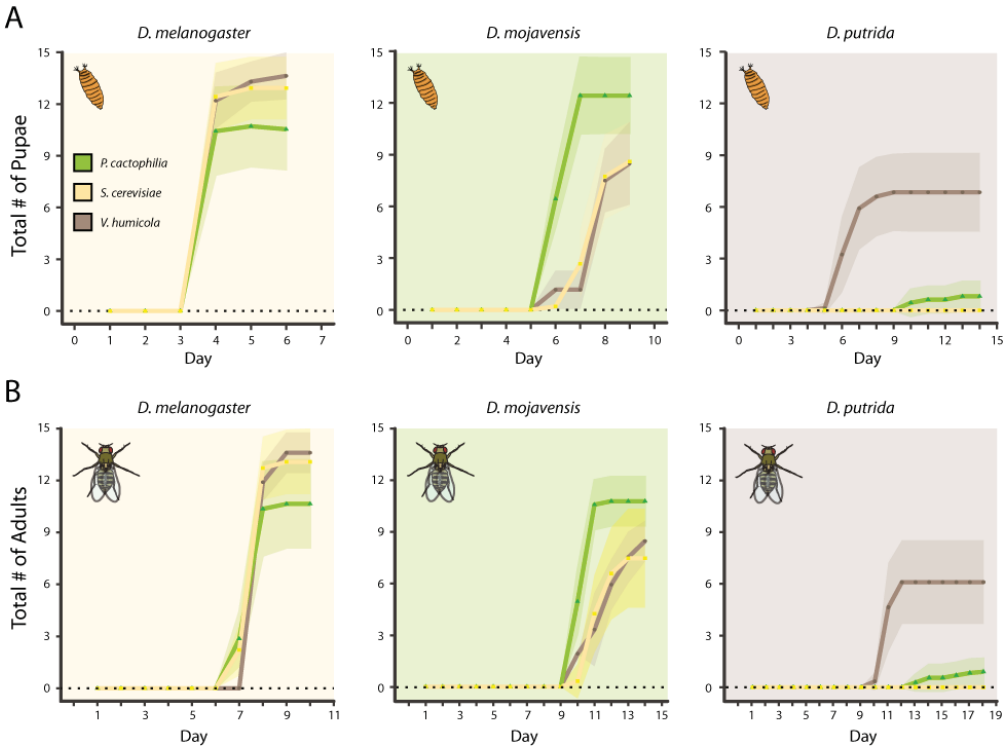


Figure 4 | Effects of target yeast species on larval developmental time metrics and survivorship. (A) Average numbers of pupae (n=15 replicates with each 15 first instar larvae) that were counted during each day in vials with different yeast species. Ribbons around line plots represent the standard deviation for the pupae number per day. (B) Average numbers of adults emerging from pupae in vials with different yeast species. Ribbons show the standard deviation from the average per day.

headspace of the three yeast species to compare their odor profiles and possibly identify any yeast species-specific odorants (**Figure S3**). From the three tested yeast species, *P. cactophilia* produced odorants in the highest abundance and variety while *V. humicola* emitted volatiles in the lowest abundance and diversity (**Figure S3 B-D**). In general, *V. humicola* was also the only yeast species in whose headspace we did not find evidence for the presence of 2-phenyl-1-ethanol. This odorant is known to be highly attractive for several *Drosophila* species and is associated with fermentation processes (**Figure S3 D**, (Becher *et al.*, 2012, 2018)). For the vinegar fly *D. melanogaster*, beside 2-phenyl-1-ethanol, eight additional volatile compounds produced by yeasts have been identified to induce upwind attraction leading to landing behavior at the source (Becher *et al.*, 2018). In our analysis of the volatiles emitted by *S. cerevisiae* we found four out of these nine compounds (**Figure S3 C**), while the headspace of *P. cactophilia* contained six odorants described as attractive to flies and *V. humicola* only produced two out of these nine volatiles. Most of the additional odorants identified solely in *P. cactophilia* samples were esters that are naturally also found in fruity bouquet such as ethyl propionate, isobutyl acetate and ethyl butyrate (**Figure S3 B**).

The three *Drosophila* species showed antennal responses in electroantennogram (EAG) recordings towards each yeast species indicating that all tested *Drosophila* species were capable of detecting the odor bouquets emitted by each of the different yeast samples (**Figure S4 B-D**). However, the three species differed in their antennal sensitivity towards the individual yeast headspaces, corresponding to observed *Drosophila* species-specific differences in their respective yeast preference.

Effects of yeast presence on host decay

After we had studied the attraction, oviposition, larval development and progeny fitness of our three *Drosophila* species towards selected yeast species, we next aimed to learn if and how our target yeast species affected host decay. Here, we wanted to draw conclusions about a possible release and provision of nutritional factors for *Drosophila* adults and larvae via host decomposition through the presence of the associated yeast species. Furthermore, we examined indicators for differences in the yeast performance itself on the three different *Drosophila* hosts. First, for each host, we qualitatively analyzed the rate of decay after exposure to males of each *Drosophila* species, where flies were allowed to feed on one of the three selected yeast species for 24 h prior to the assays. We observed the formation of an opaque biofilm on host material and surrounding artificial diet in vials containing males that had fed on our different yeast species (**Supp. Figure S5**). In control vials with host material and without flies, no such biofilm was

visible; instead, the control host samples were often rapidly overgrown with mold. In the presence of *Drosophila* males and a visible biofilm, the host material appeared to be broken down faster than the control samples and show fewer signs of desiccation (**Supp. Figure S5**).

Next, we wanted to assess if the three target yeast species are capable of differentially accelerating fermentation processes across our host material. We expected that changes in sugar contents, especially of the monosaccharides, in the host purée could be used as an approximate indicator for fermentation processes, yeast performance and propagation on the respective host material.

We analyzed sugar contents (fructose, glucose, an unknown monosaccharide, sucrose and two unknown disaccharides) of host purée, which had been inoculated with one of the three tested yeast species. Host samples were incubated for 72 h at 25˚C, and the results were compared to sugar contents of host purée that had been mixed with sterile YM medium before and after 72 h incubation. Performance levels and metabolic activity of our three tested yeasts differed depending on the available host material. In the case of banana purée and *S. cerevisiae*, where we know most about yeast metabolism, host association and host composition, our data indicated that a yeast species linked to a specific habitat induced fermentation processes and converted available sugars at a faster rate than yeasts from other ecological niches (**Supp. Figure S6 B**). However, for cactus and mushroom purée results were not conclusive enough to allow for informative statements about yeast performance and propagation (**Supp. Figure S6 C, D**).

DISCUSSION

Pioneering research as well as recent studies on the interaction of *Drosophila* species and their naturally associated yeast species have started to uncover the general principles that underlie interactions between these fly species and two other trophic levels; their yeast communities and their hosts (Cooper, 1960; Becher and Guerin, 2009; Anagnostou, Dorsch and Rohlfs, 2010; Becher *et al.*, 2012; Stamps *et al.*, 2012; Buser *et al.*, 2014; Bellutti *et al.*, 2018; Grangeteau *et al.*, 2018; Murgier *et al.*, 2019).

In our experiments, we found that the host range of tested *Drosophila* species correlates with the acceptability of different yeast species in food resources and at oviposition sites (**Figure 6**). The generalist *D. melanogaster* can populate a broad range of host substrates. We found that this fly species accepts a variety of yeast species from different groups at breeding sites and the larval performance is consistently high in the presence of diverse yeasts. However, the specialists, *D. mojavensis* and *D. putrida,* either favor yeast species from their natural environments for oviposition or their larval survivorship is negatively affected by yeasts from unfamiliar habitats, keeping their host range narrow. Thus, we propose that the larval development of more specialized *Drosophila* species is more sensitive to changes in associated yeast communities than that of generalist *Drosophila*. It would be interesting in the future to further investigate if specialist species are also more susceptible to pathogens than generalists.

On the basis of previous work (Scheidler *et al.*, 2015), we initially predicted that *Drosophila* species from different

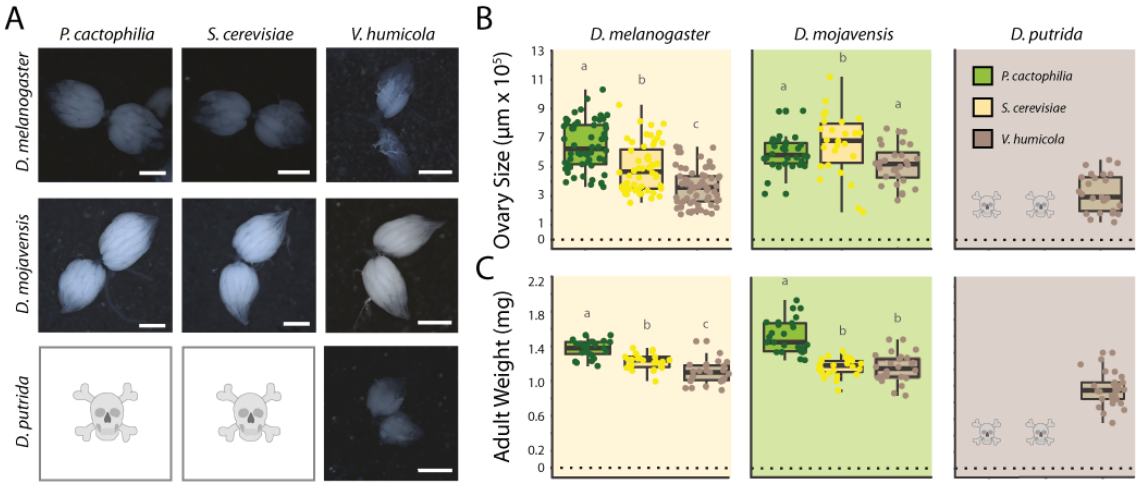
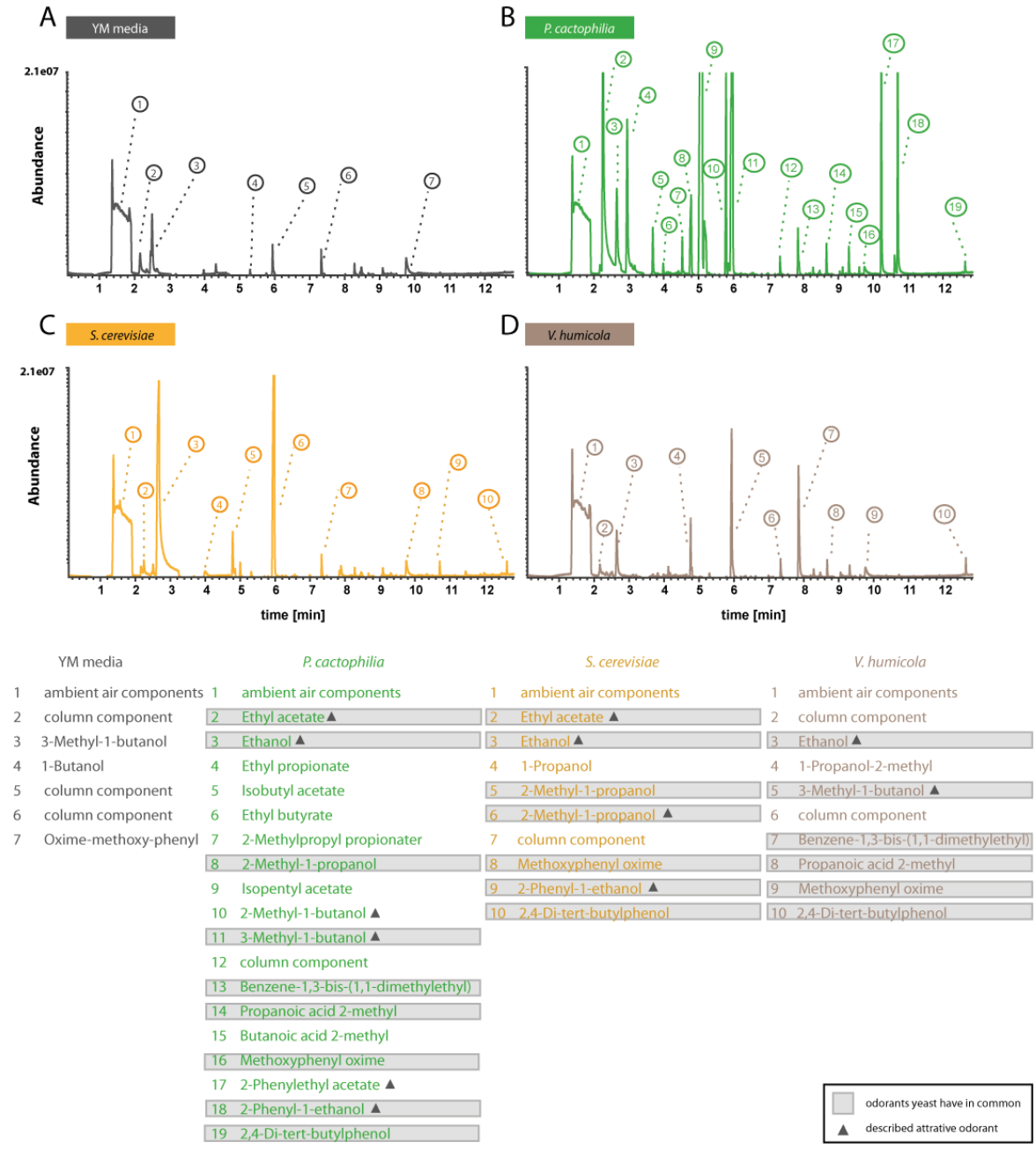


Figure 5 | Ovary size and adult weight of flies reared in association with different yeast species. (A) Representative images of dissected ovaries from adult females of all three *Drosophila* species raised on an artificial diet supplemented with one of the indicated yeast species (scale bar 500 µm). Scull symbols indicate treatments in which no larvae survived, or no females hatched. (B) Measurements from ovary surface area of female flies (n=25 to 30 ovaries per species and yeast treatment). Letters above the box plots indicate significant differences in ovary size between the yeast species treatments (Kruskal-Wallis test followed by Dunn's Multiple Comparison post test). Box plots show the median (bold horizontal lines) and whiskers the interquartile range. (C) Weight of females used for the ovary dissection in (B). Letters above the box plots indicate significant differences (One-way ANOVA followed by Tukey-Kramer Multiple Comparison post test). For each data set the median is shown in a bold horizontal line and the interquartile range is represented by the whiskers of the box plots. Scull symbols were used if all larvae died before eclosion or if adults which emerged had been males only.

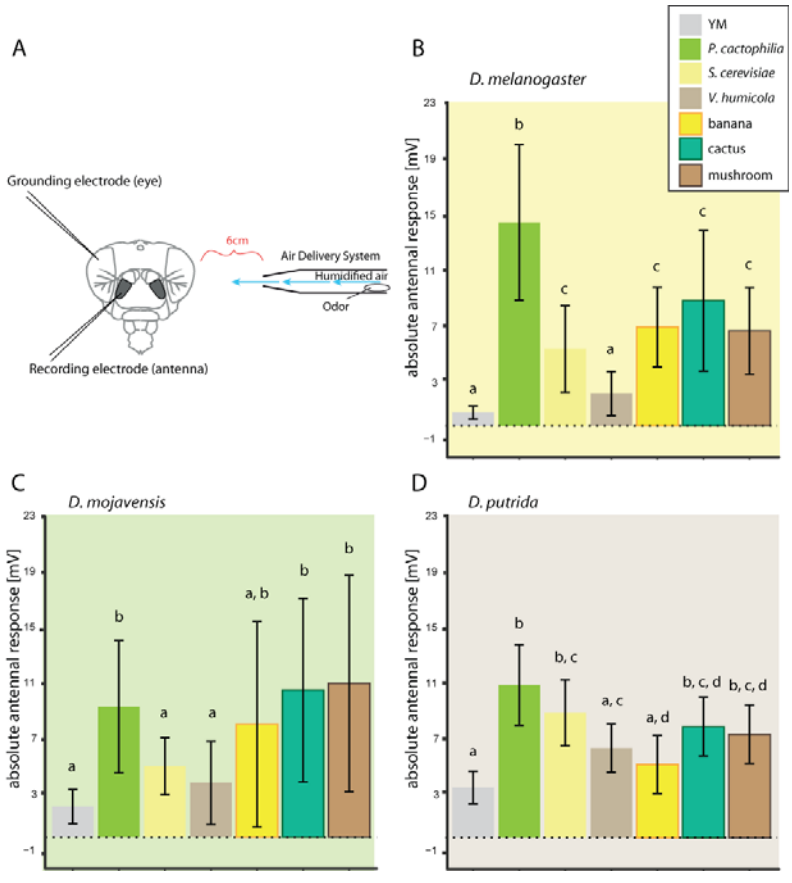


YM media	*P. cactophilia*	*S. cerevisiae*	*V. humicola*
1 ambient air components	1 ambient air components	1 ambient air components	1 ambient air components
2 column component	2 Ethyl acetate ▲	2 Ethyl acetate ▲	2 column component
3 3-Methyl-1-butanol	3 Ethanol ▲	3 Ethanol ▲	3 Ethanol ▲
4 1-Butanol	4 Ethyl propionate	4 1-Propanol	4 1-Propanol-2-methyl
5 column component	5 Isobutyl acetate	5 2-Methyl-1-propanol	5 3-Methyl-1-butanol ▲
6 column component	6 Ethyl butyrate	6 2-Methyl-1-propanol ▲	6 column component
7 Oxime-methoxy-phenyl	7 2-Methylpropyl propionater	7 column component	7 Benzene-1,3-bis-(1,1-dimethylethyl)
	8 2-Methyl-1-propanol	8 Methoxyphenyl oxime	8 Propanoic acid 2-methyl
	9 Isopentyl acetate	9 2-Phenyl-1-ethanol ▲	9 Methoxyphenyl oxime
	10 2-Methyl-1-butanol ▲	10 2,4-Di-tert-butylphenol	10 2,4-Di-tert-butylphenol
	11 3-Methyl-1-butanol ▲		
	12 column component		
	13 Benzene-1,3-bis-(1,1-dimethylethyl)		
	14 Propanoic acid 2-methyl		
	15 Butanoic acid 2-methyl		
	16 Methoxyphenyl oxime		
	17 2-Phenylethyl acetate ▲		
	18 2-Phenyl-1-ethanol ▲		
	19 2,4-Di-tert-butylphenol		

odorants yeast have in common

▲ described attrative odorant

Supp. Figure S3 | Analysis of the volatile compounds found in the headspace of the three target yeast species. (A-D) Representative GC-MS chromatograms of the headspace collected over 30 min from glass vials containing either 5 mL of pure yeast malt (YM) liquid medium or 5 mL of indicated yeast cultures grown in YM medium. A color-coded identity of numbered peaks is given under the chromatograms for each treatment. Gray boxes highlight odorants found in all or minimum two yeast species. Triangles highlight described attractive odorants for *D. melanogaster*.

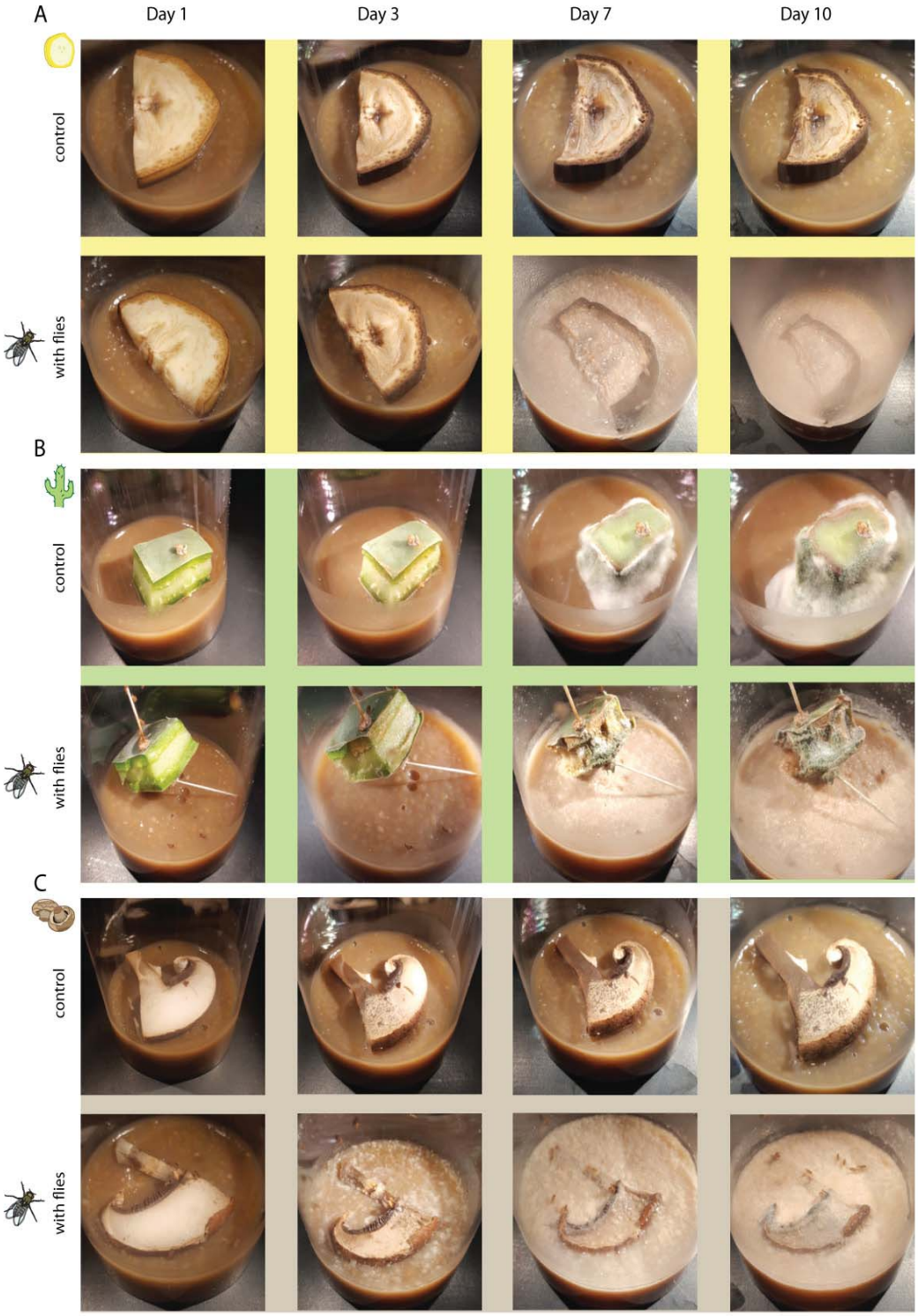


Supp. Figure S4 | Electroantennogram recordings from *Drosophila* species towards the headspace of their host material or different yeast cultures. (A) Schematic drawing of the experimental setup for the recording of electroantennograms (EAGs). (B-D) EAG responses to the different odors normalized to the smallest response. Bar plots represent mean antennal response in mV towards different stimuli (n=7-8), while error bars indicate standard deviation. Letters above the box plots indicate significant differences (Repeated measures ANOVA followed by Tukey-Kramer Multiple Comparison post test).

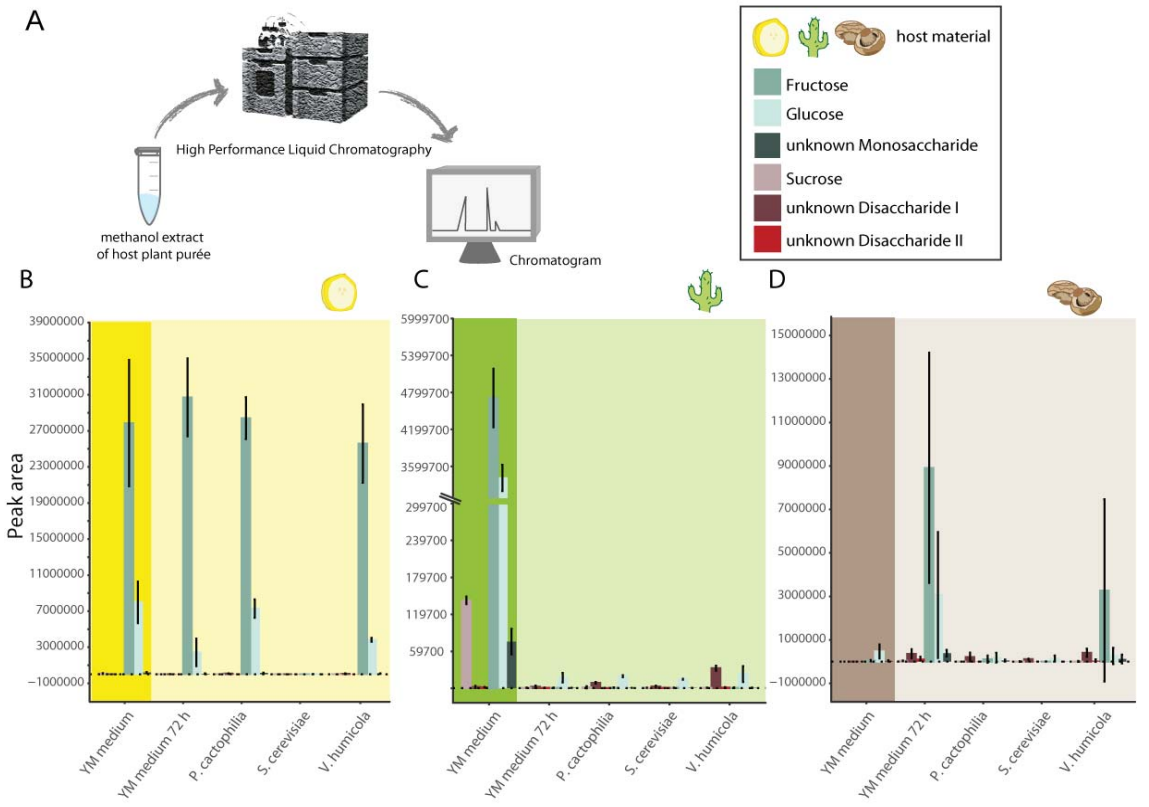
ecological niches would be most attracted to and prefer yeast species associated with their respective habitat or host. Our reasoning behind this assumption was that yeasts would want to ensure transfer to suitable habitats by producing a specific composition of chemosignals especially attractive to *Drosophila* species linked to their ecological niche and in exchange, the flies would optimize their performance. In agreement with previous studies (Palanca *et al.*, 2013; Scheidler *et al.*, 2015), we here obtained further evidence that *Drosophila* species can differentiate between different yeast species based on chemosignals. However, our data suggest that *Drosophila* flies do not necessarily prefer the yeast species associated with their natural habitat and that the attractiveness of a yeast species correlates instead with the number of attractive volatiles in the headspace of that yeast culture (**Figure 2 B, S3 B-D**). Here, a summation of the activity of attraction-mediating channels in the olfactory system might have led to an enhanced attractiveness of the respective yeast species. Additional experiments need to be conducted with a broader selection of yeast species from inside and outside of *Drosophila* ecological niches to make further predictions about the general applicability of these observations.

The fact that different *Drosophila* species can be attracted to the headspace of a yeast that the flies most likely do not encounter in nature, indicates that the attraction to yeast-generated chemosignals is an evolutionarily shared trait across a wide array of *Drosophila* species (Scheidler *et al.*, 2015; Becher *et al.*, 2018).

Of our three tested yeast species, *V. humicola* was the one with the lowest level of attractiveness for each of our selected *Drosophila* species (**Figure 2 B**). In comparison to the other two yeasts tested, *V. humicola* produced noticeably fewer of the volatiles that are known to be generally attractive to *Drosophila* species (**Figure S3 B-D**). As mentioned before, the production of attractive volatile compounds mediates yeast-insect interaction and promotes mutualistic relationships with *Drosophila* flies or other insects (Nout and Bartelt, 1998; Becher *et al.*, 2010, 2018; Christiaens *et al.*, 2014). However, mushrooms are generally poor in nutrients and yeast species relying on this substrate as a host might not be able to invest resources into the production of chemosignals that are not byproducts of its necessary metabolic activity. Consequently, the habitat and associated yeast communities of *D. putrida* and other mycophagous *Drosophilidae* might be devoid of many attractive odorants. Therefore, yeast species emitting only a few attractive volatiles could already stand out in the odor background, thus allowing that yeast to invest only minimal resources in the production of attractive chemosignals for



Supp. Figure S5 | Comparison between host decay of samples without and with *Drosophila* males. Since we did not find a visible difference in the rate of decay between the three yeast species, we generally compared the progress of host decay from samples with flies to controls without flies. (A-C) Representative images of the decomposition progress of banana (A), cactus (B) and mushroom (C) samples without and with 20 *Drosophila* males which were fed with our target yeast species. The *Drosophila* species used for the assays were as follows: Banana-*D. melanogaster*, cactus-*D. mojavensis* and mushroom-*D. putrida*.



Supp. Figure S6| Performance of yeast species on the host material of different *Drosophila* species. (A-D) Abundance of selected sugars found in *Drosophila* host material after inoculation with different yeast species. Homogenized host material of different *Drosophila* species was inoculated with target yeast species or mixed with YM medium and incubated for 72 h at 25 °C. Bar plots show the mean peak area of indicated sugars in methanol extracts from host purée inoculated with depicted yeast species or supplemented with growth medium run as High Performance liquid Chromatography (HPLC). All samples were run in triplicate. The error bars represent the standard deviation from the mean peak area of the corresponding sugar.

successful insect dispersal. Ultimately, when comparing the amount of attractive chemosignals in the headspace of our target yeast species, it is important to consider that all species were grown on a standard yeast growth medium (Yeast Malt medium). Therefore, it is a possibility that, for example, *V. humicola* might emit a different odor profile when grown on a substrate closer to its natural conditions (Becher *et al.*, 2018).

Drosophila preference for yeast species is based on a multi-chemosensory assessment

We found that the initial attraction of the three *Drosophila* species towards the headspace of the three yeast species differed from the final decision for yeast acceptance at oviposition sites of the female flies (**Figure 2**). While the yeast preference in the trap assays fully depended on volatile cues, during oviposition behavior *Drosophila* females had the opportunity to base their decision both on smell and taste. Interestingly, for *Drosophila* females we observed changes in the attractiveness of the different yeast species when the flies had the option to evaluate the properties of the yeasts using gustatory aspects (**Figure 2 B, D**). We conclude that *Drosophila* flies assess the attractiveness of a yeast species evaluating multiple chemosensory information and that an initial attraction based solely on olfactory cues can be re-evaluated following gustatory input, as has been suggested previously (Karageorgi *et al.*, 2017). In addition, for *Drosophila* flies, it has been shown that volatiles emitted by a yeast can mediate attraction and oviposition preference while host volatiles play only a secondary role in these fly behaviors (Becher *et al.*, 2012). We found that the combination of a familiar yeast and a naturally occurring host material has an impact on yeast preference and oviposition in *Drosophila* species that have a restricted host association (*D. mojavensis*, *D. putrida*; **Figure 2 D, Figure 3 C-E and Figure S2**). Especially for these *Drosophila* specialist species the preference of one yeast species over another is perhaps context dependent and is influenced by the substrate on which they grow (**Figure 2 D, Figure 3 C-E**). Thus, it appears that the simultaneous presence of yeast and host material synergistically stimulates the oviposition decision,

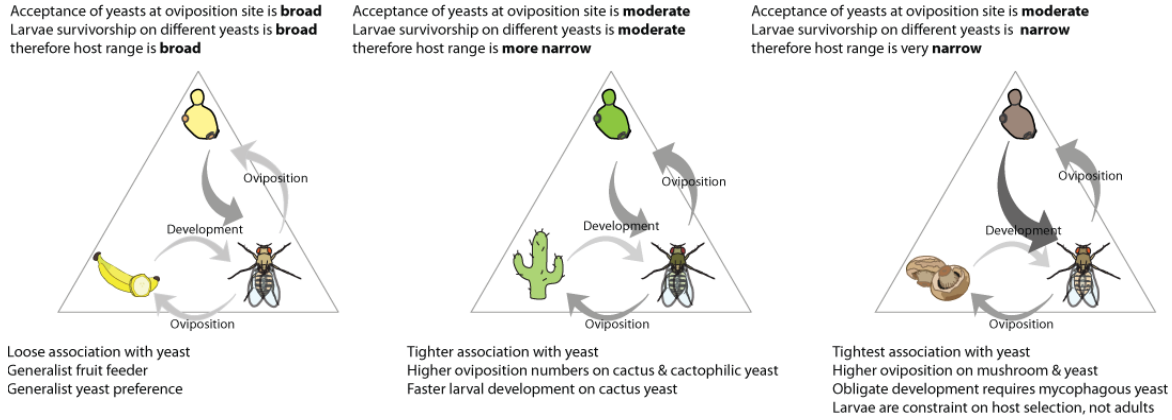


Figure 6 | Model of *Drosophila*-host-yeast interactions. Schematic drawing describing the dependency of target *Drosophila* species on the association with specific yeast communities and the presence of certain host material for their oviposition preference and larval performance.

possibly through the increased quantity of attractive odorants and other chemosignals perceived by the *Drosophila* females when host and yeast are combined.

Additionally, the different yeast species might have processed their substrate in a species-specific manner, further enhancing the attractiveness of their growth medium as a species-specific oviposition site (e.g. detoxification of secondary metabolites, (Fogleman, Duperret and Kircher, 1986)). Lastly, based on our data, we exclude the possibility that increased egg numbers in oviposition assays with host and yeast presence are a result of changes in the consistency of the presented substrate since we did not find a consistent preference of *D. mojavensis* or *D. putrida* for oviposition on the purée itself.

Larval fitness benefits from association with yeast from natural habitats

Oviposition preference by our tested adult *Drosophila* species for various yeasts was not directly mirrored by the performance (survivorship and developmental rate) of their larvae with those yeasts. Although *Drosophila* females might prefer to oviposit in the presence of yeast species from different habitats, their larvae did not benefit from this new association, and this may represent an evolutionary constraint on adaptation to a novel niche. In fact, adult preference for yeast species that are not linked to the ecological niche of a *Drosophila* species over yeast from shared habitats might be fatal for the entire next generation, as seen by the example of *D. putrida* (**Figure 4**). Contradictory to the assumption that females would seek oviposition sites which are optimally suited in nutrition and yeast community composition for high larval survivorship numbers and a fast larval development (preference-performance hypothesis, (Jaenike, 1978; Thompson, 1988)), previous studies (Mayhew, 2001; Anagnostou, Dorsch and Rohlfs, 2010) and our present findings indicate that mother does not always know best. Mounting evidence suggests that insect females generally favor substrates as oviposition sites that they themselves prefer as a food source and which promote adult fitness (fecundity and fertility; **Figure 2 D, Figure 3 C-E, Figure 5**), putting the needs of their offspring secondary to their own (Scheirs, De Bruyn and Verhagen, 2000; Mayhew, 2001; Anagnostou, Dorsch and Rohlfs, 2010). According to this hypothesis, higher numbers of eggs laid could outweigh possible losses in larval survivorship and disadvantages from the slower developmental rates of progeny. As an underlying principle for this “bad-mother” behavior, a trade-off between female fitness and fitness of their progeny has been proposed, making adult performance on host material the predominant factor influencing adaptation (Mayhew, 2001; Anagnostou, Dorsch and Rohlfs, 2010). Furthermore, differences between larval and adult performance in association with a certain yeast species are likely caused by differences in nutritional needs, which may be an explanation for distinct yeast preferences between developmental stages (Cooper, 1960). In addition, we propose that differences in yeast preference between adults and larvae in turn could reduce cross-generational competition for nutrition and resources in *Drosophila*.

How did the presence of *S. cerevisiae*, a yeast species that serves as a food source for many *Drosophila* species, have such a negative impact on the survivorship rate of *D. putrida* larvae? We know little about the ecology of *D. putrida* other than that this *Drosophila* species is mycophagous. Mushrooms in turn offer only low amounts of sugars (Portabella Mushroom per 100 g: approx. 2.5 g sugars (U.S. Department of Agriculture)) which makes them generally a bad resource for alcohol fermentation and thus alcohol levels found in mushroom are expected to be naturally rather low. The yeast *S. cerevisiae,* however, produces high amounts of alcohol while obtaining energy from the fermentation of sugars found in its host substrates, such as fruits. We propose that the production of alcohol via *S. cerevisiae* in the diet led to levels toxic to *D. putrida* larvae that presumably only tolerate low concentrations of alcohol, as they would naturally not encounter high concentrations in their mushroom host. Experiments in the past have shown a general trend, with exceptions, correlating *Drosophila* ethanol tolerance to the suitability of their breeding sites for fermentation processes (David and Van Herrewege, 1983). However, additional work is

still needed to fully understand the nutritional and metabolic consequences of larvae reared on nutrient poor substrates, such as mushrooms.

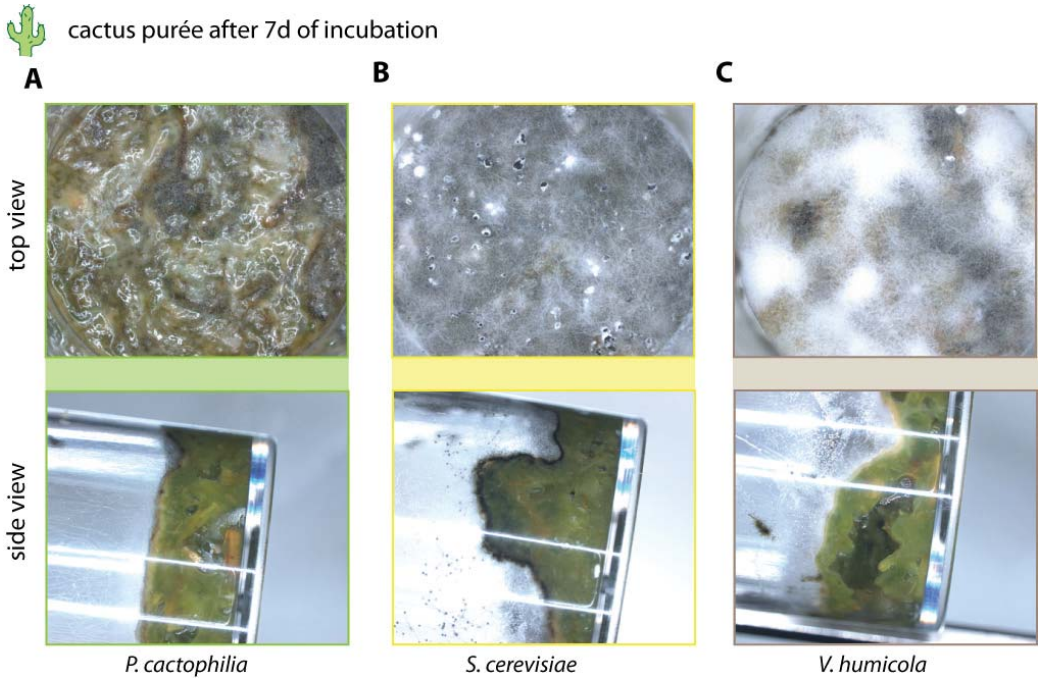
The dietary value of yeast species differs for *Drosophila* species

Ovary size correlates with ovariole number and therefore determines potential female fecundity (Boulétreau-Merle *et al.*, 1982; R'kha *et al.*, 1997; Klepsatel *et al.*, 2013; Mendes and Mirth, 2016). Moreover, well-fed females with enlarged fat storage will withstand a shortfall of food resources longer than malnourished flies. Both weight and ovary size, are affected by nutritional conditions during the adult female's larval development (Hodin and Riddiford, 2000; Tu and Tatar, 2003; Green and Extavour, 2014; Qiao *et al.*, 2019). Our data indicate that the tested yeast species had differential dietary values for our three *Drosophila* species. We observed that a yeast species could enhance ovary and female size in one species, while there would be no beneficial impact on adult traits in other *Drosophila* **(Figure 5**). Thus, each *Drosophila* species must gain different nutritional effects from the same yeast. At least in *D. melanogaster* and *D. mojavensis* the size of ovaries and weight of females of the next generation grown on our selected yeast species roughly correlated with the sequence of yeast preference at oviposition sites of the *Drosophila* females from the parental generation (**Figure 2 D, Figure 3 C-E, Figure 5 B**). This finding provides additional evidence that *Drosophila* females prefer yeast species and substrates that enhance their adult fecundity and fitness.

Drosophila flies and yeast together modify their ecological niche

In our study, we provide evidence that *Drosophila* flies and associated microorganisms accelerate the decomposition of their host material (**Supp. Figure S6 and Figure S7**). In agreement with previous studies our results suggest that *Drosophila* flies inoculate new host material with microorganisms such as yeast spores that are transferred on the flies' bodies and/or through the oral-fecal route (Ganter, 1988; Starmer, Peris and Fontdevila, 1988; Stamps *et al.*, 2012). According to our results, flies and microbes together broke down the host material faster than either group alone probably through activities associated with niche construction. Both interaction partners likely released nutritional factors or enzymes that benefited their mutual performance through degradation of the host. Furthermore, we found that the presence of flies and their microorganisms kept host samples from being overgrown by filamentous fungi, which further supports niche construction arguments (**Supp. Figure S5 and Figure S7**).

We expected that yeast species with a specialist lifestyle would outperform yeast species not commonly found in that habitat (e.g. cactophilic yeast performance would be optimized on cactus substrates). However, overall our data were not conclusive enough to evaluate yeast performance and growth on all tested host purées and future work needs to be done to refine the assay used to assess these parameters in such a complex set-up. Nonetheless, on banana purée it appears that *S. cerevisiae* converted available sugars faster than the yeast species that are naturally not found on this host (**Supp. Figure S6**). Additionally, yeast species naturally associated with



Supp. Figure S7 | Effects of yeast presence on the growth of filamentous fungi on cactus purée. Cactus purée was inoculated with 400 µL of yeast pre-culture at an OD_{600} of 2 and incubated at room temperature. (A-C) Representative images of cactus purée seven days after inoculation with three different yeast species; yeast species are indicated at the bottom of the images.

a host were able to prevent or hinder the growth of molds on their respective host substrate (**Supp. Figure S7**), which is presumably linked to the competitive performance of that yeast species on its natural host.

Association with yeasts from new habitats as a chance for speciation in *Drosophila*

Our initial hypothesis predicted that the preference of *Drosophila* flies for yeast species from their native habitats would be well established and maintained to ensure mutual benefits for both interaction partners. However, based on our findings and recent work (Günther *et al.*, 2019), we know now that certain *Drosophila*-yeast associations are less established than we initially assumed and that the flies are not only attracted to yeast from their own ecological niche but can even favor yeast species from completely different niches. This preference is mediated via chemosignals emitted by those yeasts that are universally attractive to *Drosophila* species, which are generally known for their shared preference for microbial fermentation. In agreement with Starmer 1981, we suspect that *Drosophila* flies were capable of populating extremely diverse habitats as a result of an association with resident yeast communities that have unique physiological adaptations to their environment. We propose that the association of adult *Drosophila* flies with new yeast species has the potential to allow the flies to adapt to new environments and that this insect-yeast association is a starting point for evolutionary processes leading to speciation events. However, one of the constraining factors for the adaptation of *Drosophila* to new habitats appears to be the larval performance in presence of a new yeast species and the performance of the larvae on a novel host (Markow, 2019). Our data suggest that *Drosophila* larvae are more sensitive to changes in the composition of yeast communities than adults, and a new association of fly, yeast and host can only be formed if juveniles can thrive in the new dietary situation.

CONCLUSION

In summary, our study provides evidence that after the vectoring of a yeast species by *Drosophila* flies to a new food source or breeding site, flies and yeast together accelerate the breakdown of their host material while they simultaneously hinder the growth of filamentous fungi. Thus, flies and yeast both participate in activities of niche construction, while reproductive benefits for both insect and microbe seem to have initiated a partnership that is mediated predominantly by chemosensory cues. Furthermore, the number of insect-attracting chemosignals released by a yeast species seemingly depends on the amount of nutrition found in the yeasts' natural growing substrate. We propose that yeasts associated with fruit or cacti, which are habitats rich in nutrition, can metabolically invest more resources into the production of attractive chemosignals for insect vectors, while yeasts from niches that are poor in nutrition, such as mushrooms or leaves (Starmer, 1981), can only produce a few attractive cues.

In our experiments we found that yeast preference of *Drosophila* flies appears to be context dependent and is affected by the yeasts' growth substrate. Moreover, the level of larval performance of *Drosophila* flies in association with a partner yeast species depends on co-adaptation processes between fly and yeast, most likely due to exchange of nutrients. Our tested fly species had a higher reproductive success on substrates inoculated with a yeast species from their natural ecological niche, and on substrates where yeast and host were provided together. Additionally, our results support the "bad mother" hypothesis (Mayhew, 2001), where *Drosophila* females do not appear to choose their oviposition sites in regards to an optimized larval performance. This observation further suggests that the attractiveness of a yeast species does not always correlate with a short-term fitness advantage for the attracted *Drosophila* species. Furthermore, we propose that in *Drosophila,* life-stage dependent changes in yeast preference could allow for a reduction in cross-generational resource competition between adults and larvae.

Opposite to our initial hypothesis, we found that adult *Drosophila* can be attracted to yeast species from profoundly different ecological niches, and that there is perhaps a broad acceptance for various yeast species across this insect genus. This may arise from the fact that the number of attractive odorants produced by a yeast in our experiments correlates more strongly with yeast preference of *Drosophila* flies than a common habitat. Based on our results, we propose that the propensity for *Drosophila* adults to associate with yeast species from unfamiliar habitats can lead to local adaptation of those flies towards new environments, which ultimately can drive speciation events, but only if the progeny of those flies can also survive this new host or yeast association. Consequently, we propose that novel associations with yeast from new habitats can promote *Drosophila* evolutionary events, such as adaptation to a new environment or host.

MATERIAL AND METHODS

Fly stocks

Experiments were performed with wild-type strain *Drosophila melanogaster* Canton-S (WTcs, stock #1), *Drosophila mojavensis mojavensis* (Cornell stock # 15081-1352.10, Ithaca, NY, USA) and *Drosophila putrida* (Cornell stock # 15150-2101.00, Ithaca, NY, USA). *D. melanogaster* and *D. mojavensis* flies were maintained on standard diet (**Supplementary Table S3**). For the rearing of the mycophagous *Drosophila* species, *D. putrida* standard diet was supplemented with store-bought, organic, Portabella mushroom slices (*Agaricus bisporus* (Grimaldi, 1985)). The *D. melanogaster* stock was kept at 25 °C and 70 % RH, with a photoperiod of 12 h:12 h Light:Dark (Stökl *et al.*, 2010). *D. mojavensis* and *D. putrida* flies were reared at room temperature with ambient light and humidity conditions (approximately 22°C and 16 h: 8 h Light:Dark with 40 % RH).

Yeast maintenance

Yeast species were selected based on published association with the three target *Drosophila* species and their hosts (banana, cactus and mushroom). According to a literature search, the yeast species *Pichia cactophilia* and *Vanrija humicola* were assumed to be unique for their host association (cacti and mushrooms, respectively (Gilbert, 1980; Grimaldi, 1985)), while *Saccharomyces*

cerevisiae is known to be rather ubiquitous, with a cosmopolitan distribution similar to its vector, *D. melanogaster* (Piskur *et al.*, 2006; Rozpedowska *et al.*, 2011). Yeast strains were purchased from the National Collection of Yeast Cultures (NCYC, Norwich, United Kingdom; *S. cerevisiae* NCYC 505, *P. cactophilia* NCYC 1492) and the Centraalbureau voor Schimmelcultures (CBS, Utrecht, Netherlands; *V. humicola* CBS 571). Aliquots of each yeast species were stored in 20 % glycerol solutions (Sigma Aldrich, St. Louis, MO, USA) at -80 °C for long term storage. Fresh yeast cultures were maintained on Yeast Malt (YM) agar plates (yeast extract 3 g/L (Carl Roth), malt extract 3 g/L (Carl Roth), D-glucose 10 g/L (Carl Roth), peptone 5 g/L (Carl Roth) and 2 % agar (Carl Roth, Karlsruhe, Germany)) at 25 °C and 80 % RH. Liquid cultures of all yeast species were freshly prepared every alternate day by inoculating 40 mL of YM medium (yeast extract 3 g/L, malt extract 3 g/L, D-glucose 10 g/L, peptone 5 g/L) with a single isolated colony and grown in 50 mL reaction tubes at 25 °C with 250 rpm. The liquid yeast cultures served as pre-cultures for experiments and were grown until they reached stationary phase conditions with an OD_{600} of 2 (optical density at 600 nm). The pre-cultures were then spun down in a centrifuge at 320 g for 3 min, the supernatant was discarded, and the pellet was resuspended in 15 mL fresh, sterile YM medium.

Larval development assays

Larval development was evaluated as the amount of days needed for reaching two milestones in a fly's life, (A) pupation and (B) adult eclosion, across diet and the presence of target yeast species. Prior to the assays, small plastic vials (28.5x95 mm, Steinfurt, Germany) were prepared by adding 5 mL of modified standard diet to the vials, which lacked brewer's yeast and the antifungal substance nipagin. Under a sterile bench, the surface of the modified standard diet was broken up for an easier access of yeast culture and larvae, then 200 µL of yeast preculture at an OD_{600} of 2 (details see above) were added to the diet. For each of the three target yeast species, 15 replicates were set up and incubated for 24 h at 25 °C. From oviposition plates a total of 15 first instar larvae of one *Drosophila* species were transferred per rearing vial. The oviposition plates consisted of 2 % agar and had the addition of some broken up pieces of modified standard diet supplemented with a yeast extract/peptone mixture (1:1) in the center of the plate. The yeast extract/peptone mixture served as an oviposition stimulus for the adults. After the addition of first instar larvae, vials were checked twice per day for the appearance of pupae, and after pupation for the eclosion of adults until zero new flies emerged for two consecutive days.

Host purée

Organic banana (Carmita Bio Organic, Ecuador) and Portabella mushrooms were purchased at a local organic grocery store while the cactus material (*Opuntia phaecantha v. tenuispina*) was ordered from Uhlig Kakteen (Kernen-Rommelshausen, Germany). These organic materials were homogenized with a hand-held blender, then transferred into 50 mL reactions tubes and kept at -20 °C until further use.

Fly phenotyping

The influence of diet and the presence of selected yeast species on the performance of the different *Drosophila* species was measured in regards to ovary size as a proxy for fecundity, and the weight of adult female flies was used as an assessment of overall adult fitness. Flies for the measurements originated from the larval development assays and were taken from the vials after emergence for the determination of adult weight and for the dissection of the developed ovaries in *D. melanogaster*, *D. mojavensis* or *D. putrida* respectively.

Adult weight

In the case of *D. melanogaster* female flies were measured two days after emergence, and in the case of *D. mojavensis* and *D. putrida,* six days after eclosion. Weighing of flies preceded ovary dissection but happened in succession to enable statements about a possible correlation between adult weight and ovary size. The two *Drosophila* species *D. mojavensis* and *D. putrida* (unpublished data) mature slower than *D. melanogaster* (Carracedo, Casares and Miguel, 1989) and their ovaries would have been underdeveloped independent of dietary reasons if dissected too early. Flies were removed from vials of the larval development assays and sorted on a CO_2 pad by their respective sex. Female flies from the same species were pooled together by treatment and cooled on ice in 1.5 mL reaction tubes. After 30 min on ice individual flies were weighed on an analytical scale (Sartorius Weighing Technology GmbH, Goettingen, Germany). For every treatment 25 females randomly selected from the different replicates were measured where available; otherwise, all females that hatched were weighed.

Ovary size

After the weight of flies had been determined, ovaries of these females were dissected. For each treatment and each species, ovaries of 15 females were dissected. The flies were cooled on ice and dissected in 1 M phosphate-buffered-saline (PBS) with 0.1 % Triton X 100 (Sigma-Aldrich, St. Louis, MO, USA). Images of the ovaries were taken with an Axio Zoom V16 (ZEISS, Germany, Oberkochen). All ovaries were scanned at a magnification of 50 (PlanApo Z 0.5x objective, 0.125 Numerical Aperture (NA)). The ovary size was measured in FIJI (ImageJ version 1.51 k; NIH) by tracing the outline of individual ovaries and calculating the size of the regions of interest after predefining the pixel by pixel size in the settings according to the scan information from the original image taken in the Zeiss software (ZEN 2 Blue edition, Version 2.0.0.0).

Attraction assays

In trap assays, the ability of each of the three target *Drosophila* species was tested to differentiate between the target yeast species using their sense of olfaction. Furthermore, the trap assays provided information about the attraction of the target *Drosophila* species towards the different yeast species. In every assay 40 flies of the same species at a 1:1 sex ratio had the choice between four traps, each equipped with either 2 mL YM medium (control) or 2 mL YM medium inoculated with one of the yeast species grown to an OD_{600} of 2. The plastic traps consisted of 20 mL containers (Specimen container, SAMCO6-0181, VWR, Darmstadt, Germany) which had a paper cone lid to keep the flies from leaving the container once they had entered it. In addition, 200 µL of mineral oil (Sigma-Aldrich, St. Louis, MO, USA) was pipetted onto the surface of all four treatments to

help capture flies upon contact. Traps were placed at corners of a square with a side length of 10 cm and in the middle of a 50x50 cm mesh insect rearing cage (**Figure 2 A**). Assays were run in a climate chamber at 25 °C, 70 % relative humidity and a 12 h: 12 h light-dark cycle. Flies were allowed to choose between the different traps for 24 h. After 24 h, the amount of flies outside of the traps was documented as well as the numbers of flies in the traps. Additionally, the sex of flies trapped was noted.

Oviposition assay

Prior to the oviposition assays, seven to eight-day old flies were separated into groups of 20 females each, which were then paired with five males per group. These flies were kept 24 h on food with standard diet that was supplemented with a yeast extract/ peptone mixture (1:1) to guarantee the availability of gravid females and reliably high egg counts for these assays. The oviposition plates had a diameter of 60 mm (83.3901, Sarstedt, Nürnbrecht, Germany) and consisted of 7 mL standard diet, which lacked brewer's yeast and the antifungal growth component nipagin. Additionally, the plates were inoculated with 400 µL of stationary phase yeast pre-culture at an OD_{600} of 2 and incubated for 24 h at 25 °C, all while flies were kept on the yeast extract/ peptone mixture. For these assays, three oviposition plates, each inoculated with one of the target yeast, were placed in the middle of a 50x50 cm mesh insect rearing cage and spaced 10 cm apart (**Figure 2 C**). Females were allowed to oviposit for 24 h. Assays were kept under controlled temperature and humidity conditions (25 °C and 70 % RH with a light-dark cycle of 12 h: 12 h). For all three *Drosophila* species, 14 replicates were conducted. After 24 h, the oviposition plates were removed from the mesh cages and immediately imaged for subsequent egg counts. Images of the plates were taken with an Axio Zoom V16 (ZEISS, Germany, Oberkochen) at a magnification of 12.5 (PlanApo Z 0.5x objective, 0.125 NA). For this, the tile scan function of the Zeiss software (ZEN 2 Blue edition, Version 2.0.0.0) was applied. The magnification of 12.5 led to a total amount of 28 tiles per imaged oviposition plate, which were each processed into a final image using the software's built-in stitching method with a tile overlap of 10 %. The resulting images were analyzed in FIJI (ImageJ version 1.51 k; NIH) utilizing the "multi-point" option, which marked every counted egg with a symbol and helped avoiding miscounts.

Standard diet supplemented with host purée and inoculated with yeast

Flies were prepared as described for the oviposition assays with standard diet inoculated with yeast. This time the entire surface of modified standard diet (no brewer's yeast and no nipagin) oviposition plates was first inoculated with 400 µL of stationary phase yeast pre-culture (OD_{600}=2). Then 2 g of host purée were evenly spread into a thin layer on approximately one-half of the plate's surface. Finally, 200 µL additional yeast pre-culture were added onto the half with the host purée. Plates were incubated for 24 h at 25 °C. By only partially covering the surface of the oviposition plates with the host purée it was possible to observe potential preferences of the flies towards either modified standard diet inoculated with yeast alone, or towards host purée in the presence of yeast culture (**Figure 3 A, B**). Assays were set up and run as mentioned above. Likewise, oviposition plates were processed as explained previously, with the addition that for each plate two final images from two focus planes were compiled. These two imaging heights were necessary, as the extra layer of host purée on top of the modified diet did not allow capturing all eggs in focus using just one image.

Documented host decay

Prior to the assays, 30 *Drosophila* males per replicate were allowed to feed on 50 µL of pre-culture (OD_{600}=2) from one of our selected yeast species, respectively. Host material was added on top of standard artificial fly diet. At the beginning of the assays, male flies were transferred into vials containing samples of the host material. *D. melanogaster* males were kept on banana slices, *D. mojavensis* males on pieces of cactus, and *D. putrida* males were transferred to vials with mushroom slices. As a control, vials were prepared that only contained standard diet and host material, and not flies. The course of decomposition of the host material was documented for ten days, with pictures being taken on day 1, 3, 7 and 10 (**Supp. Figure S5**).

Gas chromatography-mass spectrometry (GC-MS) headspace analysis

After confirming that the tested *Drosophila* species were able to distinguish between the target yeast species based on olfactory cues (**Figure 2 B**), the odor profile of target yeast species and YM medium was characterized to quantitatively compare relative abundance of odorants and to possibly identify species-specific yeast odorants. For this, headspace of 5 mL YM medium or yeast culture at an OD_{600} of 2 was collected for 30 min with a solid-phase microinjection (SPME) fiber (StableFlex™, DVB/CARBOXEN-PDMS, Supelco, Bellefonte, Pennsylvania, USA). After manual injection, samples were analyzed via a GC-MS (Agilent technologies GC 6896N interfaced to an Agilent technologies 5975B inert XL MSD unit) device with an installed HP-5MS UI column (19091S-413U, Agilent technologies, Santa Clara, CA, USA). For each GC-MS run, the temperature of the oven was initially held at 40 °C for 3 min, and then increased in increments of 20 °C min^{-1} up to 260 °C. Mass spectra were recorded in scan mode from 33 to 350 m/z with Electron impact (EI) ionization at 70 eV and 300 mA. Resulting GC-MS profiles were characterized by matching the ion profiles of identified odorants to a standard library (NIST Mass spectrum library) using MSD ChemStation (F.01.02.2357, Agilent, Santa Clara, CA, USA). For all three yeast species as well as the growth medium, three replicates were produced for verification.

Electroantennogram (EAG) recordings

In electroantennograms (EAGs), the summed activity of sensory neurons on the antenna of target *Drosophila* species was recorded in response to each yeast pre-culture, the YM medium and the host purée. Recordings were used to ascertain within each yeast species whether the flies perceived the different odor profiles in a discriminative manner. For these recordings, flies of seven to nine days of age were immobilized in truncated 200 µL pipette tips. The Ringer solution filled Ag-AgCl glass capillary reference electrode was placed into the fly's compound eye and of the recording electrode (filled with the same solution) was placed against the antenna's third segment, which

all took place under a microscope (10x magnification, 0.30 numerical aperture [NA], Olympus BX51W1) and with the help of a micromanipulator (Märzhauser DC-3K/PM-10, Wetzlar, Germany). Ringer solution consisted of 1.0 mM $CaCl_2$ (Sigma Aldrich, St. Louis, MO, USA), 22.5 mM glucose (Biomol, Hamburg, Gemany), 1.5 mM HCl (Sigma Aldrich), 171.9 mM KCl (Sigma Aldrich), 9.2 mM KH_2PO_4 (Sigma Aldrich), 10.8 mM K_2HPO_4 (Sigma Aldrich), 3.0 mM $MgCl_2$ • 6 H_2O (Biomol), 12 mM NaCl at a pH of 6.5 and an osmolarity of 475 mOsmol/L. The odor stimulus elicited antennal response was generated using a 10 x preamplified signal headstage (Syntech Universal AC/DC probe, Buchenbach, Germany), converted via an amplifier (Syntech IDAC-4, Buchenbach, Germany) and then recorded, visualized and analyzed via Syntech Autospike v3.7. For the odor stimulus, 10 µL of yeast culture (OD_{600}=2) or YM medium or 30 mg of host purée was used. For each stimulus, seven to eight biological replicates were recorded per *Drosophila* species.

Sugar content measurements

In order to indirectly asses yeast fitness and performance on the respective *Drosophila* host material, the capability of the target yeast species to induce or even accelerate fermentation progress was assessed by measuring sugar contents of host purée inoculated with each yeast species or the growth medium. For these measurements, 2.0 g of purée were weighed and transferred into small, sterile petri dishes (35 mm TC dish, Sarstedt, Nürnbrecht, Germany). The purée was then inoculated with 250 µL of yeast pre-culture at OD_{600} of 2 or supplemented with 250 µL YM medium. For each yeast species and the control (YM medium), three replicates per host purée were prepared. The purée was well mixed and incubated at 25 °C and 80 % RH for 72 h. After incubation, the purée was mixed again, and 100 mg of material was removed and transferred into 1.5 mL polypropylene reaction tubes. Subsequently, 1 mL of 70 % methanol (Carl Roth, Karlsruhe, Germany) was added to the host material and samples were thoroughly vortexed for 2 min. Next host material was centrifuged at 16000 rcf for 5 min to separate the extract from the solid phase. The supernatant was carefully removed, without disruption of the solid phase, and then transferred into 1.5 mL reaction tubes. Then, the supernatant was concentrated under N_2 at 35 °C. Samples were resuspended in 100 µL of water and diluted 1:5000 before injection. Sugars were analyzed on an HP 1200 series (Agilent Technologies, Santa Clara, CA, USA) coupled to an API 5000 triple-quadrupole mass spectrometer (Applied Biosciences, Thermo Fischer Scientific, Waltham, MA, USA) with an apHera™ NH_2 Polymer column (150 × 4.6 mm, 5 µm, Supelco Analytical, Munich, Germany). A chromatographic gradient of water (Solvent A) and acetonitrile (Solvent B) with a flow rate of 1 mL•min^{-1} at 20 °C was used, and the following program was run: 80 % B (0.5 min), 80-55 % B (12.5 min), 55-80 % B (1 min), hold at 80 % for 4 min. The mass spectrometer was operated in the negative mode with collision-activated decomposition, using a curtain gas pressure of 35 psi, with a collision gas pressure of 70 psi, an ion spray voltage of -4500 eV and a turbogas temperature of 700 °C. Compounds were detected using the scheduled multiple reaction monitoring (MRM) outlined in **Supplementary Table S1**. The MRM window was 300.0 s, the target scan time was 1.00 s and the cycle time was 1.00 s, with 1081 cycles. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing.

Figure design and statistics

All figures were generated in RStudio version 1.2.1335 (RStudio Team, 2018) and then adjusted for layout and graphical design in Adobe Illustrator CS5 version 15.0.0. Statistics were performed in GraphPad InStat version 3.10 and the outcome of statistical analysis was added to the pre-processed figures.

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AUTHOR CONTRIBUTION

S.K. and I.W.K. generated the original hypotheses for this study, supported by B.S.H. and M.K. Experiments were designed by S.K. in conjunction with I.W.K. Microbiological work was executed by S.K., as were the measurements of ovary development, adult weight, and survivorship tables. S.K. was responsible for all photographs and microscopy of raw data, with support from I.W.K. The attraction assays, oviposition and all other behavior data were collected and analyzed by S.K., with support from I.W.K. Sugar content measurements were performed and analyzed by M.E. supervised by J.G.. All statistical analyses were performed by S.K. Diagrams, illustrations and figures were created by S.K. and I.W.K., as was the original written manuscript with support from M.E.. Each author subsequently improved the final version of the manuscript as well as the figures, and all authors assisted with any revisions towards the final publication.

DECLARATION OF INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY INFORMATION

Table S1 | Outline of the schedule for the multiple reaction monitoring (MRM) of sugars in host material with and without yeast inoculation; DP (de-clustering potential), EP (entrance potential), CE (collusion energy),CXP (cell exit potential)

Sugar type	Retention time [min]	Precursor Ion [m/Z]	Product Ion [mz]	DP [V]	EP [V]	CE [V]	CXP [V]
Fructose	5.30	178.80	89.00	- 50	- 9.5	- 10	0
Glucose	6.20	178.80	89.00	- 50	- 9.5	- 10	0
Unkown Monosaccharide	6.15	148.98	88.92	- 50	- 8.5	- 10	- 2
Sucrose	7.55	340.90	59.00	- 65	- 10	- 46	0
Unkown Disaccharide I	8.28	340.90	59.00	- 65	- 10	- 46	0
Unkown Disaccharide II	8.7	340.90	59.00	- 65	- 10	- 46	0

Table S2|Tritrophic interaction partners

***Drosophila* species**	**associated yeast**	**host**
D. melanogaster	*S. cerevisiae*	Fermenting fruits
D. mojavensis	*P.. cactophilia*	Cactus (pitaya agria, organ pipe cactus and at times cina cactus as well as barrel and prickly pear cactus)
D. putrida	*V. humicola*	Mushrooms (*Agaricaceae, Amantiaceae, Baletaceae, Russulaceae* and *Tricholmataceae*)

Table S3| Recipe for standard diet

Ingredient	per 500 mL
Molasses	59 g in 101 mL hot water
Brewer's yeast	5.4 g
Agar	2.1 g in 135 mL cold water
Polenta	47 g in 169 mL hot water
Propionic acid	1.2 mL in 54 cold water
Nipagin (30 %)	1.65 mL

Discussion

General Discussion

The manuscripts within this dissertation aimed to investigate odor-mediated communication channels for the interactions of *Drosophila* flies with each other, other *Drosophila* species, and with microorganisms. Throughout the dissertation I introduce the frass of adult *Drosophila* flies as a previously overseen intra- and possibly interspecies communication medium (manuscript I) and demonstrate the sensitivity of hardwired stereotypical behaviors, which are mediated by only a few chemosignals, to manipulation through harmful microbes (manuscript II). Furthermore, this dissertation highlights different factors that may be involved in *Drosophila* speciation events, such as niche partitioning (manuscript IV) and insect-microbe interactions (manuscript V). Finally, I worked on a previously described method that is used for the identification of ligand-receptor pairs of olfactory systems in mammals, and tried to further establish this technique for the high-throughput identification of chemosensory receptor ligands in insects on the example of the vinegar fly *D. melanogaster* (manuscript III).

I. An understudied social communication channel in *Drosophila*

Pheromones are chemosignals used by animals to communicate social cues or to mediate sexual behaviors (Karlson and Lüscher, 1959; Touhara and Vosshall, 2009). The receivers of pheromone-based signals are usually conspecifics, but pheromones can also act in interspecies communication within a genus (classically referred to as allelochemicals, (Whittaker and Feeny, 1971; Wyatt, 2003; Touhara and Vosshall, 2009)). Generally, chemosignals are considered to be a pheromone if they control hardwired stereotypic behaviors or induce endocrinological modifications in conspecifics (Karlson and Lüscher, 1959). However, the initial definition of a pheromone has been softened and is still undergoing alterations, considering now also substances as pheromones if they are used in intraspecies communication but are not inducing stereotypic behaviors (Wyatt, 2003; Touhara and Vosshall, 2009). Pheromones are not always volatile substances but can as well be non-volatile compounds including, cuticular hydrocarbons (CHCs), peptides and proteins (Touhara and Vosshall, 2009). Depending on the nature and function of the pheromone, these chemosignals can be released into the surrounding air, transferred onto the body of other individuals or deposited onto a medium (Butenandt *et al.*, 1959; Kurtovic, Widmer and Dickson, 2007; Farine, Ferveur and Everaerts, 2012; Farine, Cortot and Ferveur, 2014).

In *D. melanogaster* the repertoire of pheromones is well studied, and the role of identified, individual pheromones has been extensively described (Gomez-Diaz and Benton, 2013; Auer and Benton, 2016). However, in research on pheromone-based communication of *Drosophila* flies, frass had been overlooked or forgotten as a possible communication channel of these flies. In manuscript I, my colleagues and I found that previously described pheromones of the vinegar fly can also be found in adult frass, sometimes even in high abundance (7-T: (Z)-7-tricosene; 9-T:(Z)-9-tricosene, 7,11- HD: (7Z, 11Z)-heptacosadiene). Here, the pheromone composition of the frass resembled the sex-specific pheromone profile of the adult flies. This remained true for all other *Drosophila* species we tested. The identification of *Drosophila* pheromones, including novel compounds that might be for example involved in oviposition behavior, is still an ongoing process especially in non-*melanogaster Drosophila* species. Since adult frass so closely mirrors the pheromone profile of the corresponding animal, but often contains even much higher concentrations of these compounds, this substrate could help to contribute to the identification of yet unknown pheromones in the genus *Drosophila*.

Some of the pheromones found in the adult *Drosophila* feces had already been characterized as aggregation inducing cues such as MP and MM (detection via Or88a, (Dweck, Ebrahim, Thoma, *et al.*, 2015)) or 9-T (activates Or7a, (Lin *et al.*, 2015)), thus it was perhaps not surprising that adult frass itself was behaviorally active and acted as an intraspecies aggregation signal to the flies in our experiments. In our study we further present evidence that the attractiveness of adult frass and its role as an aggregation signal is predominantly controlled by olfactory circuits and flies with an impaired olfactory system showed reduced attraction to *Drosophila* fecal matter. As *Drosophila* frass also contained sex pheromones such as ML (detected by Or47b, (Dweck, Ebrahim, Thoma, *et al.*, 2015)) and cVA (ligand of Or65d) as well as other fatty acids (activation of Or47b, (Lin *et al.*, 2016)) that are all mediating courtship behaviors it seems possible that adult feces could be involved in the control of reproduction behavior in *Drosophila* flies. Do *D. melanogaster* males for example, deposit their frass on a substrate to attract females and to create arenas where female receptivity is increased, and from which competing males are repelled in close-range through the presence of cVA in their fecal matter? Besides the possible role of frass in sexual reproduction behavior the aggregation inducing effect of adult feces could be also used by female flies to mark host material as suitable food sources and oviposition sites, especially in those *Drosophila* species where larvae work together in high numbers to colonize breeding sites (Durisko *et al.*, 2014; Louis and de Polavieja, 2017). However, a contrary function of frass is equally conceivable with spots of female feces

acting as an oviposition deterrent to conspecific females or to females of competing *Drosophila* species, a behavior that can be found in the Mediterranean fruit fly *Ceratitis capitate* (Arredondo and Díaz-Fleischer, 2006). At least for *D. melanogaster*, a role in interspecies repulsion has been previously shown for some of the pheromones that are also found in the adult frass (7-T, 9-T, 7,11- HD (Thistle *et al.*, 2012; Seeholzer *et al.*, 2018)). Additionally, the sex pheromones contained in *Drosophila* frass could be involved in the prevention of interspecies mating on hosts where different *Drosophila* species are found (Fan *et al.*, 2013; Seeholzer *et al.*, 2018). Future work needs to be done to investigate possible other functions of adult frass in other social behaviors of *Drosophila* flies beside its role as an aggregation cue.

In manuscript I we additionally observed that adult feces also acted as a feeding stimulus to flies. While we did not find that this feeding stimulus was mediated by Ors, we predict that this effect could be conveyed either through Irs and/or through the gustatory system. Oral uptake of fecal matter through *Drosophila* flies could be linked to the presence of bacterial and yeast communities found in the frass. Chemical cues emitted by the fecal matter itself, as well as chemosignals released by microbes that survived the passage through the alimentary canal of *Drosophila* flies could be involved in stimulating feeding in other flies. The ingestion of adult frass could potentially provide nutritional factors to the flies and could play a role in sharing a beneficial gut microbiome within the fly population. Furthermore, it is possible that some substances found in the frass could induce endocrinological changes in the feeding flies. Thus, the uptake of fecal matter through *Drosophila* flies could have a similar role as the transfer of trophallactic fluids observed in social insects such as the ant *Camponotus floridanus* (Leboeuf *et al.*, 2016).

Finally, the deposition of fecal spots on host material that we describe in manuscript I could further be used by the *Drosophila* flies to modify the substrate to their benefit by introducing beneficial microbes that accelerate the break-down of the material, detoxify present secondary metabolites and hinder the growth of harmful microorganisms. In manuscript V, I show that indeed *Drosophila* males can inoculate host material with their associated microbes and that the combination of fly and microbial activity lead to an increased decomposition of the substrate in comparison to controls. Both, the presence of flies and their associated microbial communities, additionally supressed the growth of fungal molds. Thus, it seems likely that the frass of *Drosophila* flies could play a role in the modification of host material to the advantage of the corresponding fly species.

II. The role of the olfactory system in *Drosophila*-microbe interaction

Chemosignals mediate and regulate interactions within a species and between different species groups, phyla or even between different kingdoms and are therefore, essential communication

channels within ecological networks (Günther *et al.*, 2019). Some chemical cues have an innate valence for insects and will prompt hardwired, stereotypic behaviors, while other odorants are learned by insects to be associated with a certain context. Innate behaviors that affect the performance of an individual, of a population or of an associated species can become the target of natural selection events. In the following two sections II.I and II.II examples of *Drosophila*-microbe interactions will be highlighted that are mediated and controlled by chemosignals.

II.I Manipulation of odor-mediated stereotypic behavior through pathogens

Microorganisms that manipulate their host's physiology and behavior have always been extraordinarily fascinating to scientists and non-scientists. Consequently, the elucidation of mechanisms leading to these microbial induced changes in physiology and behavior are the focus of many research projects. A spectacular example for microbial host manipulation in the class of *Insecta* is the ant parasite *Ophiocordyceps unilateralis*, which is also referred to as the "zombie ant fungus". When an ant is infected by this fungus, against its natural behavior, it will climb up any tall structure, affix itself at the very top by biting into the substrate and ultimately succumb to the fungal infection (De Bekker *et al.*, 2014; Elya *et al.*, 2018). The "zombie ant fungus" will then feed of the body of the ant, eventually forming a fruiting insect that protrudes from the dead body and serves in the dispersal of the fungal spores (De Bekker *et al.*, 2014; de Bekker *et al.*, 2015; Elya *et al.*, 2018). Interestingly, there is a fungus that induces similar behavior in dipterans including *Drosophila* flies. This fungus called *Entomophthora muscae* will force infected flies to seek high locations shortly before their deaths (MacLeod, Mueller-Koegler and Wildling, 1976; Keller, 1984; Elya *et al.*, 2018). There, the infected flies will affix themselves to the surface via a sticky fungal fluid that is secreted through the flies' proboscis (MacLeod, Mueller-Koegler and Wildling, 1976; Keller, 1984; Elya *et al.*, 2018). Finally, with wings lifted up and away from their abdomen, infected flies will die. The *E. muscae* will then grow through weak points in the flies' cuticle and form spores that are ejected onto new hosts upon interaction (Elya *et al.*, 2018). Processes underlying both these examples of fungal host behavior manipulation are the topic of recent publications and ongoing studies (De Bekker *et al.*, 2014; de Bekker *et al.*, 2015; Elya *et al.*, 2018).

In manuscript II, my colleagues and I describe how some pathogens of *D. melanogaster* use the flies' social communication channels that are linked to hardwired stereotypic behaviors to their own advantage by increasing dispersal. Initially, we started into the project with the aim to find possible new odorants correlated to disease and infection that would possibly induce

avoidance behavior in healthy flies. For *Drosophila* flies only a few substances are known to signal the presence of harmful microbes to the fly such as geosmin and phenol, which are detected through Or56a and Or46a, respectively (Stensmyr *et al.*, 2012; Mansourian *et al.*, 2016). Both, Or56a and Or46a, are Ors that mediate innate avoidance behavior and enable the flies to perceive, and rapidly respond to possible dangers in their environment. We characterized the odor profile of *D. melanogaster* flies infected with different pathogens in order to identify possible unknown odorants indicating disease to the flies. Furthermore, we tested the behavior of healthy flies towards sick individuals. Our selected pathogens included species that were negatively affecting the health of sick flies but could be eventually fended off (*Erwinia carotovora sp. carotovora* 15 (Ecc15),(Basset *et al.*, 2000)), as well as pathogens that would cause a rapid death of the infected flies within 24 h (*Serratia marescens Db11, and Pseudomonas entomophila,* (Flyg, Kenne and Boman, 1980; Vodovar *et al.*, 2005; Liehl *et al.*, 2006)). Much to our own surprise, we observed that healthy flies would be attracted to the frass of infected flies and to the infected flies themselves, independent of the hazardousness of the corresponding pathogens. Intriguingly, when we had a look at the odor profiles and body washes of the diseased flies, we found that pheromone levels, predominantly of the volatile compounds methyl laurate (ML), methyl myristate (MM), methyl palmitate (MP), and palmitoleic acid (PA), were drastically increased. Pheromone levels of cVA however were not elevated in infected flies. Generally, in our samples we observed that the deadlier the pathogen was, the higher the abundance of pheromones detected. Non-pathogenic bacteria did not induce changes in pheromone levels. In further experiments we could show that at least for infections with *P. entomophila,* the increase in pheromone production was linked to alterations in the lipid metabolism of the flies and to immune as well as insulin response pathways.

Affected pheromones were linked to reproduction behavior and aggregation and since the corresponding Ors activate hardwired neuronal circuits, the healthy flies were not able to resist the attractiveness of infected conspecifics. Upon interaction with the sick individuals or with frass of these flies healthy *D. melanogaster* flies themselves would become infected. Even though pheromone levels, including pheromones that are mediating courtship behavior, are increased in infected flies, in mating assays we did not find a better performance in courtship behavior of those diseased individuals. Thus, by manipulating the social communication channels of *D. melanogaster,* the corresponding pathogens increase their own dispersal at the detriment of the flies, not granting any higher reproductive success to the infected host. Although the reproductive behavior of *Drosophila* flies is very effective, this hardwired circuity is sensitive to manipulations. However, as the genus *Drosophila* is very successful and species-rich, the advantages of this reproduction strategy seem to outweigh disadvantages, such as

manipulations of sex pheromone cues through pathogens. None of the tested pathogens in the study were *Drosophila*-specific species and in future experiments it would be very interesting to find out if these pathogens also manipulate social communication channels in other insects. Is the increase in pheromones a by-product of the bacterial infection and the associated damage in *D. melanogaster* alone, or is the hijacking of hardwired sexual behaviors a general mechanism used by these pathogens to increase transmission rates and dispersal? For the widespread arthropod symbiont *Wolbachia* it is known for example that this bacterium does use their hosts' reproductive system and linked behaviors to their own advantage, impacting mate choice, reproduction and affecting the evolution of their hosts' population (Arbuthnott, Levin and Promislow, 2016).

As seen with the example of *Wolbachia*, host manipulations are not necessarily correlated with negative or detrimental effects for the host. Do beneficial fly associated bacterial and yeast communities equally influence choices and stereotypic behaviors of their hosts? It would be interesting to find out whether beneficial microbes change foraging preferences of their associated *Drosophila* species, manipulating the flies to prefer host material as food sources or breeding sites that are best suited as their own growing substrates. For example, do *Drosophila* flies that ingested and matured on cactophilic microbes prefer cactus material over other food sources and choose this host as a subsequent breeding site? It has been already described that *Drosophila* individuals that developed in the presence of certain bacteria or yeast will often prefer these microbes as food sources once they are adults (Farine, Cortot and Ferveur, 2014; Fischer *et al.*, 2017; Grangeteau *et al.*, 2018; Murgier *et al.*, 2019; Qiao *et al.*, 2019). However, it remains to be ascertained if this also leads to preference of the microbes' growth medium or host environment.

II.II Chemosignal-mediated interaction of *Drosophila* flies with yeast communities

The association of *Drosophila* flies with selected yeasts species comes with benefits for both, the insect and the yeast alike. The sessile, immobile yeasts are transported by *Drosophila* flies to new substrates, which benefits the microbes' dispersal (Ganter, 1988; Starmer, Peris and Fontdevila, 1988). In the new substrates the flies promote the growth of their associated yeast species for example through the deposition of nutritious, microbe-rich frass, and after passage through the insect's alimentary canal, the yeasts are enabled to sexually reproduce (see introduction section VI (Reuter, Bell and Greig, 2007; Coluccio *et al.*, 2008; Becher *et al.*, 2012; Stamps *et al.*, 2012; Stefanini, 2018). In turn the yeasts provide the *Drosophila* flies with

essential nutritional factors, detoxify secondary metabolites in the flies' food sources and at breeding sites (Tatum, 1939; Fogleman, Duperret and Kircher, 1986; Becher *et al.*, 2012; Dweck, Ebrahim, Farhan, *et al.*, 2015), and increase the flies' resistance towards pathogens and parasitoids (Vass and Nappi, 1998; Rivera *et al.*, 2003; Li *et al.*, 2007; Lee, Simpson and Wilson, 2008; Anagnostou, LeGrand and Rohlfs, 2010). Many aspects of *Drosophila*-yeast interactions match the criteria of mutualistic relationships, thus it has been hypothesized that the production of volatiles that are attractive to *Drosophila* flies is a yeast-specific trait, which evolved to maintain and ensure the association of yeasts with certain insect species, including *Drosophila* flies. There is accumulating evidence for this hypothesis, such as the occurrence of chemosignal production in yeasts, and the development of their corresponding detecting units in insects at an overlapping evolutionary time scale, as well as the identification of metabolic pathways in yeasts that are not necessary for the microorganism's survival but produce odorants attractive to these insects (Engel and Grimaldi, 2004; Dujon, 2006, 2012; Davis *et al.*, 2013; Nel *et al.*, 2013; Christiaens *et al.*, 2014; Becher *et al.*, 2018). An opposing hypothesis is that volatiles released by yeasts did not evolve to attract insects but are the by-products of the yeast's metabolic activity for example from the detoxification of fermentation intermediates (Palanca *et al.*, 2013). Thus, *Drosophila* flies would have then secondarily evolved to follow these chemosignals as they are indicative of suited food sources and breeding sites (Palanca *et al.*, 2013).

In manuscript V, we predicted, that *Drosophila*-yeast interactions with a classic mutualistic nature would be positively selected for, leading to a consistent attraction of the flies towards a yeast-specific chemosignal profile. In particular we expected *Drosophila* flies to prefer yeast species from a shared habitat that are associated with benefits to adult and larval performance over yeasts from unfamiliar ecological niches. However, while in agreement with previous work (Palanca *et al.*, 2013; Scheidler *et al.*, 2015) we found that flies can distinguish between different yeast species based on the yeast's corresponding chemosignal profile, we did not see a consistent attraction of *Drosophila* flies towards yeasts from their natural habitat. Instead the attractiveness of a yeast species correlated with the number of attractive odorants emitted by the yeast. Consequently, the more attraction mediating channels in the olfactory system were activated the higher the attractiveness of a yeast species was. As the headspace of a yeast can even attract *Drosophila* species that naturally do not occur in the same habitat as that yeast, we suspect that the corresponding detecting olfactory receptors are relatively conserved across a broad range of *Drosophila* species. Once all individual odorants that convey this attraction have been identified, the corresponding olfactory receptors can be characterized

in *D. melanogaster* and in comparison, with the amino acid sequence of homologs in other *Drosophila* species our hypothesis can be tested.

Furthermore, we found that the preference of *Drosophila* flies for a specific yeast species was not exclusively mediated via olfactory cues but was based on a multi-chemosensory assessment of the yeast, as flies evaluated the attractiveness of yeast also under gustatory aspects. As previously suggested (Karageorgi *et al.*, 2017), we observed that an initial olfactory-based preference for a yeast species could change upon an evaluation of gustatory cues emitted by that yeast. Moreover, we provide evidence that the presence of host material influences yeast preference and oviposition choice of *Drosophila* flies, especially in those *Drosophila* species with a specialized lifestyle. We propose that chemosignals from the yeast and its associated host work synergistically in shaping the attractiveness of a yeast species and in controlling oviposition, possibly through the activation of a multitude of attraction mediating channels.

In our study, we further noticed that yeast species from habitats that lack nutrients, especially carbohydrates, produced fewer insect-attracting volatiles than those yeasts from hosts rich in nutrition. In future work with a wide array of yeast species it would be interesting to find out whether this trend holds true and whether indeed the availability of nutrition in a yeasts' host defines the number of metabolic pathways that lead to the production of insect-attracting volatiles.

Overall, our work in manuscript V shows that the association of *Drosophila* species with beneficial yeasts from a shared habitat is less stable and less established than we initially expected. Instead *Drosophila* adults can become attracted to yeast species from new, unfamiliar niches through chemosignals released by those yeasts that are universally attractive to *Drosophila* flies (for interpretation of this phenomenon see discussion section III.II). Seemingly there is no conserved aspect to the association of *a Drosophila* species with a certain yeast species but rather to the attraction of the flies to certain chemosignals in a seemingly additive manner and not to a yeast-species specific composition. Future work should follow up on the question of whether *Drosophila*-yeast associations co-evolved to form mutual partnerships or whether *Drosophila* flies evolved to associate yeast-emitted chemosignals with suited food sources and breeding sites.

III. Speciation in the genus *Drosophila*

There are not many habitats where *Drosophila* flies cannot be found, including such extreme environments as caves in Brazil or the excretory glands of land crabs (Gilbert, 1980; Tosi *et al.*, 1990; Markow, 2019). What factors enabled the rapid radiation of *Drosophila* species onto such a variety of different food sources and breeding sites (Markow, 2019)? And what mechanisms determine the success of this remarkable species-rich genus? In section III.I and III.II I introduce two hypotheses that explain possible mechanisms underlying *Drosophila* speciation events.

III.I Modification of sensory modalities as a driver of *Drosophila* speciation

What are the ecological and evolutionary principles that shape the organization and function of the neuronal circuitry that control animal behavior? And is there a relation between the architecture of neuronal circuits, their function and behavioral relevance? Numerous studies do indeed provide evidence for a correlation between structural size and morphological specialization with the functional significance of the corresponding sensory modality (McGaugh *et al.*, 2014; Moran, Softley and Warrant, 2015). For example, the eye degeneration in cavefish like the Mexican tetra *Astyanax mexicanus*, correlates with a decreased size of brain regions that are processing visual information and therefore, visual stimuli prove to be of little importance for the fish. In insects, often a reduction or an enlargement of glomeruli in the animal's antennal lobe in comparison to related species or conspecifics can be found when the corresponding stimulating odorants play an important role in the ecology of that individual. For example, *Lepidoptera* males possess an enlarged macroglumerular complex (MGC) that processes sex pheromone cues (Kazawa *et al.*, 2009; Namiki *et al.*, 2018). Moreover, in *Drosophila suzukii* the glomerulus DA1 that is activated upon cVA detection is reduced in size compared to *D. melanogaster* as *D. suzukii* itself only produces trace amounts of this compound (Dekker *et al.*, 2015). Furthermore, in *D. sechellia* the size of the DM2 glomerulus that detects important odorants of the flies' particular food source and breeding site, the morinda fruit, is increased in comparison to the corresponding glomerulus in the close relative *D. melanogaster* (Dekker *et al.*, 2006).

However, why does one exaggerated sensory structure often coincide in a trade-off with another sensory trait? Why is there no *Drosophila* species that does it all; produce a vast number of sex pheromones, has an intricate body pigmentation and complex wing-display sequence for reproduction purposes? One reason behind an inverse relationship between different sensory modalities is that the investment into a sensory stimulus and its corresponding morphological structures as well as circuitry comes with high energetic costs. A reduced

anatomical investment into other sensory stimuli thus helps to minimize the overall energetic costs of the organism. In primates for example the emergence of trichromacy (three independent channels for the mediation of color information) is linked to a high number of olfactory genes with non-sense mutations and olfactory pseudogenes. In manuscript IV, we tested whether we find a similar phenomenon in the genus *Drosophila* between species that rely heavily on visual stimuli versus species that are more olfactory driven. However, we did not observe a similar correlation between the quantity of olfactory pseudogenes to eye morphology or visual bias in the analyzed *Drosophila* species. We cannot exclude though that expression levels of the corresponding olfactory or visual genes differ and this remains to be studied in the future.

In conjunction with the energetic aspects of a sensory trade-off, we propose in manuscript IV, that in the genus *Drosophila* the observed inverse prioritization of visual stimuli over olfactory stimuli or vise versa, promotes speciation, reduces competition between different *Drosophila* species that share overlapping habitats (sympatric species) and allows through niche partitioning for different species to coexist in a common environment (see also (Keesey et al. 2019)). Thus, there seem to be positive selection processes supporting these sensory trade-offs between related species within the *Drosophila* genus.

In a large-scale comparison of 62 *Drosophila* species my colleagues and I consistently found that in fact, evolutionary events led to the occurrence of either an olfactory or visual bias within this genus. The inverse relationship between the olfactory and visual system of *Drosophila* flies we traced back at least in parts to a restricted resource allocation between the two sensory modalities due to a shared imaginal disc during larval stages. From this one shared imaginal disc both adult sensory systems will develop. Mutations in the genes that control the balance between the eye and antennal region of the imaginal disc can favor either an olfactory or visual bias and, in agreement with previous work (Ramaekers *et al.*, 2018), we show that already single gene mutations can cause an inverse variation in the quantity of ommatidia (single units composing a compound eye) and sensilla in *D. melanogaster*. Future work needs to be done to identify all players in control of the push-pull between both sensory modalities and whether similar genes control the balance between the eye and antennal region in the imaginal disc within different *Drosophila* species.

In our study we were especially interested in investigating to what extent differences in the olfactory and visual system correlated with reproductive behavior and host preferences across *Drosophila* flies. Based on our results and earlier findings, we conclude that indeed in

Drosophila species, the importance of vision for sexual behaviors or host navigation is mirrored in exaggerated visual structures including the corresponding neuronal circuits. In turn, when courtship behavior of a *Drosophila* species was mostly mediated through sex pheromones, we found that the flies in comparison had enlarged antennae with a high number of trichoid sensilla, i.e. those sensilla that are known to detect pheromones. In those fly species with an olfactory bias we additionally found that host navigation would be notably olfactory driven. The vinegar fly *D. melanogaster*, as aforementioned, relies on the sex pheromone cVA for its courtship behavior, has a high density of trichoid sensilla on its antennae and visual cues are so negligible to the fly that reproduction does not require light in this species (Sakai *et al.*, 1997, 2002; Auer *et al.*, 2019). In contrast, *D. suzukii* only produces low amounts of cVA, does not require this pheromone for its reproduction behavior and only possesses a low number of trichoid sensilla (Dekker *et al.*, 2015). Concurrently, as *D. suzukii* is considered a member of the spotted wing *Drosophila*, this fly species uses wing pigmentation in its courtship behavior and without light the flies will not successfully mate. Strikingly, the compound eyes of D. suzukii are distinctly enlarged and are up to 2.5 times bigger than the eyes of *D. melanogaster*.

In manuscript IV we further observed that in a manner of character displacement, especially *Drosophila* species within a clade or subgroup displayed large differences in olfactory and visual structures. We predict that in the genus *Drosophila* under the high selection pressure of courtship and mate selection as well as through host competition, repeated speciation events favored an inverse relationship between vision and olfaction, which led to a competitive release.

III.II Do associated yeast communities shape host specialization in *Drosophila*?

Drosophila flies encounter and interact with yeast communities in every aspect of their lives. Beneficial yeasts produce micro- and macronutrients for the flies and their offspring (Loeb and Northrop, 1916; Tatum, 1939; Becher *et al.*, 2012; Dweck, Ebrahim, Farhan, *et al.*, 2015; Bellutti *et al.*, 2018), modify the chemical composition of the flies' host material (Fogleman, Duperret and Kircher, 1986; Starmer and Fogleman, 1986; Goddard, 2008) and influence sexual behaviors in these insects, for example by affecting female fecundity or cuticular hormone synthesis (Grangeteau *et al.*, 2018; Murgier *et al.*, 2019). The yeast communities *Drosophila* flies are exposed to are equally diverse as the plethora of ecological niches that *Drosophila* species populate.

As aforementioned, in manuscript V, we initially predicted that the mutual benefits of certain fly-yeast interactions led to an established partnership between these two species

groups, that is initiated and maintained through chemosignals produced by the yeasts, which act as attractants to the corresponding *Drosophila* species. When we noticed that the tested flies did not consistently prefer yeast from a shared ecological niche but would also be attracted to yeasts from unfamiliar habitats, we wanted to understand what the ecological implications of this behavior might be. Previous studies already proposed a “bad mother” hypothesis (Mayhew, 2001), especially for herbivorous insects; but also for *Drosophila* species (Scheirs, De Bruyn and Verhagen, 2000; Anagnostou, Dorsch and Rohlfs, 2010). Nevertheless, we were surprised to find that female *Drosophila* would make oviposition choices for the presence of yeasts at breeding sites that negatively affected their offspring’s development and survivorship rates. We observed that often, only the *Drosophila* females themselves would profit from their yeast preference, through for example the uptake of nutritional factors or through increased fecundity.

Furthermore, in our study the host breadth of *Drosophila* species correlated with the capability of the species to tolerate and utilize a range of yeast species in its host material. The cosmopolitan *Drosophila* species, *D. melanogaster*, can form beneficial affiliations with various yeast species, and their offspring perform equally well in association with numerous different yeasts. Hence, *D. melanogaster* flies can be found in a wide array of host substrates. In contrast, when larval development and survivorship numbers were sensitive to changes in the composition of interacting yeast communities, and when the adult flies had a narrow yeast species preference, the host range of the corresponding *Drosophila* species is equally narrow, as seen in the specialists, *D. mojavensis* and *D. putrida.*

While larval performance is a constraining factor in the host range of a *Drosophila* species, the preference of *Drosophila* females for unfamiliar yeasts could lead to the colonization of new ecological niches and subsequently to local adaptation events. It seems possible that in small steps, through the acquirement of new yeast community compositions that have similar or shared physiological abilities, flies could slowly transfer to new hosts. The local adaptation of a *Drosophila* subpopulation if followed by host acceptance and host utilization, could then ultimately culminate in a speciation event (Markow 2019). Thus, we propose that interactions of *Drosophila* flies with yeasts from new niches and habitats represents a chance for speciation in the genus *Drosophila*. In agreement with earlier work (Starmer, 1981), we suggest that the enormous and rapid increase in species diversity (radiation) in the genus *Drosophila* could be linked to the manifold metabolic abilities of the interacting yeast communities.

IV. Characterization of ligand- chemosensory receptor pairs in insects

The identification of excitatory or inhibitory ligands of olfactory receptors in insects is indispensable for progress in understanding how chemosensory signals mediate insect behaviors, and in deciphering the underlying neuronal circuits. While in *D. melanogaster* for most functional chemosensory receptors, agonistic and antagonistic ligands have been found and described (Münch and Galizia, 2016). This process has been a great, as well as time-consuming endeavor and could not have been achieved without the efforts of several laboratories (de Bruyne, Clyne and Carlson, 1999; de Bruyne, Foster and Carlson, 2001; Hallem and Carlson, 2004, 2006; Mansourian and Stensmyr, 2015; Dweck *et al.*, 2018). Overall this data collection took more than two decades thus far. For other insect species that do not have genetic tools available, such as those found for the vinegar fly (e.g. the “empty” neuron system or binary expression systems), the progression of chemoreceptor deorphanization has been even slower and more complicated. When von der Weid and colleagues published a method for the *in vivo* deorphanization of ORs in mammals, and for a set of ORs in *D. melanogaster* that only required the transcriptome of chemosensory receptors, hopes were high that this technique could be the breakthrough for a fast, *in vivo*, high-throughput identification of ligand-receptor pairs in all insect models. The deorphanization technique described by von der Weid et al. is based on the phenomenon that upon prolonged odorant exposure at high concentrations, the transcript levels of the interacting olfactory receptors would respond with a decrease in overall quantity. Consequently, the technique was named DREAM method (**D**eorphanization of **r**eceptors based on **e**xpression **a**lterations of **m**RNA levels).

In manuscript III, using *D. melanogaster* and its comprehensive database of odorant and olfactory receptor interaction partners, we evaluated whether the DREAM method is a universal tool for a reliable identification of olfactory ligand-receptor pairs in a high-throughput manner in insects. The in-depth knowledge available for our selected ligand-receptor interaction partners allowed us to have precise expectations for the outcome of the DREAM application, providing that the technique functions as suggested in insects. First, we assessed whether the DREAM method could be used to predict interacting ligands of olfactory receptors in insects, including IRs. Secondly, we were interested to find out whether the direction of the alterations in receptor transcript levels was indicative of the nature of ligand-receptor interactions. Do excitatory odorants cause a downregulation in mRNA levels of olfactory receptors as implied from DREAM applications in mammals in the original publication, and do inhibitory ligands thus trigger an upregulation in transcript levels? From our eight agonist-receptor pairs, we only found a change in receptor mRNA levels for five pairs following the DREAM method. These changes were not

all downregulations but also in two cases increases in receptor transcript levels. However, for a considerable amount (69 %) of all observed alterations in receptor mRNA levels in our experiments, we could show a correlation between regulatory event and ligand-receptor interaction (excitatory or inhibitory) in SSR experiments. At the same time, we did not observe a correlation between the direction of alterations (decrease or increase) in receptor transcript levels and the mode of ligand-receptor interaction (activation vs. inhibition).

In our study we differentiated between two categories of false predictions after application of the DREAM technique: false positive and false negative ligand-receptor predictions. We defined false positive predictions as incidences where olfactory receptor mRNA levels were changed upon odorant treatment even though there was no verifiable ligand receptor interaction in electrophysiological measurements. We traced these false positive predictions back to the high, non-ecologically relevant odorant concentration used in the DREAM method that presumably led to unspecific ligand receptor interactions. False positive interaction partner predictions could be subsequently debunked using traditional deorphanization techniques like “decoder systems” in *D. melanogaster* or heterologous expression systems for non-*melanogaster* insect species. Although false positive predictions do not severely affect the applicability of the DREAM method, as they can be falsified, the occurrence of unreliable results makes the DREAM technique unsuited for a rapid identification of unknown ligand-receptor pairs in *D. melanogaster*. More severe than the false positive predictions were false negative interaction predictions where the transcription of receptors were unchanged after DREAM treatment even though the receptors are naturally activated or inhibited by the compound. These false negative predictions would interfere with the elucidation of the entire ligand spectrum of olfactory receptors and possibly prevent the discovery of olfactory circuits that control important insect behaviors. Thus, both, the positive and negative false interaction predictions, led to the conclusion that without further adjustments the DREAM technique was not the anticipated silver bullet for a precise, high-throughput deorphanization of olfactory receptors in *D. melanogaster* or other model insect species.

Thereupon, we wanted to make progress in understanding the mechanisms underlying the DREAM technique in order to potentially modify parameters which determine the reliability of ligand- receptor interaction predictions. A popular hypothesis for the downregulation of receptor transcript levels seen after DREAM application was that the OSNs, which express the corresponding interacting receptor, would respond to the overstimulation by rendering the

neuron less sensitive to the odorant stimulus. However, we would also find upregulation of receptor mRNA levels after long time excitatory odorant stimulation or downregulation of receptor transcript numbers after odorant treatment with inhibitory compounds. Furthermore, we did not observe changes in the dose-response curve of tested olfactory receptors in SSR experiments. Thus, we excluded this hypothesis as the mode of action of the DREAM technique.

Perhaps, ligands that induce either a decrease or increase in receptor transcript levels share binding properties that modulate the corresponding receptor in a way that lead to a certain direction of the induced mRNA level changes. After performing SSR measurements for our selected ligand-receptor pairs and after conducting GC-MS analysis of the odorant concentration in the DREAM setup we further eliminated inadequate ligand-receptor pairs as well as insufficient odorant stimulation as causes for a lack of regulatory events. Next, we had a look at all incidences of a successful application of the DREAM method and compared chemical properties of the odorant treatments or characteristics of the responsive, interacting olfactory receptors. However, we did not identify common ligand qualities or receptor features that would correlate with regulatory events in receptor transcription levels upon DREAM treatment. Eventually, based on data from the original publication and after additional experiments we determined that the duration of the odorant exposure in the DREAM method is a critical factor for the outcome of its application. The fact that the length of the odorant exposure might have to be adjusted for different ligand-receptor pairs and different insect species, adds up further to the limitations of the DREAM method.

Although we evaluated the DREAM technique as unfit for a universal, high-throughput chemoreceptor deorphanization, I propose that this method could still be valuable in narrowing down possible candidates for ligand interaction partners from a big repertoire of olfactory receptors. Especially, when used with RNA sequencing or new technologies such as the NanoString nCounter gene expression system (Geiss *et al.*, 2008) where the expression of 200 or more genes can be simultaneously analyzed, the DREAM method could be applied to identify a set of possible interaction partners that in conjunction with traditional deorphanization techniques can be further characterized. However, users should be aware that not all interacting receptors may be found through the application of the DREAM method.

V. Conclusion

The physiology, genetics and neuroethology of *D. melanogaster* have been extensively studied for over a century now. However, scientists still discover previously unknown principles

and mechanisms in this model species on an almost daily basis. The ecology of the vinegar fly and of its close as well as distant relatives in the genus *Drosophila*, has become a focal point of scientists in the last decade. Throughout the projects of my dissertation I described possible mechanisms that underlie *Drosophila* speciation as well as *Drosophila* host use and localization. Furthermore, I uncovered an overseen intra- and interspecies communication channel and investigated the role of olfaction in *Drosophila*-microbe interactions.

By closely observing the *Drosophila* species in our laboratory, my colleagues and I recognized that in our studies of the flies, we were neglecting a fly product with an important role in the flies' life, being the adult frass. Subsequently, we were able to show that the frass of adult *Drosophila* flies acts as an intra- and potentially an interspecies social communication channel, mediating intraspecies aggregation as well as sexual behaviors and presumably interspecies repulsion. Moreover, I investigated whether adult frass might be involved in accelerating the decomposition of host material, which would release essential macro- and micronutrients for the flies. Here, I further focused on the role of chemosignals in the interaction of *Drosophila* species with their associated beneficial yeast communities. Our data indicated that the odor-mediated preference of *Drosophila* flies for certain yeast species is less established than one might expect from a mutually beneficial fly-microbe interaction. Instead, the association of flies with novel yeast species from new hosts seems to hold the potential for local adaptation processes of *Drosophila* flies and might eventually lead to speciation events. Besides the potential involvement of associated yeast communities in *Drosophila* radiation, I propose that mate selection and host competition might have favored niche partitioning in the genus *Drosophila,* and as a competitive release, led to character displacement in the flies' olfactory and visual system. When we compared olfactory and visual structures, including the corresponding neuronal circuits of 62 *Drosophila* species we consistently found an inverse relationship between the two sensory modalities of olfaction and vision that occurred repeatedly but independently of each other. The inverse relationship between the olfactory and visual system of *Drosophila* species could be the consequence of a restricted resource allocation between these two sensory modalities through a shared developmental structure during larval stages of the flies. Additionally, I show how stereotypic, developmentally preprogrammed odor-mediated behaviors of *Drosophila* flies can be exploited through pathogens that in turn benefit via increased dispersal and transmission rates. We observed that *D. melanogaster* flies infected with selected pathogens, would display an increase in the production of aggregation and sex

pheromones, which attracted healthy flies. These healthy individuals themselves would become infected upon interaction with sick flies and with contaminated frass.

Finally, I aimed to introduce a new technique for a fast, effective characterization of the ligand spectrum of chemosensory receptors to the olfactory research in insects. This method is referred to as DREAM (**D**eorphanization of **r**eceptors based on **e**xpression **a**lterations of **m**RNA levels), was initially developed for the deorphanization of olfactory receptors in mammals and only requires transcriptomic chemosensory gene information for its application. We wanted to use the DREAM method to identify odorants and their interacting olfactory channels that mediate and control important insect behaviors, such as mate selection or host navigation. However, after a thorough examination of the possibilities, mechanics and limitations of the DREAM technique, we concluded that this method is neither reliable nor time efficient enough to replace traditional deorphanization methods in insects. Only in conjunction with other deorphanization techniques the DREAM method can be used to identify possibly candidates for ligand interaction partners that would then need to be further characterized in additional experiments.

General Summary

The astonishing capabilities and properties of the sense of smell in insects are fascinating to scientists and non-scientists alike. If you open a bottle of wine or fruit juice and pour yourself a glass, you can be almost certain that soon after there will be some *Drosophila* flies buzzing around your drink. How do these insects find the source of their interest so quickly and what compounds in the odor bouquet of our drinks help the flies to pinpoint the location of the beverage? Scientists have studied the molecular and neuronal mechanisms underlying these and other odor-mediated insect behaviors for over a century now. Hereby, researchers have learned much about ecological and evolutionary principles that shape the architecture and function of the neuronal circuits governing insect behavior. Furthermore, knowledge was gained about the processes starting from the binding of an odorant molecule to olfactory receptors and the subsequent conversion of the chemical signal into an electrical output, up to the processing of the olfactory information, and the corresponding behavioral output. Already, progress in understanding the olfactory systems of insects has led to ample practical applications in the fight against agricultural insect pest species and disease vectors. Moreover, our knowledge of the olfactory sense of individual insect species now allows for research on the communication between different insects and other species groups within entire ecological networks.

Within this dissertation I contributed to projects that broaden our knowledge about chemosignal-mediated intra- and interspecies communication in *Drosophila* (manuscripts I and II), with a special focus on social behaviors, such as aggregation and reproduction, that are controlled and governed by odorants.

In a chemical analysis of adult *Drosophila* frass, my colleagues and I characterized the chemical profile of this fecal matter (manuscript I). We found that the pheromone composition in the frass closely resembled the sex-specific pheromone profile of the corresponding *Drosophila* fly. The insect feces contained pheromones in high abundance that are associated with intraspecies aggregation or sexual behaviors. In behavioral experiments, flies were attracted to fecal matter of conspecifics and closely-related *Drosophila* species, but less so to the frass of distantly-related species. This aggregation behavior was mediated via olfactory channels. Apart from an involvement in aggregation and sexual behaviors, we predict that the pheromone profile of female frass could mark oviposition and breeding sites, attracting conspecific females or deterring competing *Drosophila* females within one species or interspecifically. Moreover, as flies also ingested adult frass, we suspect that fecal matter might

contain nutritional factors and could represent a source for a beneficial gut microbiome, shared between the flies of a population. Lastly, through the deposition of fecal spots on host material the flies might inoculate the substrate with beneficial microbes that in turn aid in host degradation, release micro- and macronutrients, detoxify noxious host metabolites and prevent the growth of fly pathogens.

Furthermore, I contributed to the discovery that pathogens can exploit pheromone-mediated predictable behaviors of *D. melanogaster* to promote their own transmission rates and dispersal (manuscript II). Here, the pathogens take advantage of the fact that *Drosophila* flies are not able to resist certain social cues linked to aggregation and courtship behavior due to the hard-wired nature of the controlling circuitry. Upon interaction with diseased conspecifics and their contaminated frass, healthy flies would become infected with the respective pathogen. When we studied wild-type and mutant vinegar flies that were infected with the pathogen *Pseudomonas entomophila*, we traced the heightened pheromone production back to alterations in the lipid metabolism of the corresponding flies, and showed an involvement of immune as well as insulin response pathways. Although infected *D. melanogaster* flies would produce high quantities of social chemosignals, sick individuals did not gain the benefit of a higher reproductive success rate. Instead healthy and infected flies were manipulated at their own detriment. Throughout manuscript II, we demonstrate how initially effective stereotypic behaviors that are controlled through only a few olfactory channels and corresponding neuronal circuits can become the target of pathogenic manipulations. However, as *Drosophila* flies represent an immensely successful and species-rich insect genus we predict that the benefits of this reproduction strategy prevail.

Next, I studied the role of the olfactory system in yeast preference of *Drosophila* species and aimed to understand what factors influence the attractiveness of yeast-emitted chemosignals (manuscript V). I investigated whether *Drosophila* flies learned to associate specific chemosignal profiles with familiar, beneficial yeast species, which in turn would ensure stable fly-yeast communities. However, while in behavioral experiments *Drosophila* flies distinguished between different yeast species based on chemosensory cues, there was no consistent attraction of the insects towards familiar yeasts from their natural habitat. Instead, in our assays the attractiveness of a given yeast was linked to the number of yeast-emitted odorants that activate attraction mediating olfactory channels, and elicit attraction behavior. Besides olfactory cues, *Drosophila* flies based their yeast preference on additional gustatory signals released by the yeasts. Thus, *Drosophila* species evaluate the quality of a yeast species based on a multi-chemosensory assessment. Even though yeast-produced chemosignals are

the key players in host attraction and navigation of *Drosophila* flies, and host odors only play a secondary role in these behaviors, we provide evidence that chemosensory cues from yeasts and host synergistically shape the yeast preference of flies and mediate oviposition behavior in *Drosophila*. Moreover, we noticed a correlation between the number of insect-attracting volatile cues produced by a yeast species and the availability of nutrition, in particular carbohydrates, in the yeasts' natural growing substrate. In future work it would be interesting to find out whether the growing substrate of a yeast species indeed defines the amount of chemosignals produced by the respective microbe.

Another important topic of the projects within my dissertation were the principles that underlie the enormous species-richness and adaptive radiation of the genus *Drosophila*, which members can be found in a broad range of hosts and habitats with approximately 2000 species total (Markow, 2019). In manuscript IV and V, I propose two factors that might be involved in driving speciation events in *Drosophila* flies.

Firstly, repeated, independent evolutionary events led to the occurrence of an inverse relationship between the sensory modalities of olfaction and vision in the genus *Drosophila* (manuscript IV). In a comparison of 62 *Drosophila* species, my colleagues and I consistently found that the insects would either possess exaggerated olfactory or visual structures, including the respective neuronal circuits, but never both. The differences between the olfactory and visual systems of *Drosophila* species were largest within species of one clade or subgroup. We concluded that on the one hand the flies would minimize overall energic costs by anatomically investing mostly into one sensory modality, and thereby reduce the investment into the other modalities. On the other hand, an olfactory or visual bias in a manner of character displacement, allows for a competitive release between *Drosophila* species that populate overlapping habitats and enables the species to coexist in a common geographical location. The inverse relationship between olfactory and visual structures we attributed at least in part to a restricted resource allocation between the olfactory and visual system as a consequence of a shared developmental structure during larval stages. Neither of these two sensory modalities can change without causing a bias. In experiments with *D. melanogaster* wild-type and mutant flies, we provide evidence that already single gene mutations can affect the quantity of ommatidia and sensilla in an inverse relation. We hope that our study in the future will help to facilitate the identification of the factors in control of the balance between the visual and olfactory system in the genus *Drosophila.*

Secondly, I propose that *Drosophila*-associated beneficial yeast communities may play a key role in the rapid increase of species diversity in this genus (manuscript V). Specific *Drosophila*-yeast associations are less stable and more uncommon than we intuitively presumed. Flies would instead prefer yeasts from an unfamiliar habitat over familiar, beneficial yeasts. *Drosophila* oviposition choice, in particular for the presence of specific yeast species at breeding sites, did not always correlate with benefits for larval developmental rates or survivorship. In agreement with the "bad mother" hypothesis (Mayhew, 2001), females would often prefer yeasts that would only positively affect their own performance through an optimized nutrient supply and through an enhanced fecundity. We predict that this phenomenon could allow for the colonization of new hosts and may ultimately lead to local adaptation processes. In the case that the corresponding *Drosophila* larva could utilize present yeast communities and host material to their own advantage, the local adaptation of subpopulations could favor speciation events. Thus, the species-richness and host breadth of the genus *Drosophila* could be promoted through the diversity and properties of interacting yeast communities.

In order to identify ligand-chemosensory receptor pairs that regulate and control *Drosophila* behaviors I observed in the different manuscripts of my dissertation, I wanted to establish the aptly named DREAM technique (**D**eorphanization of **r**eceptors based on **e**xpression **a**lterations of **m**RNA levels, (von der Weid *et al.*, 2015)) for use in insects, which was originally developed for olfactory research in mammals. The DREAM method is based on the phenomenon that in mammals olfactory receptors respond with an alteration in transcript levels upon extended odorant exposure at high concentrations. Instead of the traditional, time-consuming pairwise characterization of olfactory receptors and interacting ligands, the DREAM method could potentially allow for a rapid high-throughput deorphanization. However, while we observed for the majority of analyzed ligand-receptor pairs a correlation between changes in receptor transcript levels and electrophysiologically measurable ligand-receptor interaction, we also obtained false positive and false negative predictions from our DREAM applications. Furthermore, we found that parameters of the DREAM method, particularly exposure time, might have to be adjusted for different ligand-receptor pairs, which adds to the limitations of the applicability of the DREAM method in insects. As the occurrence of false predictions would require additional experiments to verify or falsify said predictions, and as a consequence of its limitations, we conclude that the DREAM technique alone is no reliable tool for a rapid, high-throughput identification of olfactory or other chemosensory receptors in insects. However, we expect that in conjunction with traditional deorphanization methods, the application of DREAM

could be utilized to narrow down possible ligand interaction partners from a bigger set of olfactory receptors.

In comparative studies within the genus *Drosophila* we can not only learn much about the neuroethology of sensory modalities in insects but also about *Drosophila* intra- and interspecies communication. Moreover, if we include additional trophic levels, such as microbial communities and hosts, we can broaden our understanding of multi-trophic interactions within entire ecological networks.

Zusammenfassung

Die erstaunlichen Fähigkeiten und Eigenschaften des Geruchssinns von Insekten faszinieren Wissenschaftler und Nicht-Wissenschaftler gleichermaßen. Öffnet man zum Beispiel eine Flasche Wein oder Fruchtsaft und gießt sich ein Glas ein, ist es sehr wahrscheinlich, dass kurz darauf *Drosophila*-Fliegen um das Getränk schwirren. Wie finden diese Insekten so schnell die Quelle ihres Interesses und welche Verbindungen im Geruchsbukett unserer Getränke helfen den Fliegen, den Standort des Glases zu lokalisieren? Wissenschaftler untersuchen seit über einem Jahrhundert die molekularen und neuronalen Mechanismen, die diesen und anderen geruchsvermittelten Insekten-Verhaltensweisen zugrunde liegen. Dabei haben die Forscher bereits viel über ökologische und evolutionäre Prinzipien gelernt, die Architektur und Funktion der neuronalen Netzwerke, welche Insektenverhalten regulieren, formen. Darüber hinaus wurden Erkenntnisse über Prozesse von der Bindung eines Geruchsmoleküls an chemosensorische Rezeptoren und der anschließenden Umwandlung des chemischen in ein elektrisches Signal bis hin zur Verarbeitung der Geruchsinformationen und der entsprechenden Verhaltensweisen gewonnen. Schon jetzt haben Fortschritte im Verständnis des Geruchssinns von Insekten zu umfangreichen praktischen Anwendungen im Kampf gegen agrarwirtschaftliche Insektenschädlinge und Krankheitsvektoren geführt. Des Weiteren ermöglicht unser bisheriges Wissen über den Geruchssinn einzelner Insekten nun die Erforschung der Kommunikation zwischen verschiedenen Insektenarten bis hin zu unterschiedlichen Phyla innerhalb ökologischer Netzwerke.

Im Rahmen dieser Dissertation habe ich an Projekten mitgewirkt, die unser Wissen über Chemosignal-vermittelte, innerartliche und artenübergreifende Kommunikation in *Drosophila* erweitern (siehe Manuskripte I und II), mit einem besonderen Fokus auf soziale Verhaltensweisen, wie Intraspezies-Aggregation und Reproduktionsverhalten, welche beide von Geruchsstoffen kontrolliert und gesteuert werden.

In einer chemischen Analyse des Kots erwachsener *Drosophila*-Fliegen, haben meine Kollegen und ich das chemische Profil dieser Fäkalien charakterisiert (Manuskript I). Wir fanden heraus, dass die Pheromon-Zusammensetzung im Fliegenstuhl dem geschlechtsspezifischen Pheromon-Profil der jeweiligen *Drosophila*-Fliege entsprach. So stellte sich heraus, dass die Fäkalien der Insekten, Pheromone in hohen Überfluss enthielten, die für die Regulation der innerartlichen Aggregation und des Sexualverhaltens verantwortlich sind. In Verhaltensexperimenten suchten Fliegen die Fäkalien von Artgenossen und eng-verwandten

Drosophila-Arten auf, zeigten sich dagegen aber weniger von dem Kot entfernter *Drosophila*-Arten angezogen. Dieses Aggregationsverhalten wurde durch den Geruchssinn der Insekten gesteuert. Wir nehmen an, dass der Insektenkot nicht nur an einer Regulierung von Aggregations- und Sexualverhalten beteiligt ist, sondern dass *Drosophila*-Weibchen den Kot weiterhin zur Markierung von Eiablage und Brutplätzen nutzen. In diesem Kontext dienen die Fäkalien möglicher Weise dazu, konspezifische Weibchen zu rekrutieren oder konkurrierende intra- sowie interspezifische Weibchen abzuschrecken. Da *Drosophila*-Fliegen zudem aber auch Fäkalien zu sich genommen haben, vermuten wir darüber hinaus, dass der Kot zusätzlich essentielle Nährstoffe für die Fliegen enthielt und eine Quelle für ein gesundes Darmmikrobiom darstellt, das zwischen den Fliegen einer Population geteilt wird. Schließlich ist es denkbar, dass die Fliegen durch ihre Fäkalien Wirtsmaterial mit nützlichen Mikroben animpfen. Diese Mikroorganismen könnten wiederum bei der Zersetzung des Wirts helfen, dabei Mikro- und Makronährstoffe freisetzen, schädliche Wirtsstoffwechselprodukte entgiften und das Wachstum von Fliegen-Pathogenen verhindern.

Weiterhin trug ich zu der Entdeckung bei, dass *Drosophila*-Krankheitserreger Pheromon-vermittelte, stereotypische Verhaltensweisen der Fliege ausnutzen können, um Übertragungsraten zu erhöhen und ihre Ausbreitung zu vergrößern (Manuskript II). Hierbei profitieren die Pathogene von der Tatsache, dass *Drosophila*-Fliegen aufgrund der vorprogrammierten Natur der jeweiligen Verhaltensweisen nicht in der Lage sind, bestimmten chemischen Signalen im Zusammenhang mit Aggregations- und Werbeverhalten zu widerstehen. Durch die Interaktion mit erkrankten Artgenossen und mit kontaminierten Fäkalien steckten sich gesunde Fliegen mit dem jeweiligen Erreger an. In Experimenten mit Wildtyp- und Mutanten Essigfliegen, die mit dem Erreger *Pseudomonas entomophila* infiziert waren, führten wir die erhöhte Pheromon-Produktion auf Veränderungen im Fettstoffwechsel der entsprechenden Fliegen zurück und zeigten eine Beteiligung des Immun- und Insulinsystems. Obwohl infizierte *Drosophila*-Fliegen große Mengen chemischer Signale produzierten, die mit Sozialverhalten verbunden sind, profitierten kranke Individuen nicht durch erhöhte Fortpflanzungserfolgsraten. Statt dessen wurden gesunde und infizierte Fliegen zu ihrem eigenen Nachteil manipuliert. In Manuskript II, zeigen wir, wie ursprünglich effektive, stereotype Verhaltensweisen, die durch nur wenige olfaktorische Kanäle und neuronale Schaltkreise gesteuert werden, zum Ziel pathogener Manipulierungen werden können. Da *Drosophila*-Fliegen jedoch eine immens erfolgreiche und artenreiche Insektengattung darstellen, nehmen wir an, dass die Vorteile dieser Reproduktionsstrategie letztendlich überwiegen.

Ein weiterer Aspekt meiner Forschungsarbeit war die Untersuchung der Rolle des Geruchssystems in der Präferenz von *Drosophila-Arten* für bestimmte Hefenarten. Dabei wollte ich verstehen, welche Faktoren die Attraktivität von Hefe-produzierten Chemosignalen bestimmen (Manuskript V). Ich untersuchte, ob *Drosophila* -Fliegen gelernt haben, bestimmte chemische Profile mit vertrauten, nützlichen Hefearten zu assoziieren, was wiederum gefestigte Fliegen-Hefe-Gemeinschaften gewährleisten würde. Auch wenn *Drosophila-Fliegen* in Verhaltensexperimenten verschiedene Hefearten auf Grund artspezifischer, chemischer Signale unterscheiden konnten, waren die Insekten nicht konsistent zu nützlichen Hefen aus einem gemeinsamen Lebensraum hingezogen. Statt dessen korrelierte in meinen Assays die Attraktivität einer Hefe mit der Anzahl an Hefe-produzierten Geruchsstoffen, die ausgewählte olfaktorische Kanäle aktivierten, welche im Gegenzug Attraktions-Verhalten auslösten. Neben olfaktorischen Signalen fundieren *Drosophila-Fliegen* ihre nämlich Präferenz für bestimmte Hefen auf zusätzliche gustatorische Hinweise, die von den Hefen freigesetzt werden. Auf diese Weise bewerten *Drosophila*-Arten die Qualität einer Hefenart auf Grundlage vielzähliger, chemischer Signale.

In Insekten bestimmen Hefe-produzierte Signale primär die Attraktivität eines Wirts und die Geruchsstoffe des Wirts selbst haben nur eine untergeordnete Rolle in der Regulierung dieses Verhaltens. In Manskript V liefere ich jedoch Beweise dafür, dass chemosensorische Signale von Hefe und Wirt synergistisch die Präferenz der Fliegen für Hefearten und Eiablageplätze beeinflussen. Darüber hinaus zeigen wir in unserer Studie, dass die Anzahl von chemischen Signalen, die von einer Hefenart produziert werden und zusätzlich eine Insekten-anziehende Wirkung besitzen, mit der Verfügbarkeit von Nährstoffen, insbesondere von Kohlenhydraten, im natürlichen Nährboden der Hefen zusammenhängt. Zukünftig wäre es interessant herauszufinden, ob generell das Wachstumssubstrat einer Hefeart die Anzahl der Chemosignale bestimmt, die von einer Hefe produziert werden können.

Ein weiteres wichtiges Themengebiet der Projekte in meiner Dissertation waren die Prinzipien, die dem enormen Artenreichtum und der adaptiven Radiation der Gattung *Drosophila* zugrunde liegen, deren Mitglieder in einem breiten Spektrum von Wirten und Lebensräumen mit insgesamt ca. 2000 Arten gefunden werden können (Markow, 2019). In Manuskript IV und V schlage ich zwei Faktoren vor, die an Artenentstehungsereignissen in *Drosophila-Fliegen* beteiligt sein könnten.

Zum einen führten wiederholte, unabhängige evolutionäre Ereignisse zum Auftreten einer inversen Beziehung zwischen den Sinnesmodalitäten des Riechens und des Sehens in

der Gattung *Drosophila* (Manuskript IV). Denn in einem Vergleich von 62 *Drosophila-Arten* beobachteten meine Kollegen und ich immer wieder, dass die Insekten entweder vergrößerte olfaktorische oder visuelle Strukturen besaβen, einschließlich der jeweiligen neuronalen Schaltkreise, aber nie beides. Die Unterschiede zwischen den geruchs- und visuellen Systemen der verschiedenen *Drosophila-Arten* waren zwischen den Arten einer monophyletischen Gruppe oder zwischen Untergruppen am größten. Wir schlussfolgerten, dass einerseits die Fliegen Energiekosten minimieren können, indem sie hauptsächlich in eine Sinnesmodalität investieren und dabei die die anderen Modalitäten untergeordnet behandeln. Anderseits ermöglicht eine Spezialisierung in olfaktorische oder visuelle Strukturen, dass ähnliche *Drosophila*-Arten überlappende Lebensräume bevölkern können und an einem gemeinsamen geographischen Ort nebeneinander leben. Die inverse Wechselwirkung zwischen dem olfaktorischen und visuellen System konnten wir zumindest teilweise auf einen begrenzten Zugriff auf Entwicklungsanlagen während der Larvenstadien zurückführen. Keine der beiden Sinnesmodalitäten kann sich in der Größe ändern, ohne die Größe der jeweils anderen Sinnesmodalität zu beeinträchtigen. In Experimenten mit Wildtyp- und Mutanten *D. melanogaster* liefern wir den Nachweis, dass bereits einzelne Genmutationen die Menge an Ommatidien und Sensillen invers beeinflussen. Wir hoffen, dass unsere Studie in der Zukunft dazu beitragen wird, die Identifizierung der Faktoren zur Kontrolle des Gleichgewichts zwischen dem visuellen und olfaktorischen System in der Gattung *Drosophila* zu *erleichtern.*

Des weiteren prognostiziere ich auf Grundlage unserer Forschungsergebnisse, dass *Drosophila*-assoziierte, nützliche Hefegemeinschaften eine Schlüsselrolle in der raschen Zunahme der Artenvielfalt in dieser Gattung spielen können (Manuskript V). Spezifische *Drosophila*-Hefe-Assoziationen sind weniger etabliert und seltener, als wir intuitiv angenommen haben. In unseren Experimenten zogen *Drosophila*-Fliegen Hefen aus unbekannten Lebensräumen vertrauten, nützlichen Hefen vor. Hierbei brachte die Entscheidung von *Drosophila*-Weibchen für bestimmte Eiablageplätze und deren Hefegemeinschaften nicht immer Vorteile für die Entwicklung ihres Nachwuchses mit sich. In Übereinstimmung mit der "bad mother" Hypothese (Mayhew, 2001) bevorzugten Weibchen oft Hefen, die nur ihre eigene Nährstoffversorgung und Fruchtbarkeit positiv beeinflussten. Wir vermuten, dass dieses Phänomen die Kolonisierung neuer Wirte ermöglicht und letztlich zu lokalen Anpassungsprozessen führen könnte. In Fällen, in denen *Drosophila*-Larven die vorhandenen Hefegemeinschaften und das Wirtsmaterial nutzen können, ist es denkbar, dass, die lokale Anpassung der Teilpopulationen Artenentstehungsereignissen begünstigt. So könnte der

Artenreichtum und das Wirtspektrum der Gattung *Drosophila* durch die Vielfalt und Eigenschaften der interagierenden Hefegemeinschaften gefördert werden.

Um Liganden chemosensorischer Rezeptoren zu identifizieren, die die *Drosophila*-Verhaltensweisen regulieren und kontrollieren, die ich in den verschiedenen Manuskripten meiner Dissertation beobachtet habe, wollte ich die in Säugetieren verwendete, sogenannte DREAM-Technik (**D**eorphanization of **r**eceptors based on **e**xpression **a**lterations of **m**RNA levels, (von der Weid *et al.*, 2015)) für eine Anwendung in Insekten etablieren. Die DREAM-Methode basiert auf dem Phänomen, dass in Säugetieren olfaktorische Rezeptoren auf eine andauernde, hochkonzentrierte Geruchseinwirkung mit einer Veränderung ihrer Transkriptionsrate reagieren. Anstelle der traditionellen, zeitaufwendigen, paarweisen Charakterisierung olfaktorischer Rezeptoren und deren interagierenden Liganden könnte die DREAM-Methode potentiell eine schnelle Deorphanisierung mit hohem Durchsatz ermöglichen. Während ich bei der Mehrheit der analysierten Liganden-Rezeptorpaare eine Korrelation zwischen Veränderungen der Rezeptor-Transkriptlevel und elektrophysiologisch-messbarer Liganden-Rezeptor-Interaktion beobachtete, erhielt ich jedoch auch falsch positive und falsch negative Vorhersagen in meinen DREAM-Anwendungen. Darüber hinaus fanden wir heraus, dass einzelne Parameter der DREAM-Methode, insbesondere die Dauer der Duftbehandlung, für verschiedene Liganden-Rezeptor-Paare, angepasst werden müssten, was zu einer beschränkten Anwendbarkeit der DREAM-Methode in Insekten führt. Das Auftreten falscher Vorhersagen würde zusätzliche Experimente erfordern, um die Vorhersagen zu verifizieren oder zu falsifizieren. Als Folge ihrer eingeschränkten Anwendbarkeit schlussfolgerte ich, dass die DREAM-Technik alleine kein zuverlässiges Werkzeug für eine schnelle, hochdurchsatzigfähige Identifizierung olfaktorischer oder anderer chemosensorischer Rezeptoren bei Insekten darstellt. Ich erwarte jedoch, dass in Verbindung mit traditionellen Deorphanisierungsmethoden die Anwendung von DREAM genutzt werden könnte, um mögliche Kandidaten für Liganden-Interaktionspartner aus einem größeren Repertoire olfaktorischer Rezeptoren zu bestimmen.

Durch vergleichende Studien innerhalb der Gattung *Drosophila* können wir nicht nur viel über die Neuroethologie der Sinnesmodalitäten von Insekten lernen, sondern auch über innerartliche- und artenübergreifende Kommunikation in *Drosophila* Spezies. Darüber hinaus können wir unser Verständnis von multitrophischen Wechselwirkungen innerhalb ökologischer Netzwerke erweitern, wenn wir zusätzliche trophische Ebenen, wie mikrobielle Gemeinschaften und *Drosophila*-Wirte mit in unsere Analysen einbeziehen.

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Declaration of independent assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich-Schiller-University, Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller-University, Jena or to any other university.

Place/Date

Signature

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