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Review

Evolution of Insect Olfaction

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Neuroethology utilizes a wide range of multidisciplinary approaches to decipher neural correlates of natural behaviors associated with an animal's ecological niche. By placing emphasis on comparative analyses of adaptive and evolutionary trends across species, a neuroethological perspective is uniquely suited to uncovering general organizational and biological principles that shape the function and anatomy of the nervous system. In this review, we focus on the application of neuroethological principles in the study of insect olfaction and discuss how ecological environment and other selective pressures influence the development of insect olfactory neurobiology, not only informing our understanding of olfactory evolution but also providing broader insights into sensory processing.

Introduction

In order to locate and evaluate food, shelter, mates, and breeding substrates as well as to avoid predators and other dangers—or simply just to move around—animals rely on a wide range of sensory systems. These senses supply the animal's nervous system with information subsequently used to generate a simplified internal representation of the complex external world, which in turn allows the animal to decide upon and execute the appropriate behavioral response given the situation. Identification and functional dissection of neural circuits underlying specific behaviors is currently a hot topic in neuroscience, an interest in part fuelled by recent methodological advances allowing for in vivo manipulation of activity from precisely defined neuronal circuits, or even from single neurons. Technical advances aside, prerequisites for these types of endeavors are (1) that the behavioral repertoire of the animal under scrutiny is understood and (2) an understanding of which external stimuli or situations cause the behavior of interest to be elicited. In principle, one needs accordingly to have at least a rudimentary grasp of the ecology of the species under study. For example, if one wishes to study innate fear in the house mouse (*Mus musculus*), it is obviously important to know what a mouse would be scared of, i.e., to know which potential dangers and predators a mouse would face in the arid regions of the northern Indian subcontinent, the evolutionary cradle of the species (Boursot et al., 1996).

The study of neuronal circuits in a behavioral context, specifically in a comparative, ecological, and/or evolutionary framework, is usually termed neuroethology. Typically, neuroethological studies are concerned with natural behaviors and are often performed in less established "model" systems. Although a species like the duck-billed platypus (*Ornithorhynchus anatinus*) might be impractical as a model overall, or offer no direct general advantage over established systems, species like this may offer unique insights with respect to specific questions, in this instance mammalian electroreception (Scheich et al., 1986). Moreover, expanding neuroscientific studies beyond established laboratory models is naturally also of importance to verify the generality of processes and functions. Comparative approaches, as in exploring a given trait with differing importance across closely related taxa, can also be an efficient way to identify the functional significance of specific features, be they genes or neurons, correlated with the trait under study. Knowing the ecology of the study animal can provide clues as to the natural context in which a given set of neurons comes into importance, and to relevant external stimuli, in turn providing access to specialized circuits underlying specific behaviors. The ecology can moreover assist in creating improved behavioral assays, better reflecting the behavioral complexity of animals operating in a natural setting, yielding improved behavioral readout possibilities.

Neuroethological approaches have provided significant insights into mechanisms underlying a wide variety of neural processes. A classic example is the auditory map of the barn owl (*Tyto alba*) (Knudsen and Konishi, 1978). The nocturnal barn owls are masters at localizing prey through auditory information and are capable of hunting in complete darkness (Payne, 1971). By recording from the midbrain, while presenting sounds akin to those an owl would encounter in its natural habitat, from various locations in space, Knudsen and Konishi managed to localize an area in the inferior colliculus, housing a set of neurons, so called space-specific neurons, which would only fire once auditory stimuli were delivered from a specific spatial position. The cells in this region were found to be organized in a precise topographic array, with cell clusters arranged to represent the vertical and horizontal location of the sound. Although the barn owl is a highly specialized animal, showing some neuronal features with respect to auditory processing not present in other brain regions or species, the owl's auditory system nevertheless relies on neural strategies for, e.g., coincidence detection and enhancement of reliability, which are probably essential for the operation of many other circuits within as well as outside the owl auditory system. The barn owl and its auditory localization pathway have also provided fundamental insights into neuronal computation and in particular how these computations are affected by experience.

The neuroethological approach is, however, not without its drawbacks. The disadvantage of working with natural behaviors is that these are indeed natural behaviors, and as such in some cases only exhibited by free-ranging animals, i.e., wild animals

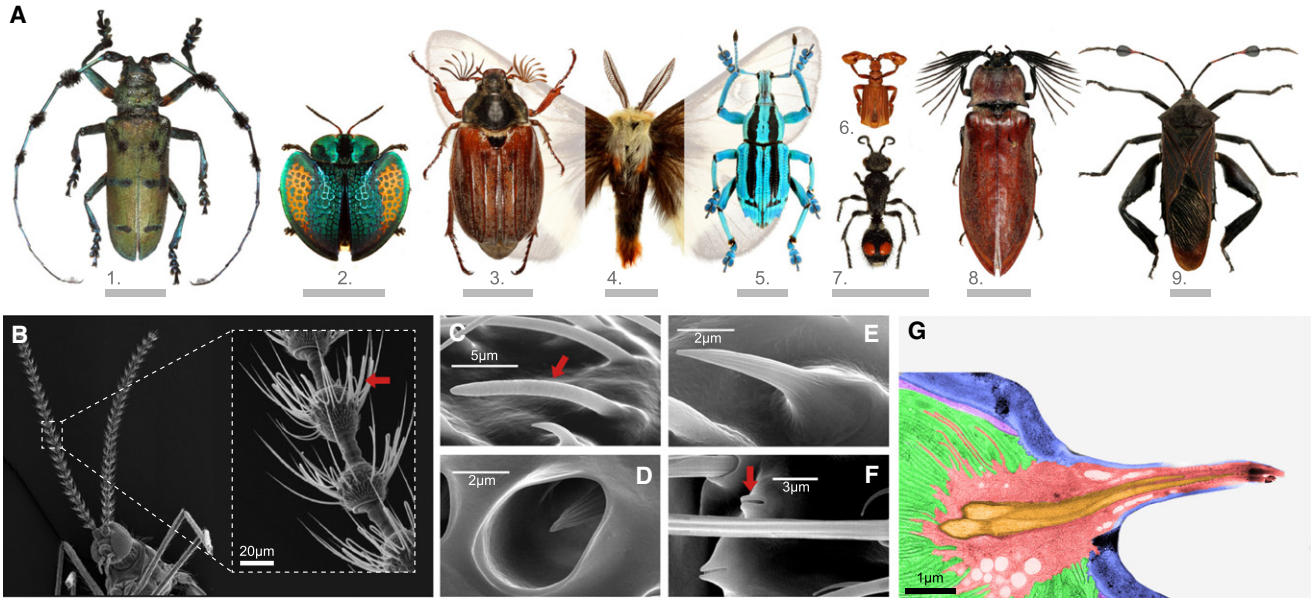

Figure 1. Diversity of Insects, Antennae, and Olfactory Sensilla

(A) Insects come in a plethora of shapes and forms. Elaborate antennal structures are found across many different insect taxa and highlight the general importance of olfaction in this group of animals. Depicted species: (1) longhorn beetle, *Diastocera wallichi* (Coleoptera; Cerambycidae); (2) tortoise beetle, Cassidini tribe, undetermined (Coleoptera: Chrysomelidae); (3) European cockchafer, *Melolontha melolontha* (Coleoptera: Scarabaeidae); (4) Nevada buck moth, *Hemileuca nevadensis* (Lepidoptera: Saturniidae); (5) snout beetle, *Euphotos bennetti* (Curculionidae), (6) Ground Beetle, undetermined (Coleoptera: Paussidae); (7) velvet ant, undetermined (Hymenoptera; Mutillidae); (8) click beetle, undetermined (Coleoptera: Elateridae); and (9) leaf-footed bug, undetermined (Hemiptera: Coreidae). Scale bar represents 1 cm. All photos by M.C.S.

(B) A male swede midge, *Contarinia nasturtii* (Diptera: Cecidomyiidae) with impressively sized antenna. Insert shows the peculiar sensilla circumfila (arrow), which are composed of multiple fused and bifurcated sensilla that form elongated loops. The s. circumfila are unique to gall midges and their functional significance remains unclear (Boddum et al., 2010). Image courtesy of Y. Hillbur, SLU Alnarp.

(C–F) Examples of principal types of olfactory sensilla, here from the antennae of the mosquitoes *Anopheles gambiae s.s.* and *An. Quadriannulatus*. (C) blunt sensilla trichodea (arrow), (D) large s. coeloconia, (E) s. basiconica, and (F) small s. coeloconica (arrow). Adapted from Pitts and Zwiebel (2006), with permission from the authors.

(G) Longitudinal section through a s. trichodea from *D. melanogaster*. Color code as per Figure 2A. Adapted from Shanbhag et al. (1999), with permission from Elsevier.

roaming their habitat. Simply observing animals in nature is often a complex task; to carefully monitor behaviors and subject these to experimental manipulation is often a herculean task. In addition, natural behaviors are typically complex composites of distinct subroutines. Even a fairly simple creature like the honeybee worker *Apis mellifera* shows a considerable behavioral repertoire, with at least 59 distinct and recognizable behaviors on the menu (Chittka and Niven, 2009). Differentiating among the behaviors and determining which stimuli elicit which behavior is in many cases challenging. Even if distinct behaviors can be discerned, monitored, and subjected to manipulation, finding the neural correlates might often be hard. Neuroscience tools readily available in established systems, such as the fly or the mouse, are in many instances not directly transferable to other species, at least not without considerable efforts. Insects, however, in spite of their minute size, display a wide span of behaviors of which most are stereotype and executed in an obligate manner pending the presentation of the correct stimulus, even in a laboratory setting. Insects in addition comprise a remarkably diverse group of organisms. Within a given family, one can often find a wide variety of lifestyles and habitats (Grimaldi and Engel, 2005), thus providing excellent entry points for comparative studies within a narrow and defined phylogenetic framework. Insects are in short ideal for neuroethological studies and have consequently also received considerable attention in this respect. In particular, insects have proven a particularly successful model in studying the sense of smell. Here we aim to review work addressing insect olfaction from a neuroethological perspective, highlighting particularly salient findings that inform our broader understanding of olfactory evolution and neurobiology specifically and sensory processing more generally. Specifically, we will cover how insects decode their chemical environment, how the peripheral olfactory system adapts and evolves, and in turn how this reflects the adaptive forces acting on the system over evolutionary time.

Environment and Function Drive Variability in Peripheral Olfactory Morphology

The sense of smell is of pivotal importance to most insects (Dethier, 1947). The importance of olfaction is evident from the elaborate antennal structures, the functional equivalents of the human nose, found in many insects. Apart from antennae, insects also detect odors with their maxillary palps and/or labial palps. The antennae (and palps) come in a multitude of shapes (Figure 1A) but nevertheless conform to the same basic principles (Schneider, 1964). The distal segment of the antennae is

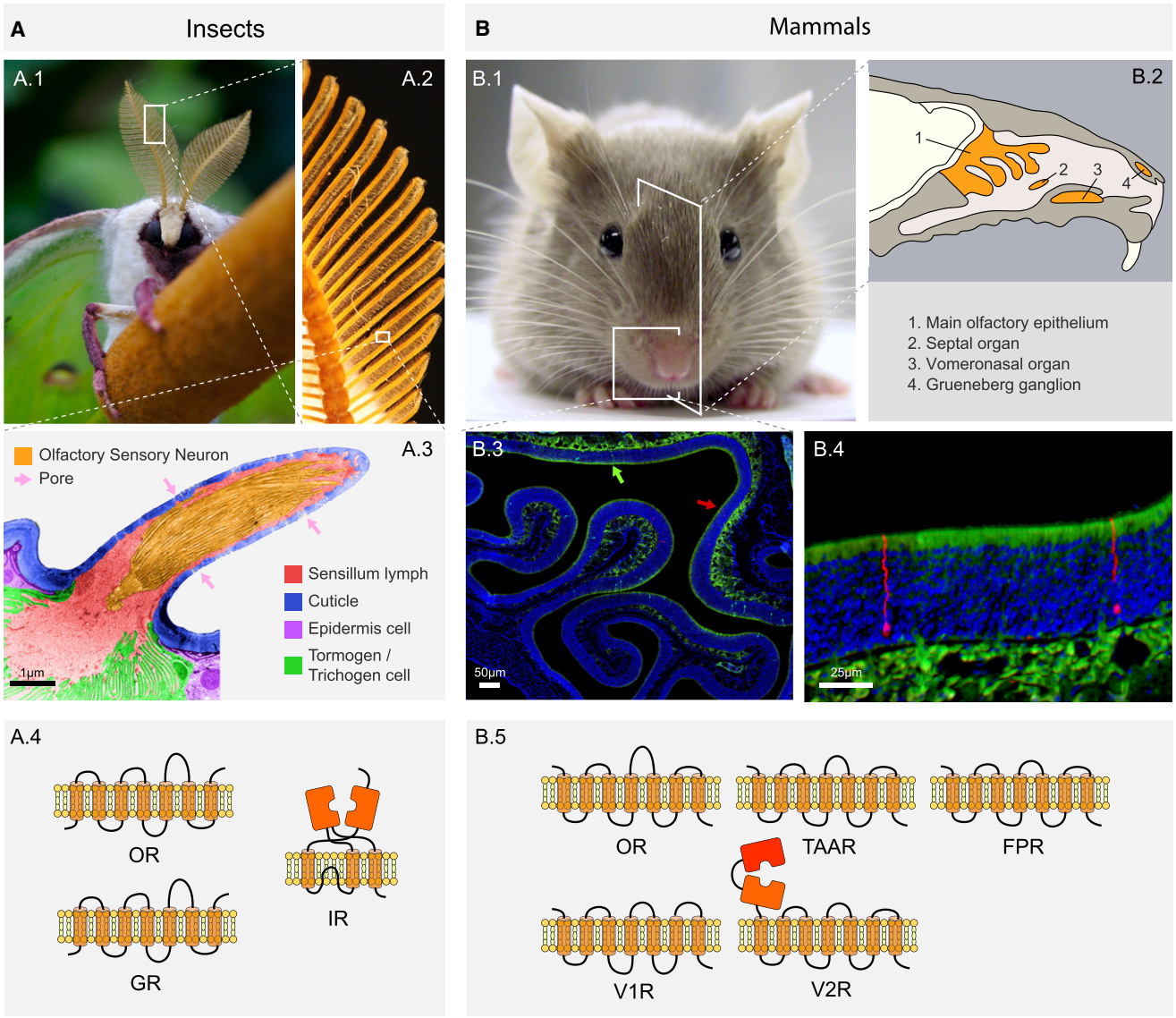

Figure 2. The Anatomy of the Insect and Mammalian Peripheral Olfactory Systems

(A) In insects, the primary olfactory detecting organs are the antennae, here illustrated by a male luna moth *Actias luna* (Lepidoptera; Saturniidae) (*A1*) (Photo: B. Bumgartner). Although not visible on this image, insects, also detect odors with their palps (shown in Figure 1B). The antennae are in several insect orders highly branched structures (*A2*) (Photo: M.M. Cordts); densely covered in olfactory sensilla (*A3*). The sensilla, here illustrated by a longitudinal section through a s. basiconica from *D. melanogaster*, houses the olfactory sensory neurons (OSN) whose dendritic part express the chemosensory receptors that binds the odor molecules. Volatile chemicals are in insects primarily detected by two different receptor types (*A4*); the odorant receptors (ORs) and the ionotropic receptors (IRs). The gustatory receptors (GRs), as the name implies, mediate taste; however, members of this gene family also detect CO_2. Image *A3* is adapted from Shanbhag et al., 1999, with permission from Elsevier.

(B) Mammals, here illustrated by Harvey the house mouse (*B1*), detect odors via multiple organs located in the nasal cavity (*B2*). The main olfactory epithelium and the septal organ detect general odorants, whereas the vomeronasal organ and the Gruenenberg ganglion primarily detect compounds mediating sexual and social behaviors. The main olfactory epithelium is a highly convoluted structure (*B3*), with the outer cell layer composed of closely packed OSNs (*B4*). Images *B3* and *B4* comes from a cross between two gene-targeted mice expressing fluorescent reporters from the M71 and the P2 odorant receptor loci respectively. Neurons expressing the M71 receptor are labeled by RFP (red arrow) and P2 expressing neurons by GFP (green arrow). Mammals rely on a variety of odor receptors, of which the vomeronasal receptor (V1R and V2R), the trace amine associated receptor (TAAR), and the formyl peptide receptor (FPR) molecules are involved in social and sexual communication and assessment. Images *B1*, *B3*, and *B4* courtesy of professor P. Mombaerts, MPI Biophysics, Frankfurt, Germany.

covered, to various extents with olfactory sensilla, which show a wide variety of shapes and structures (Schneider and Steinbrecht, 1968) (Figures 1B–1F). Irrespective of form, the olfactory sensilla all share the same function, namely, to encapsulate and protect the sensitive dendrites of the olfactory sensory neurons (OSNs) (Zacharuk, 1980) (Figure 2A). Although fulfilling the same role, the organization of the peripheral olfactory system of insects is quite different from that of mammals (Figure 2B).

The insect antennae have presumably evolved from structures that predominantly mediated mechanosensory input. In primitive

terrestrial arthropods, the antennae have great flexibility of movement due to the presence of intrinsic musculature, but owing to the small number of sensilla, quite a poor capacity for chemoreception. The sensillum-rich flagellar antennae found in most insects are, however, void of intrinsic muscles, and are in most lineages specialized structures for detecting odor molecules (Schneider, 1964). Exemptions are naturally found, such as in the aquatic water scavenger beetles (Coleoptera: Hydrophillidae), whose antennae actually lack an olfactory function altogether and instead serve as "snorkels," which are used to refill internal air reservoirs (Schaller, 1926). Whether antennal architecture is shaped by the evolutionary necessity to detect certain odor molecules is uncertain. Most likely, the variability in antennal shapes (as seen in Figure 1A) reflects constraints imposed by the physical, rather than the chemical environment of the insects. For example, the delicate plumose antennae of the volant Nevada buck moth in Figure 1A has very likely evolved to capture volatile molecules with high efficiency in air, but would be ill suited to fulfilling the same function for a ground- or soil-dwelling insect.

As to why insects are equipped with a second nose, i.e., the maxillary and/or the labial palps, remains unclear. In several insect species, including the hawk moth *Manduca sexta* (Lepidoptera: Sphingidae) and the African malaria mosquito *Anopheles gambiae* (Diptera: Culicoidae), these organs serve a distinct function as they house OSNs detecting CO_2, which in both species is a crucial sensory cue for locating resources (Thom et al., 2004; Lu et al., 2007). However, in the vinegar fly *Drosophila melanogaster* (Diptera: Drosophilidae), CO_2 detection is accomplished via OSNs on the antennae, and the palp's OSNs show overlapping response spectra with those of the antennae (de Bruyne et al., 1999). In the vinegar fly, the palps have instead been suggested to play a role in taste enhancement (Shiraiwa, 2008). How general such a function would be across insects remains to be investigated. Whether sensillum architecture confers a functional advantage in detecting a specific class of chemicals also remains unclear. Trichoid sensilla (Figure 1C) in many insects, including vinegar flies and most (if not all) moths, house OSNs tuned to pheromones (van der Goes van Naters and Carlson, 2007; Kaissling et al., 1989). However, whether the trichoid structure itself is advantageous for the detection of this type of chemicals is uncertain. Likewise, OSNs housed in coeloconic sensilla (Figure 1G) respond mostly to water-soluble amines and acids (see below), but what role, if any, the actual coeloconic architecture play is unknown.

The peripheral olfactory system of insects shows a remarkable morphological diversity at all levels. The role of this diversity remains unclear but probably reflects selection pressures for high sensitivity, phylogenetic and/or developmental constraints, and imposed by the physical environment, rather than adaptations to detect specific volatile chemicals.

The Molecular Machinery of Insect Olfaction Is Uniquely Composed

The odor molecules pass through pores or slits in the sensillum cuticle and enter the sensillum lymph (Steinbrecht, 1997). From here on, the typically hydrophobic chemicals that constitute odor ligands on land interact with members from multiple gene families, of which only two will be discussed here. The odor molecules initially bind to so-called odorant binding proteins (OBPs, Vogt and Riddiford, 1981). OBPs are secreted in large quantities by support cells surrounding the OSNs and show specific binding properties (Swarup et al., 2011). Although their exact function remains to be elucidated (but see Laughlin et al., 2008, for their role in pheromone communication), these proteins are probably involved in transporting the odor ligands to the receptor sites, situated in the dendritic membrane of the OSNs. The OBPs form a large insect-exclusive gene family with conserved structure, but which otherwise shows a high degree of sequence diversity. The OBP family is possibly as old as the insects themselves, having evolved in response to demands imposed by the conquest of land (Vieira and Rozas, 2011, but see Forêt and Maleszka, 2006). So-called odorant binding proteins are also found in vertebrates; these, however, belong to the lipocalin family and show no structural similarity to the insect OBPs (Bianchet et al., 1996). The OBP family in the vinegar fly comprises 51 members (Hekmat-Scafe et al., 2002), and similar numbers have been found in other insects so far investigated. Although subfamilies can be discerned within the OBP family, examination of these genes across broader taxonomic range reveals that the OBPs largely cluster according to phylogeny, with groupings representing independent, lineage specific radiations of specific OBPs. Clear orthologs, present across different insect orders, are hence essentially lacking (Vieira and Rozas, 2011). Analyses of the OBP repertoires from the 12 complete *Drosophila* spp. genomes have shown that in ecologically specialized species, OBPs evolve more rapidly than in their generalist relatives (Vieira et al., 2007), suggesting that OBPs play an important role in ecological diversification. In our view, elucidating the precise role of this interesting gene family should be a prioritized task for the field.

Like the OBPs, the gene family encoding odorant receptors (ORs) in insects is also an insect exclusive radiation. The ORs form a large and highly divergent gene family (Clyne et al., 1999; Vosshall et al., 1999), which shows no homology to the OR families of vertebrates and nematodes. The insect ORs and the related gustatory receptors (GRs, Clyne et al., 2000; Scott et al., 2001) together form an arthropod-specific chemoreceptor superfamily, in which the ORs constitute a single highly expanded branch (Robertson et al., 2003). Members of this superfamily essentially share no homology to any known gene family, and encodes for seven transmembrane-domain receptors with an inverted transmembrane topology as compared to the G protein-coupled olfactory receptors of vertebrates (Benton et al., 2006). In contrast to the ORs of vertebrates, the insect ORs form heteromeric complexes typically composed of a single ligand-binding OR (Störtkuhl and Kettler, 2001; Dobritsa et al., 2003, but see Goldman et al., 2005) and the OR coreceptor Orco (Vosshall et al., 2000; Larsson et al., 2004). Orco acts as a chaperone (Larsson et al., 2004) and also takes part in signal transduction (Sato et al., 2008; Wicher et al., 2008). The rise of the OR family is assumed to date back to the early Devonian and the first insects as an adaptation to terrestrial life (Robertson et al., 2003). However, one could also envision that the OR radiation occurred at a later stage (perhaps first with the rise of Neoptera); being driven by the diversification of vascular plants

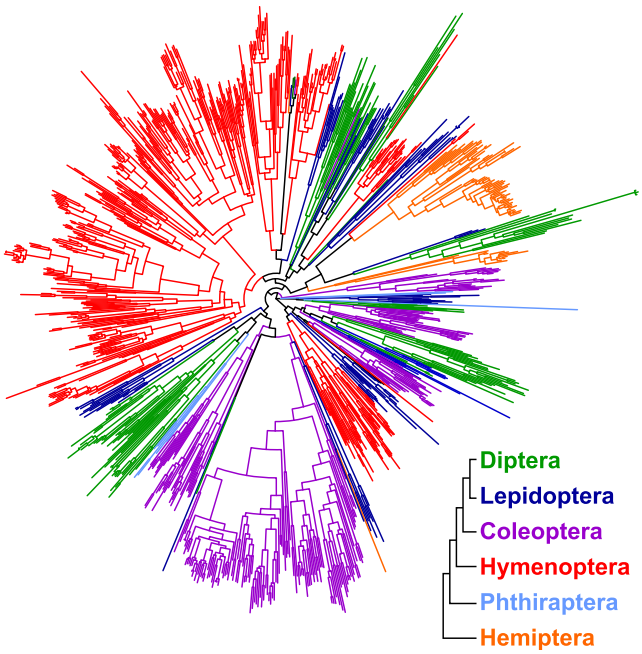

Figure 3. Phylogenetic Relationships within the Insect Odorant Receptor Family

The insect ORs forms a highly divergent gene family. With the exception of the coreceptor *Orco*, close orthologs and conserved subfamily structures are absent across insect orders. The shown tree was computed (using FastTree based on a MAFFT-nsi alignment) from 1069 OR genes from nine species belonging to six different orders.

and the increasing abundance of volatile chemicals in the environment. The latter scenario is in our view more likely.

Insect ORs form a large and highly divergent gene family, with no close orthologies (apart from *Orco*) or apparent subfamily structure conserved across insect orders Figure 3). Thus-far-identified OR repertoires range in size from ten in Phthiraptera (i.e., lice, Kirkness et al., 2010) to ~200–400 in Hymenoptera (i.e., bees, ants, and wasps; Robertson et al., 2010; Wurm et al., 2011). As with the OBPs, the OR family is characterized by species-specific expansions of single genes or gene subfamilies. Recently duplicated OR loci gain novel functions through positive selection, presumably driven by needs arising from host shifts or host specializations (see below, Gardiner et al., 2008). These processes may also render previous adaptations in the chemosensory repertoire void, resulting in the loss of OR genes that no longer serve a functional purpose. Analysis of the OR repertoires of the five closest relatives of *D. melanogaster* also revealed that in the food source specialist species examined, the OR family evolve faster as well as show a higher rate of pseudogene conversion as compared to generalist siblings (McBride et al., 2007). However, by extending the analysis to all 12 sequenced *Drosophila* species, Gardiner and colleagues (2008) found that the proportion of pseudogenized genes did not differ between the specialist and generalist taxa, whereas the endemic species showed significantly more losses than the mainland species. In their view, small effective population size and genetic drift may rather account for OR gene loss than ecological specialization. Firmly categorizing these species in terms of ecology and demography is however difficult. For example, although *D. erecta* is specialized upon fruit from *Pandanus* spp. screwpines, this resource is not continuously available in the habitat. Accordingly, this species must also utilize other resources. Moreover, *D. erecta* has a restricted and patchy distribution and may thus in fact have a small effective population size (Lachaise et al., 1988). Consequently, examining OR repertoires of additional drosophilid taxa is undoubtedly necessary before any firm conclusions can be drawn.

In short, the molecular basis of insect olfaction shows a number of unique features and is characterized by two large gene families, the OBPs and the ORs, which are presumably exclusive to this group of animals. When these two gene families first appear in the insect lineage and whether the initial conquest of land or the diversification of land plants drove their evolution remains to be determined. All insect genomes to date stem from derived orders. Deep sequencing of species from basal insect orders, as well as from allied hexapod orders is thus needed in order to understand the evolutionary history of these gene families.

The Insect Olfactory System Selectively Detects Signature Features of the Habitat

Insects have to detect specific volatile information in a very complicated chemical environment. How is this feat accomplished? In the vinegar fly and the African malaria mosquito, more or less the complete OR repertoires have been deorphaned, i.e., their key odorant stimuli have been identified. In both species, the ORs display a varying degree of specificity, with certain receptors showing a high degree of selectivity, while others respond to a broad spectrum of compounds (Carey et al., 2010, Hallem and Carlson, 2006). Response profiles of OSNs, obtained through single sensillum recordings (SSRs) from numerous other insects also suggest a spectrum of OR binding affinities. Perhaps the most well-known specialist OSNs are those detecting pheromones, where OSNs capable of separating two enantiomers with a specificity spanning over more than four decadic concentration steps have been found (Wojtasek et al., 1998). Highly specialized OSNs tuned to host volatiles have been identified from a number of insect species (e.g., Mustaparta et al., 1979; Todd and Baker, 1993; Tanaka et al., 2009). OSN response spectra are however dependent on concentration, number of, and the ecological relevance of the screened volatiles to the organism under scrutiny. Most, if not all OSNs can be triggered to respond to almost any compound if presented with high enough doses. Thus screening of volatiles at inappropriately high concentrations would give misleading results, as would a screen with a too small stimulus battery or one comprised of chemicals of no relevance to the animal as key ligands might be missing.

A solution to this problem is to use gas chromatography (GC) for stimulus delivery, which enables rapid screening of large numbers of compounds selected from the habitat and ecology of the species (Figure 4). GC-linked SSR (Wadhams, 1982) experiments indeed also suggest a very high degree of specificity of ORs across many insect species (e.g., Wibe et al., 1997; Kristoffersen et al., 2008; Ghaninia et al., 2008). In these

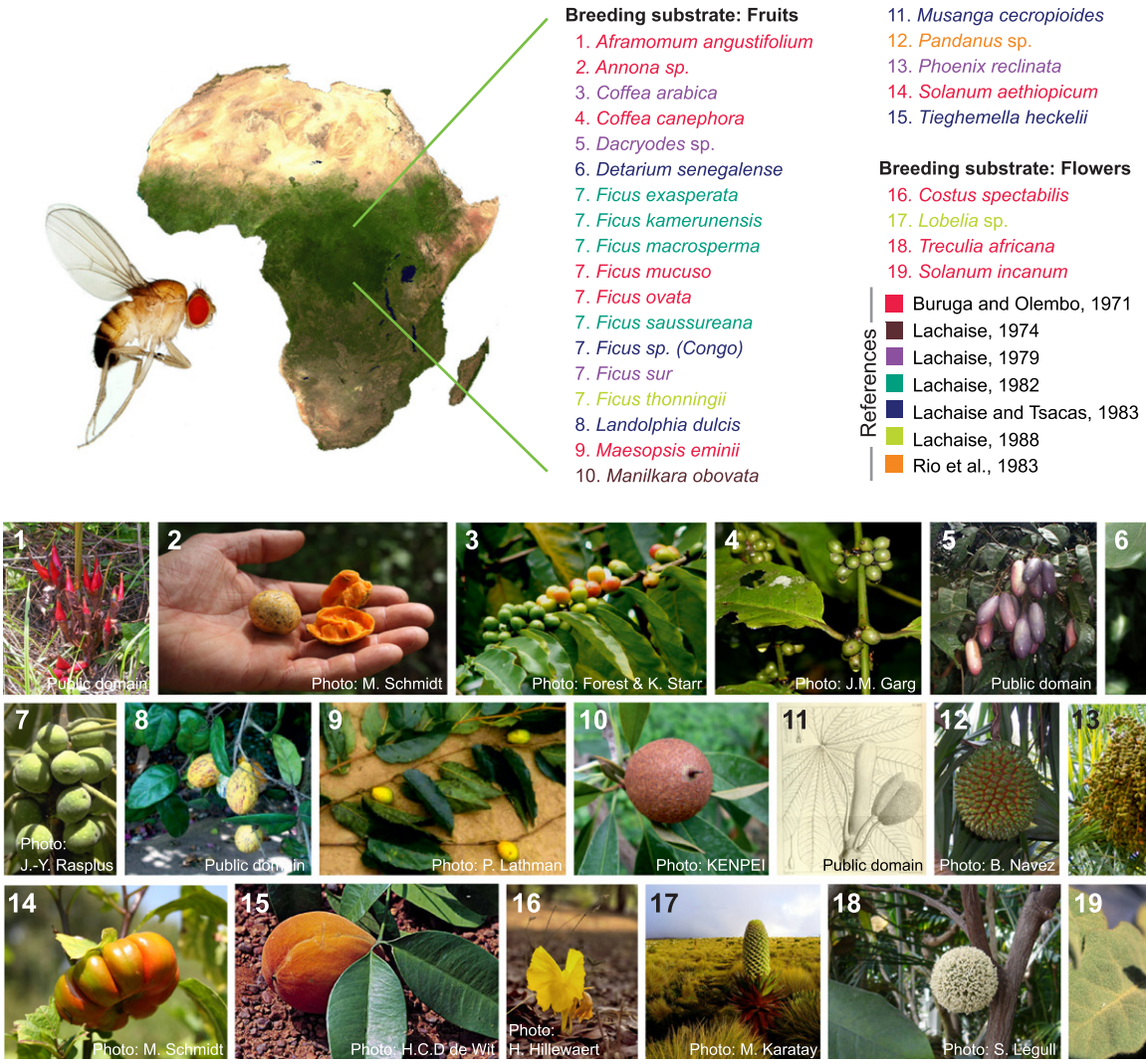

Figure 4. The Native Breeding Substrates of *D. melanogaster*

The vinegar fly, although today cosmopolitan and a human commensal has its ancestral home in east equatorial Africa. Odor ligands that the fly's nose has evolved to detect should be found among the listed hosts, of which all are native to equatorial Africa and reportedly utilized by semi-wild populations of D. melanogaster as breeding (and feeding) sites (Buruga and Olembo, 1971; Lachaise, 1974, 1979; Lachaise et al., 1982; Lachaise and Tsacas, 1983; Rio et al., 1983). Of note is the surprisingly high number of flowers used. Very few of the listed plants have had their volatile profile examined.

experiments, hundreds (or more) volatiles were screened; however, only a minute fraction produced responses, with each OSN typically responding to few compounds of structural proximity. The detected compounds also make sense in light of the examined animal's ecology. OSNs in the vinegar fly for example, which feeds on fermentative yeasts (typically from fruit), accordingly detect volatiles associated with microbial activity and alcoholic fermentation, as well as compounds, which even though more generally occurring in nature, nevertheless are typical for fruit (Stensmyr et al., 2003a). The two African scarabs *Pachnoda marginata* and *P. interrupta* (Figure 5A), which both can be found on a wide variety of flowers and rotting fruits, hence also display OSNs narrowly tuned to compounds typical of these resources (Figures 5B and 5C). The scarabs are also equipped with selective OSNs indicative of aspects representative of unsuitable and avoided objects, such as unripe fruit, foliage, and mammals. The former group of compounds elicits positive chemotaxis when screened individually, whereas the latter are either ignored or repellent (Larsson et al., 2003; Bengtsson et al., 2009) (Figure 5C). Selective OSNs detecting odorants inhibiting host attraction have also been found in many other insects, such as the spruce bark beetle (*Ips typographus*) (Andersson et al., 2009).

Assuming the fraction of insect species examined so far is representative, the ORs appears to be largely divided into those that detect chemicals specifically associated with key aspects of the host (or of unsuitable hosts) and to those that detect compounds of more general nature. The ORs tuned to specific host odors also appear to be the most selective. In the African malaria mosquito, the most narrowly tuned ORs detect volatiles

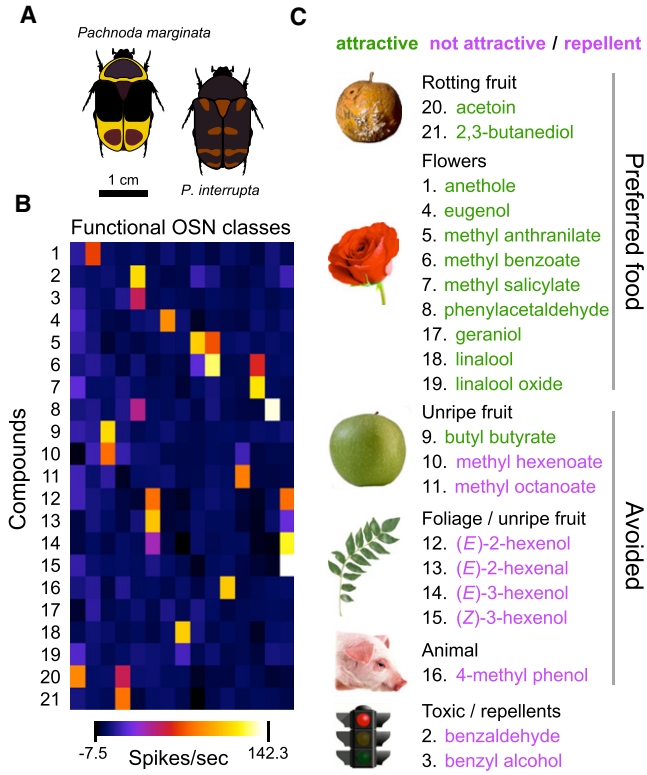

Figure 5. Scarab Noses in Tune with Their Environment

(A) The fruit chafer *Pachnoda marginata* and the sorghum chafer *P. interrupta* are native to tropical equatorial Africa and the semiarid Sahel, respectively. Both species feed on a wide variety of fruit and flowers.

(B) Response spectra of identified functional olfactory sensory neuron (OSN) classes from the two species obtained through single sensillum recordings. Most of the OSN classes are present in both species with retained function. The heatmap illustrates the high degree of selectivity of the individual OSN groups. Numbers refer to (C). Plot based on data from Bengtsson et al. (2011).

(C) Behavioral effect and the ecological correlates of the physiologically active compounds. A majority of the single compounds eliciting positive chemotaxis (green), in either field or laboratory experiments are also indicative of objects that the scarabs are known to utilize, whereas the repellent compounds (magenta) are on the other hand characteristically found in objects, which the beetles are known to avoid. Behavioral data from Larsson et al. (2003) and Bengtsson et al. (2009).

of acute biological relevance for the species, such as AgOr2 that is narrowly tuned to indole, a major component of human sweat (Carey et al., 2010). Furthermore, we can assume that ORs assigned to detect key features of what constitutes a host (or an unsuitable host), provide input to neuronal circuits mediating innate behaviors, whereas receptors tuned to more general odors likely drive behavior in a context-dependent manner. That certain nonpheromone receptors indeed mediate critical information has also been illustrated in the vinegar fly, where activation of a single OR gene is sufficient to elicit attraction toward vinegar (Semmelhack and Wang, 2009).

Insects appear to detect odors via a specific detection system, which is not configured to broadly sample chemical space, but constitutes a discriminating machinery tuned to select compounds of relevance to the animal, where each chemoreceptor has a direct ecological correlate. The mammalian olfactory system appears to show a similar level of specificity. Investigated ORs from rodents respond selectively to a small number of structurally related compounds (e.g., Araneda et al., 2000; but see Grosmaitre et al., 2009). Furthermore, GC-linked electrophysiology experiments performed in the house mouse *Mus musculus*, suggest narrow receptor tuning (Lin et al., 2006) that correlates with ecologically significant odorants, suggesting a similar evolutionary strategy shapes odorant selectivity in the mammalian olfactory system. A difference to the insect system may however be that mammalian odorant receptors to a larger extent appear to be tuned to sample select chemical features, rather than select compounds. In mammals accordingly, the identity of a specific chemical is likely to depend more on combinatorial activation of a number of ORs (Buck, 2005) than is the case in insects.

To further understand the general principles underlying insect olfactory coding, we suggest to expand the number of species investigated, particularly from poorly sampled insect orders, and to take evolutionary and ecological facts into careful consideration. When performing these experiments it is also *highly* important to use odors in relevant concentrations.

Evolutionary Trends in the Peripheral Olfactory System of Insects

How does the insect olfactory system then respond to selective pressure? A classic case of adaptation is the peripheral pheromone-detecting system often found in male insects locating mates using female-produced sex pheromones as cues. To detect the low concentrations of pheromones released by the females (often around 0.1–10 ng per hour; e.g., Lacey and Sanders, 1992), male insects have often added surface area to the antennae. This means that the antennae have become highly pectinate or multilayered leaf shaped (as seen in Figure 1A). Such an evolution can be compared to the vertebrate system, where a similar process has occurred in the nasal cavity. Animals relying heavily on high olfactory sensitivity, such as rabbits, have a highly convoluted structure (Allison and Warwick, 1949), while animals less reliant on odor information, such as humans, display much less complicated nasal cavities (Negus, 1957). All of these processes, in all types of animals, serve the basic purpose of making room for more sensor elements (OSNs), thereby enhancing capacity to detect salient environmental cues. By having more detecting neurons, the chance of capturing the information residing in an odor molecule increases. In addition to a numerical increase in detectors, male insects have also often increased the sensitivity of each detector, thereby gaining a multiplicative effect. The mechanisms behind sensitivity augmentation are still unclear, but could reside both in the number of chemoreceptors expressed on the dendritic surface and/or in the transduction mechanisms translating the signal from chemical to electrical. Analogous sexual dimorphism is also found in the other sensory modalities, such as the visual system. For example, male insects typically have both larger and morphologically more complex eyes than females (e.g., Beersma et al., 1977), reflecting the importance of visual cues for locating mates and for securing matings (Thornhill and Alcock, 1983). In certain firefly species), striking sexual dimorphism with respects to eye size is found, with one sex having

considerably enlarged eyes, and a visual sensitivity peak closely tuned to the conspecific bioluminescence flash signal (Lau et al., 2007).

Reproductive pheromone cues are not the only stimuli that can shape olfactory structure and function. For example, the fly species *Drosophila sechellia*, which is endemic to the Seychelles archipelago and a close relative to the vinegar fly, has adapted to subsist on the native *Morinda* fruit that is generally toxic to most other drosophilids. In *D. sechellia* one specific type of olfactory sensillum has been lost and instead replaced by a dramatic increase in another type of sensillum. The increased type houses OSNs tuned to the odor of the single host, whereas the lost express ORs with putative ligands not found in the fruit (Stensmyr et al., 2003b; Dekker et al., 2006). Host driven sensory augmentations are also seen in *Culex* mosquitoes. Here, the sensillum type that houses OSNs tuned to nonanal, a volatile characteristic of birds, are more numerous in ornitophilic *Culex* taxa than in mammalophilic. The OSNs in these sensilla moreover display a remarkable selectivity and sensitivity toward nonanal, on a par with or even surpassing that of pheromone OSNs found in moths. The amplified and sensitive nonanal detection system presumably provides the mosquitoes with improved long-range host detection (Syed and Leal, 2009). A high proportion of host-odor-tuned OSNs is also found in the grass-dwelling Japanese scarab beetle *Phylloperta diversa*, where the majority of the nonpheromonal olfactory sensilla contain OSNs tuned to so-called green leaf volatiles. These OSNs likewise display an extreme degree of specificity and sensitivity, and as with the mosquito, probably provide the scarab with improved long-range host detection (Hansson et al., 1999).

How these sensillum adaptations have been generated is unknown, but hints of a possible molecular mechanism involving microRNAs come from work done by Cayirlioglu et al. (2008). microRNAs are small noncoding RNA units, which posttranscriptionally suppress gene expression by binding to the nontranslated 3′ end of mRNAs of specific target genes. In the vinegar fly, loss of the microRNA, *miR-279*, which regulates expression of the transcription factor Nerfin-1, causes ectopic formation of CO_2 sensing OSNs in the maxillary palps. It is accordingly possible that other microRNAs, regulating other transcription factors are also underlying topographical reconfigurations of sensilla and OSNs of other types. Interestingly, the loss of *miR-279* creates a phenotype intermediate between that of the vinegar fly and the African malaria mosquito. If the ectopic expression of CO_2 receptors on the maxillary palps also confers a switch in behavior from repellent, as in the vinegar fly (Suh et al., 2004), to attractive, as in the mosquito (Gillies, 1980), remains unclear.

Host shifts and specialization do not however only entail increase of specific input channels but may also lead to, or even be the result of, loss of detector channels. In the fruit-piercing moth *Calyptrata thalictri* (Lepidoptera: Noctuidae), a subset of the males has been found to draw blood meals from mammalian hosts. This shift in behavior has been linked to a reduction of a specific group of OSNs tuned to repellent inducing vertebrate volatiles. Blood feeding could thus stem from a loss of innate repulsive behavior to vertebrate odors, leading to increased chance of zoophilic interactions and the opportunity to feed on blood (Hill et al., 2010). Loss of innate repulsion has also been implied as a driving force for the *D. sechellia*-noni specialization. In this case however, loss of repulsion stems from altered expression of two OBPs confined to gustatory sensilla on the legs, which have rendered *D. sechellia* taste blind to the toxic acids of its host (Matsuo et al., 2007).

Adaptations are hence observed in parts of the peripheral olfactory system that directly interfaces with key features of the species-specific host preference. However, shifts in ecology do not necessarily have to result in wide rearrangements of the olfactory system. For example, across all nine members of the *melanogaster* species group, OSNs from large basiconic sensilla have largely conserved function, in spite of these species stemming from quite a wide geographic range and occupying different habitats (Stensmyr et al., 2003b). The presence of OSNs with highly conserved function has also been observed across owlet moths with disparate ecology (Stranden et al., 2003). These core OSNs presumably detect compounds signifying key aspects of what makes up for a suitable host, regardless of the specific niche, or alternatively, detect common compounds that are of general interest. Similarly, across drosophilid flies the OR repertoire tuned to odorants connected with yeast and microbes (the staple food item of virtually all drosophilds, irrespective of detailed preference), which indeed also seems to be functionally conserved (Stensmyr et al., 2003b; de Bruyne et al., 2010; Stökl et al., 2010).

In summary, the insect olfactory system reflects the needs imposed by the taxon-specific ecology. Host shifts and specialization leads to corresponding alterations in the odor detection machinery. The adaptations noted include increase as well as decrease of select detector units. Although the olfactory systems from quite a number of insects have been examined to date, properly controlled for, comparative functional studies are actually rare. Additional examination of carefully chosen taxa of appropriate phylogenetic distance and with well-defined and contrasting ecology is accordingly needed before more solid conclusions can be drawn.

Matching the Periphery—Evolution of the Antennal Lobe

The adaptations at the antennal level are also reflected in the primary olfactory center of the insect brain, the antennal lobe (AL). The AL, homologous to the olfactory bulb of vertebrates, is composed of typically spheroid structures, called glomeruli. All OSNs expressing the same receptor converge onto one out of these usually between 50 and 200 glomeruli (Vosshall et al., 2000). The glomerulus also houses the branches of local interneurons and the dendrites of projection neurons that transmit the processed information to higher brain areas (Tolbert and Hildebrand, 1981; Distler and Boeckh, 1996). In 1924, Bretschneider was the first to report the presence of a strong sexual dimorphism in the AL; male oak eggar moths, *Lasiocampa quercus* (Lepidoptera: Lasiocampidae) displayed several enlarged glomeruli at the entrance of the antennal nerve into the AL (Bretschneider, 1924). Sixty years later, Koontz and Schneider (1987) showed that these enlarged glomeruli, termed the macroglomerular complex (MGC; Boeckh and Boeckh, 1979; Hildebrand et al., 1980) (Figure 6A), very likely served

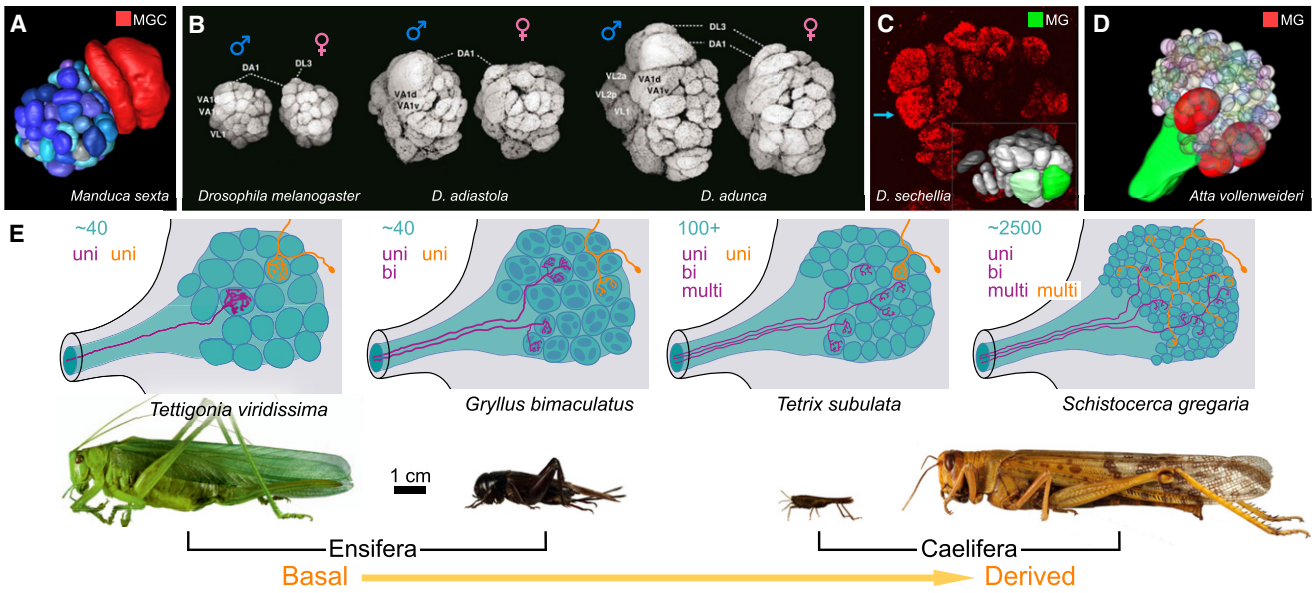

Figure 6. Evolutionary Trends in the Insect Antennal Lobe

(A) The MGC of the hawk moth *Manduca sexta* (red), showing the two main glomeruli; the cumulus at the entrance of the antennal nerve resting on the toroid, a doughnut-shaped glomerulus. Ordinary glomeruli are seen beneath the toroid in different shades of blue. Image courtesy of L. Kuebler, MPI Chemical Ecology.

(B) Although no pronounced sexual dimorphism is present in the AL of *D. melanogaster*, a range of Hawaiian drosophilids has evolved distinct male-specifc macroglomeruli, presumably processing pheromone information. Adapted from Kondoh et al. (2003), with permission from The Royal Society.

(C) Macroglomeruli have not only evolved in the context of sex pheromones, but can also be found in circuits processing host odors, as illustrated here by *Drosophila sechellia*, where the macroglomerulus process information regarding key volatiles of its sole host, the noni fruit. Adapted from Dekker et al. (2006), with permission from Elsevier.

(D) In the leaf cutting ant, *Atta vollenweideri*, three enlarged glomeruli specific to males process trail-pheromones. Adapted from Kuebler et al. (2009), with permission from Wiley-Liss, Inc.

(E) The evolutionary progression of antennal lobe morphology within the Orthoptera, from the basal suborder Ensifera to the more derived suborder Caelifera. In lower orthopterans, such as in the great green bush-cricket (*Tettigonia viridissima*), the AL looks like in most other insects; around 50 glomeruli with uniglomerular OSN projections and with uniglomerular projection neuron dendritic innervations. In the southern fieldcricket (*Gryllus bimaculatus*) each glomerulus has become divided into a number of microglomeruli, and OSNs and PNs target several of these within the same glomerulus. In the slender ground-hopper (*Tetrix subulata*) the number of glomeruli has increased substantially and OSNs target single or multiple of these, while PNs still are mainly uniglomerular. In the desert locust (*Schistocerca gregaria*) the microglomerulization has reached an extreme proportion; thousands of minute microglomeruli are packed tightly within the lobe. OSNs mostly target multiple microglomeruli, as do PNs. Schematic drawings are based on Ignell et al. (2001). Photos: M.C.S.

a purpose in receiving and processing information regarding the female sex pheromone. In 1992 Hansson et al. showed that OSNs tuned to different pheromone components target specific glomeruli of the MGC (Hansson et al., 1992). This was indeed the first clear evidence of the functional role of glomeruli as projection areas of OSNs putatively expressing the same receptor. The MGC serves as an example of how strong selection pressure, here to increase the sensitivity toward sex pheromones, can create pronounced size differences among olfactory glomeruli. Since the early 1990s a large number of moth species have been studied, and it has been shown that very often input regarding the main component of a sex pheromone mixture is processed by an enlarged glomerulus, the cumulus (e.g., Hansson et al., 1991). This MGC part can then be surrounded by a number of smaller satellite MGC-glomeruli receiving information regarding the presence of other pheromone components, or of behavioral antagonists preventing interspecific attraction (e.g., Kárpáti et al., 2008). Pheromone sensing evolved has probably evolved from simple, single-component processing, and thereby one-glomerulus MGCs, to the more complex state that we see in most species today. This means that the MGC may be created through a budding process, where new, satellite glomeruli have been added over evolutionary time. Such a process is suggested by the finding that OSNs carrying genetically similar ORs project to adjacent glomeruli in the antennal lobe of the vinegar fly (Couto et al., 2005), and a similar arrangement could be envisaged in the moth MGC. Specific factors determining glomerulus formation has been identified both morphologically (e.g., Oland and Tolbert, 1996) and molecularly (e.g., Rodrigues and Hummel, 2008). These do, however, still not provide a conclusive picture of how the glomerular array might change over evolutionary time. Interestingly, in the hawk moth and the American cockroach *Periplaneta americana* (Blattaria: Blattidae) a subdivision of the major glomerulus (the cumulus) has been observed (Christensen et al., 1995; Hösl, 1990). In both species differential innervation patterns seem to be connected to topographical representation of the antennal length axis.

Sexual dimorphism in the AL is not only restricted to the Lepidoptera. Also in drosophilid flies, sexual dimorphism with respect to specific glomeruli has been observed (Figure 6B). An investigation across 37 species of drosophilids from the Hawaiian Islands found two glomeruli enlarged in males across several of the investigated species (Kondoh et al., 2003). The

homologous glomeruli in *D. melanogaster* (the DA1 and DL3) have also been shown to receive pheromonal input (van der Goes van Naters and Carlson, 2007). A phylogenetic comparison further revealed that the noted sexual dimorphism has evolved independently in two of the lineages. Male-specific macroglomerulus/macroglomeruli have also been found in several other insect groups, such as, e.g., cockroaches, wasps (Hymenoptera: Vespidae), and bees (Hymenoptera: Apidae) (Jawlovski, 1948), but is probably a much more widespread phenomenon, having evolved wherever a need for long-distance detection of female produced volatile pheromones is present.

Other environmental selection pressures beyond pheromones, including food and oviposition site-associated odors, can also shape glomerular organization and structure. For example, the two glomeruli (DM2 and VM5d) in the fly *D. sechellia*, targeted by OSNs tuned to its singular food source, the noni-fruit, are 200% larger in both sexes relative to *D. melanogaster* (Dekker et al., 2006) (Figure 6C). Interestingly, the expansion of the noni-fruit specific detection system in *D. sechellia* not only provides higher sensitivity to the fruit odors, but it also makes the fly tolerant to much higher odor concentrations that would inhibit attraction in all other fruit flies. The mechanisms underlying this dual function are still unclear.

Similar alterations in glomerular size for odors other than sex pheromones has also been observed in workers and males of leaf-cutting ants (Hymenoptera: Formicidae), which have one and three greatly enlarged glomerulus respectively (Kleineidam et al., 2005), which presumably process trail-pheromone components (Kuebler et al., 2010) (Figure 6D). Female *M. sexta* also show two enlarged glomeruli, which are specific to a set of host plant volatiles and accordingly assumed to be involved in behaviors specific to the females, probably in locating and selecting suitable oviposition sites (King et al., 2000).

An interesting example of AL evolution is found within the order Orthoptera, which includes, e.g., grasshoppers, crickets, and wetas. When comparing the grasshopper and locust to other orthopteran insects it is clear that a strong evolutionary trend from a "normal" glomerular system with unbranched OSN axons in primitive orthopterans to a microglomerular system with branched input neurons in grasshoppers and locusts is present in the AL structure (Ignell et al., 2001) (Figure 6E). The functional significance of a system evolving from a glomerular architecture with unbranched OSNs and with most PNs targeting single glomeruli, into a system with thousands of microglomeruli innervated by highly branched OSNs and PNs is still unclear. By allowing a much more diverse interaction between OSNs and PNs such a system could potentially increase the coding capacity. The functional characteristics among orthopteran olfactory systems, however, still remain to be elucidated, and this is an area where we see progress adding significantly to our understanding of the evolution of the insect sense of smell.

In general, the insect antennal lobe offers an excellent substrate to study evolutionary processes in olfaction. Even though insects have radiated into so many different species and life forms, the antennal lobe of neopteran insects has maintained its basic architecture with incremental steps of change introduced over evolutionary time. This fact makes it possible to follow these changes and often to connect them to changes in life style. We propose intensified comparative studies of key groups, as, e.g., the orthopterans, in combination with the molecular developmental studies presently being performed in the vinegar fly. Such a combination will allow us to reach a considerably deeper understanding of evolutionary processes molding antennal lobe architecture.

Exploring Nature for Relevant Odor Ligands

To understand the relevance and significance of a given neural circuit, one needs to know the sensory stimuli that activate it. In the case of the olfactory circuitry, this initially means finding a relevant odor ligand. For the pathways mediating sexual behaviors, the ligand is typically a pheromone, and the isolation and identification of which is nowadays mostly a technical matter. Identifying odor ligands activating circuits underlying other important behaviors is however in many cases a more daunting task even if detailed knowledge of the animal's ecology is at hand. Help can however be drawn from a slightly unexpected direction, namely deceptive plants. A wide range of plant is known to trick insects into pollination without providing a reward. To accomplish this feat, these plants all rely on being able to trigger and to exploit neural circuits underlying obligate and innate attraction in the targeted insects. In short, the plants copy signals that the intended victims of the deception cannot afford to ignore. Although visual and tactile cues are in many instances important, most often the key to success resides with the plants being able to mimic odors of importance to the insects (Urru et al., 2011). Accordingly, deceptive plants can provide unique insights into what constitutes a critical resource to the targeted insect and what sensory cues mediate the attraction to this resource.

The dead horse arum (*Helicodiceros muscivorus*) and the Solomon's lily (*Arum palaestinum*) serves as excellent examples of how deceptive plants can be used to identify important odor ligands. The former produces a ghastly smell, reminiscent of rotting flesh and also attracts carrion blowflies (Diptera: Calliphoridae), the latter has in contrast a pleasant smell, similar to fruity wine and instead attracts drosophilid flies. The apparent carrion mimicry is remarkably simply accomplished, via the production of just three compounds, namely dimethyl mono-, di-, and trisulfide (Stensmyr et al., 2002). The mimicry of alcoholic fermentation is likewise accomplished via only a handful of odorants, including e.g., acetoin acetate and 2,3-butanediol acetate (Stökl et al., 2010). The deception nevertheless works since the copied odors are diagnostic for the targeted insects favored oviposition sites (i.e., decomposing animals and rotting fruit respectively), whereas they are very rarely present in other substrates. These plants hence nicely demonstrate the principle that insects rely on a select set of chemicals to localize essential resources.

Systems built on sensory deceit are thus excellent sources of information regarding key stimuli for the dupe. The mimicking flowers produce odors to which olfactory receptors in insects very likely have evolved high affinity. Having access to such ligands is of course of utmost importance when dissecting the neural function of the olfactory system, from periphery to brain, and further deepens our understanding of insect behavior. Investigations of such systems should be carefully selected among

plants duping interesting target species. Vinegar flies is a natural candidate, but, relating to our suggestions above, finding flowers that target primitive insects as pollinators would be highly valuable, as would identifying plants/flowers that could be used as deceptive traps for insects of public health (e.g., mosquitoes) and agricultural economic concern (e.g., beetles).

Conclusion

The insect olfactory system and its ability to evolve over relatively short time spans is probably an important part of the explanation why insects are such successful organisms. This success is manifested in the fact that insects have occupied almost every imaginable ecological niche. The fact that insects adapt to all these different conditions at the same time provides us with a plethora of fascinating examples of adaptations, both in the peripheral sensory organs and the brain, and it allows us to observe evolution in action. The development of sensitive peripheral detection systems seems to be important in shaping also the primary central centers. Glomeruli are added to accommodate OSNs expressing newly evolved receptor proteins, and glomeruli expand or contract as the number of OSNs expressing a certain receptor change in absolute numbers. Enigmatic architectures, such as the Orthopteran antennal lobe and its innervation do, however, still puzzle those of us studying insect olfaction and its evolution.

These differences in structure show us how relatively fast sensory systems can adapt to altered external conditions or new lifestyles. Still, however, we lack insights into how the neural circuitry, both at the micro and the macro scale, adapts to these changes. Future comparative studies must therefore make use of high-resolution techniques, combining detailed investigations of connectivity in primary olfactory centers with functional studies of the elements identified. Only then can we obtain conclusive information regarding the connection between neural function and behavior, and of the evolution of olfactory function. These kinds of data are presently being produced in the model insect, *D. melanogaster*, but we still lack any kind of detailed information from other insects. A future goal must therefore be to identify species that will provide data from both an adaptive and a phylogenetic standpoint, and use these to build a database where neuroethologically and evolutionarily relevant data can be gathered and compared.

When a system evolves toward high efficiency, it will also be highly suited to trigger innate attraction and/or repulsion. The system can be ''trusted'' to deliver reliable information regarding a resource. Such specificity also opens up for exploitation. Flowers dupe insects into doing their bidding by imitating irresistible odors. These deceptive systems offer us unique opportunities to explore how olfactory sensitivies are tuned through evolution, whereby certain odorants come to represent key behaviorally salient cues. Our aim with the present review is to generally raise awareness as to the interesting and unique cross-disciplinary neurobiological insights that can be gained from neurethological paradigms, particularly as they relate to olfaction. As is obvious from our discussion, much still remains to be discovered regarding how olfaction works and evolves, and with three million species of insects probably still not described, numerous interesting cases await to be examined. The combination of modern molecular methods, applied in model insects as the vinegar fly and the malaria mosquito, in combination with well-designed comparative studies with a well-founded phylogenetic background is what we suggest will allow us to optimize our search for deeper understanding of olfaction and its evolution. All of these investigations should always be approached with the natural behavior and habitat of the organism in mind.

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