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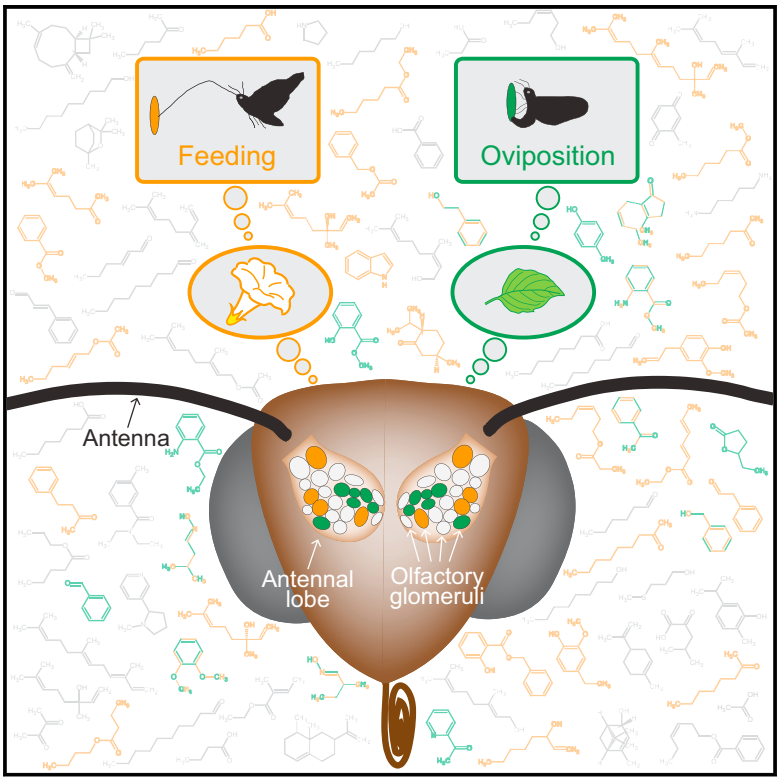
Spatial Representation of Feeding and Oviposition Odors in the Brain of a Hawkmoth

Graphical Abstract



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In Brief

The hawkmoth, *Manduca sexta*, feeds from and lays eggs on plants. Bisch-Knaden et al. show that feeding and oviposition can be elicited by monomolecular, plant-derived odorants and that odor-evoked neural activity in distinct olfactory glomeruli is correlated with these types of behavior.

Highlights

- Using 80 odors, we establish a functional atlas of a hawkmoth's antennal lobe
- We reveal the behavioral significance of these 80 odors in a wind tunnel
- Feeding and oviposition correlate to the activity of distinct olfactory glomeruli

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Spatial Representation of Feeding and Oviposition Odors in the Brain of a Hawkmoth

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SUMMARY

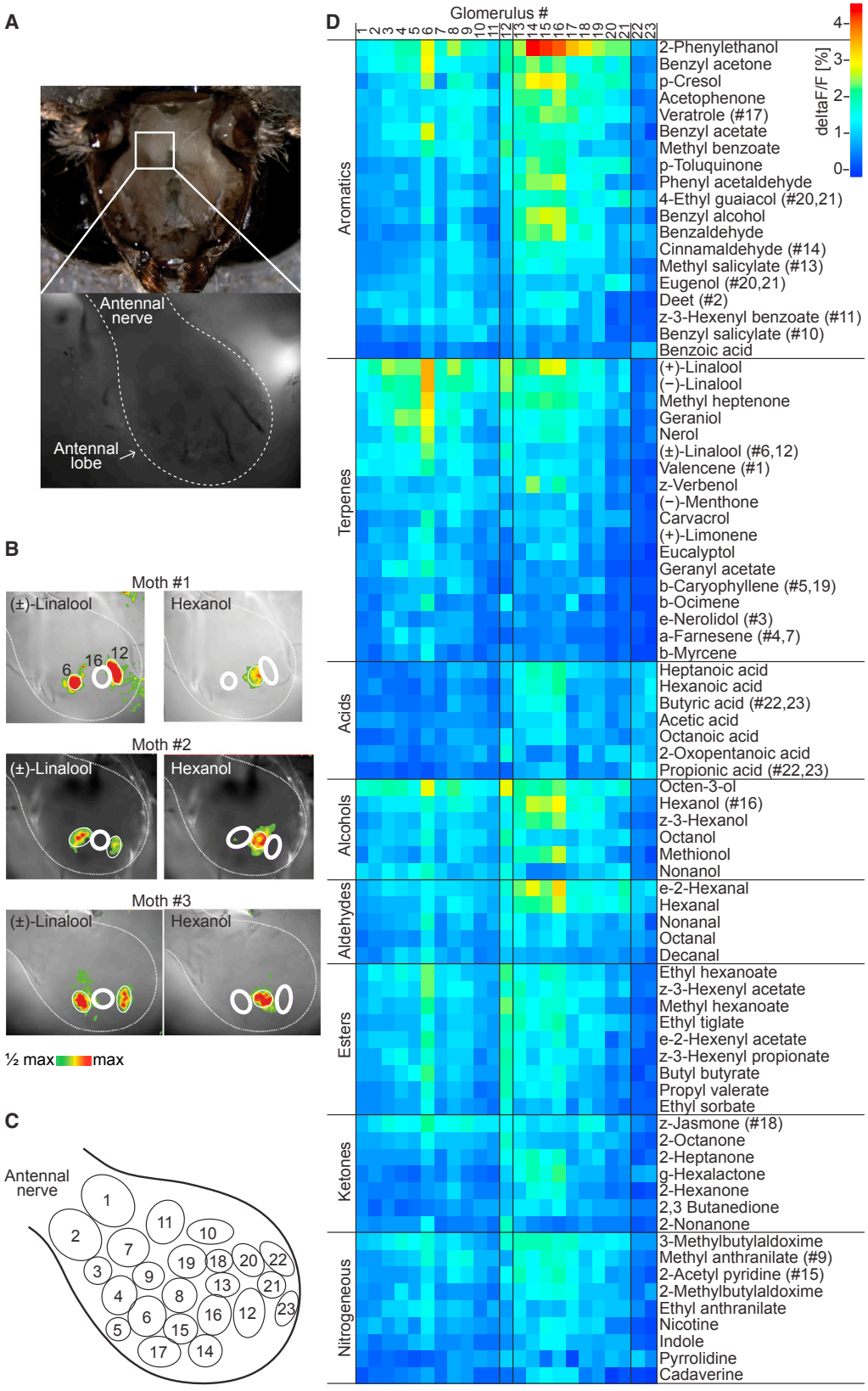
Female hawkmoths, *Manduca sexta*, use olfactory cues to locate nectar sources and oviposition sites. We investigated if the behavioral significance of odorants is represented already in the antennal lobe, the first olfactory neuropil of the insect's brain. Using *in vivo* calcium imaging, we first established a functional map of the dorsal surface of the antennal lobe by stimulating the moths with 80 ecologically relevant and chemically diverse monomolecular odorants. We were able to address 23 olfactory glomeruli, functional subunits of the antennal lobe, in each individual female. Next, we studied the relevance of the same odorants with two-choice experiments (odorant versus solvent) in a wind tunnel. Depending on odorant identity, naive moths made attempts to feed or to oviposit at the scented targets. A correlation of wind tunnel results with glomerular activation patterns revealed that feeding and oviposition behaviors are encoded in the moth's antennal lobe by the activation of distinct groups of glomeruli.

INTRODUCTION

Insects, like many other animals, use their sense of smell to locate ecologically relevant targets from a distance. Olfactory cues play an important role for nocturnal insects, like the hawkmoth *Manduca sexta*, that forage, mate, and oviposit from dusk to dawn. These moths feed on floral nectar from solanaceous plants, including jimson weed (*Datura wrightii*) and coyote tobacco (*Nicotiana attenuata*), which also serve as host plants for the herbivorous caterpillars. The adult moths also use flowers from non-hosts (e.g., Palmer's century plant [*Agave palmeri*]) as nectar sources (Alarcon et al., 2008). Hence, the life history of *M. sexta* is closely associated with different plant species, and it has been shown that its behavior to a large part is governed by olfactory cues (Haverkamp et al., 2016b; Kessler and Baldwin, 2007; Raguso and Willis, 2002; Riffell et al., 2008; Späthe et al., 2013b). Odorants are often innately attractive or aversive, thereby helping the animal to decide, for instance, to approach or avoid a potential food source or oviposition site. Odorants with a specific valence activate spatially distinct regions in higher brain centers of mammals (Rolls et al., 2003) and insects (Strutz et al., 2014), but do so even at the first processing levels, for example, the olfactory bulb in the mouse (Kobayakawa et al., 2007), or the antennal lobe of the vinegar fly (Knaden et al., 2012). Here, we investigated how different plant-derived odorants contribute to decision-making in this moth and if we find neuronal correlates of these decisions in the first olfactory processing center, the antennal lobe.

Being similarly organized, both the olfactory bulb of mammals and the antennal lobe of insects have a species-specific number of spherical structures, so-called olfactory glomeruli. Each glomerulus receives input from a subpopulation of olfactory sensory neurons expressing the same type of olfactory receptor. Hence, each glomerulus constitutes a functional subunit of the antennal lobe, and the number of glomeruli roughly reflects the number of olfactory receptors expressed in that species (Gao et al., 2000; Vosshall et al., 2000). The incoming olfactory information is modulated by local interneurons that connect to sensory neurons and to projection neurons (Heinbockel et al., 2013; Reisenman et al., 2011), the latter conveying the information to higher levels of the olfactory pathway. Studies with vinegar flies have shown that a single odorant activating a single, narrowly tuned glomerulus can mediate innate attraction or aversion (Ebrahim et al., 2015; Stensmyr et al., 2012). However, a concerted action of several input channels is commonly required to control the fly's behavior (Badel et al., 2016; Kreher et al., 2008). Odorants usually activate several glomeruli implicating a combinatorial coding strategy of olfactory information (Malnic et al., 1999).

In *M. sexta*, the antennal lobe comprises ~70 glomeruli (Grosse-Wilde et al., 2011) arranged in a single layer around a central fiber core. After opening the head capsule, the neural representation of odorants at the dorsal surface of the antennal lobe (i.e., in ~35 glomeruli) can be studied simultaneously using *in vivo* calcium imaging (Galizia et al., 1999). A bath-applied fluorescent calcium sensor allows monitoring of neural activity in the exposed brain area on stimulation of the antenna with odorant puffs. In this way, odorant-specific spatial patterns of glomerular activity can be recorded (Bisch-Knaden et al., 2012; Hansson et al., 2003). As all cell types in the antennal lobe are monitored by using a bath-applied calcium sensor, the question arises of whether the observed activation patterns reflect the response of input or



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output neurons. Each glomerulus in the antennal lobe of female *M. sexta* receives input via 4,000–5,000 olfactory sensory neurons (Oland and Tolbert, 1988). The number of projection neurons per glomerulus, however, is only four to five (Homberg et al., 1988; King et al., 2000). This convergence ratio of about 1,000:1 between input and output neurons strongly suggests that the activation patterns reflect rather the activity of incoming sensory neurons than the activity of projection neurons. The response of ~360 local interneurons, most of them arborizing in many if not all glomeruli, might additionally contribute to neural activation patterns (Homberg et al., 1988; Reisenman et al., 2011).

The volatiles from hawkmoth-visited flowers (Loughrin et al., 1990; Raguso et al., 2003a; Raguso et al., 2003b; Riffell et al., 2009a) as well as the odorants emitted by vegetative parts of host plants (Fraser et al., 2003; Reisenman et al., 2013; Späthe et al., 2013b) have been thoroughly investigated. Plant volatiles have been shown to attract moths when presented as a blend of several compounds (Fraser et al., 2003; Riffell et al., 2009a), but single chemicals can also be attractive or repellent (Haynes et al., 1991; Kessler and Baldwin, 2007; Morgan and Lyon, 1928). In our study, we analyzed the activity patterns evoked by these ecologically relevant floral and vegetative odorants in the antennal lobe of female moths and included additional odorants to cover a comprehensive area of chemical space. We next tested the behavioral relevance of the same odorants in a two-choice behavioral assay (odorant versus solvent) to determine their innate meaning for *M. sexta*.

By establishing a functional map of the antennal lobe using a set of diagnostic odorants, we were then able to correlate the odorant-evoked activation level of each glomerulus with the observed valence of the same odorant. Previous studies have given emphasis to a temporal coding mechanism of complex olfactory information by performing simultaneous recordings from a mixture of local interneurons and projection neurons in restricted areas of the antennal lobe (Riffell et al., 2009a; Riffell et al., 2009b). Although calcium imaging does not inform us about the temporal details of the neural responses, it provides a view on the spatial representation of monomolecular odorants across the full dorsal glomerular array at the sensory input level. By this means, we were also able to draw conclusions about the molecular receptive range of receptor neurons targeting these glomeruli. Furthermore, we could identify glomeruli whose activation was correlated with feeding behavior, and we found other glomeruli whose activation was correlated with oviposition behavior.

RESULTS AND DISCUSSION

Neural Representation of Odorants in the Brain of *M. sexta*

We recorded *in vivo* neural activation patterns in the antennal lobe of female moths that were stimulated with monomolecular odorants. Eighty stimuli were chosen to represent a wide range of ecologically relevant and chemically diverse odorants (Table S1). Such activation patterns are odorant specific and reproducible between animals (Bisch-Knaden et al., 2012). However, we could not directly align activity spots to olfactory glomeruli, because a thick neurolemma that prevents visible glomeruli contours in the living animal covers the antennal lobe in *M. sexta* (Figure 1A). To identify individual glomeruli and to be able to describe the molecular receptive range of their innervating olfactory sensory neurons, we chose odorants with a characteristic activation pattern, one or two consistent activity spots per diagnostic odorant (Figure 1B), and created a schematic of putative olfactory glomeruli in the antennal lobe (Figure 1C). The position of each activity spot in this schematic was defined by its position relative to other activity spots (Soucy et al., 2009). With the help of 19 diagnostic odorants (Table S1), we were able to identify 23 putative glomeruli that could consistently be recognized in the antennal lobe of each individual. That is, two-thirds of the glomeruli at the dorsal surface of the lobe have been identified in our study. The remaining dorsal glomeruli might be narrowly tuned to odorants that have not been tested here, and therefore could not be addressed.

Animal preparations were stable enough to present the diagnostic odorants in each animal to create an individual glomerular schematic and to test ~20 additional odorants. There was a correlation between the physicochemical properties of the 80 odorants and their neural representations as has been shown for vinegar flies, bees, tadpoles, and rats (Haddad et al., 2008): the more similar two odorants are in chemical space, the more similar was the representation of these two odorants in the antennal lobe of *M. sexta* ($r = 0.4$, $p < 0.0001$, Mantel test). This highly significant correlation let us assume that the identified 23 activity spots in the antennal lobe corresponded to 23 olfactory glomeruli.

In 60.2% of the 1,840 odorant-glomerulus combinations, the neural activation level was low (Figure 1D, blue cells in heatmap), a medium response (cyan to orange cells) was found in 39.2% of the cases, and a strong response (red cells) was observed in 0.6% of odorant-glomerulus combinations. Strongly responding glomeruli were mainly located in the central part of the antennal lobe, indicating that glomeruli close to the margins and at the

Figure 1. Odor-Evoked Activity Patterns in the Antennal Lobe of *M. sexta*

(A) Picture of a moth with an opened head capsule and exposed antennal lobes prepared for *in vivo* calcium imaging (photo by Marco Schubert). *Enlarged inset*, the right antennal lobe under fluorescent illumination; glomerular boundaries are not visible.

(B) Examples of calcium imaging recordings from the right antennal lobe after stimulation with 2 of 19 diagnostic odorants in three different females. The increase of fluorescence is color coded (see scale) and superimposed onto the view of the antennal lobe; the entrance of the antennal nerve is in the upper left corner. White dotted line, outline of the antennal lobe; white circles, positions of activity spots; numbers, glomerulus identification. See (C).

(C) Schematic of 23 activity spots at the dorsal surface of the right antennal lobe (same orientation as in A and B).

(D) Activity in 23 glomeruli (columns) after stimulation with 80 odorants (rows). Each cell in the heatmap represents the median response of a glomerulus (after subtraction of the solvent response) to an odorant (numerical values are given in Table S2). Horizontal lines separate chemical classes; vertical lines separate functional clusters of glomeruli (see Figure S1B). Numbers in parentheses next to odorant names depict glomeruli that could be identified with the help of the respective diagnostic odorant. See also Figure S1.

entrance region of the antennal nerve were partly masked by overlying glomeruli or the antennal nerve, respectively. Neural representations of odorants were often more similar within a chemical class than between chemical classes (Figure S1A). Aromatics, for example, evoked significantly different activation patterns than terpenes, corresponding to previous imaging studies with small sets of odorants (Bisch-Knaden et al., 2012; Hansson et al., 2003). Moreover, a hierarchical cluster analysis revealed four functional groups of glomeruli (Figure S1B). Glomeruli belonging to the same functional group were located close to each other (Figure S1C). Thus, the neural representation of odorants in the antennal lobe of *M. sexta* seems to be partially chemotopic, which is consistent with findings in honeybees (Sachse et al., 1999). However, a chemotopic representation is still under debate in vinegar flies (Couto et al., 2005; Hallem and Carlson, 2006) and mice (Soucy et al., 2009; Uchida et al., 2000).

To further validate the assignment of glomeruli, we intended to compare the observed glomerular response profiles with results obtained by other electrophysiological methods. Yet, in *M. sexta* females, there are only two glomeruli whose response characteristics have previously been described via intracellular recordings from uniglomerular output neurons: the female-specific lateral large female glomerulus (LFG) (King et al., 2000) and one glomerulus next to the lateral LFG (Reisenman et al., 2005). The latter glomerulus is located deep in the antennal lobe and therefore could not be accessed in our functional imaging study. The lateral LFG, in contrast, can be addressed by its size and because of its distinct location at the entrance of the antennal nerve into the antennal lobe (Rössler et al., 1998). Output neurons associated with the lateral LFG are mainly tuned to linalool, with a higher response to (+)-linalool than to (−)-linalool (Reisenman et al., 2004). In our imaging study, glomerulus #2 was located at a similar position in the lobe as the lateral LFG and also responded more strongly to (+)-linalool than to (−)-linalool (median response: 2.0 versus 1.2, n = 11 moths, p = 0.019, Wilcoxon matched-pairs test). Furthermore, there was a good correlation between the rank order of responses to eight odorants from three chemical classes that were tested in both studies (r = 0.86, p = 0.007, Pearson correlation). Hence, based on its similar location and its characteristic response profile, glomerulus #2 might coincide with the lateral LFG.

Sensory neurons that express the same type of olfactory receptor usually target the same glomerulus (Gao et al., 2000). That is, the response spectrum of each olfactory sensory neuron is correlated to the response spectrum of one of the olfactory glomeruli in the antennal lobe. In extracellular recordings from sensilla on the *M. sexta* antenna, however, it is difficult to separate responses from collocated sensory neurons within a sensillum (Kaissling et al., 1989; Späthe et al., 2013b). Still, olfactory sensory neurons could be identified that responded mainly to aromatics or terpenes or that were broadly tuned to odorants from several chemical classes (Ghaninia et al., 2014; Shields and Hildebrand, 2001). Accordingly, in the present imaging study, we found glomeruli responding predominantly to aromatics (e.g., #20), terpenes (e.g., #4), and also broadly tuned glomeruli (e.g., #6). On the other hand, the receptive range of glomeruli #22 and #23 could not be related to any neuron or sensillum type described in *M. sexta* (Ghaninia et al., 2014; Shields and Hildebrand, 2001; Späthe et al., 2013b). Up to now, single sensillum recordings in *M. sexta* have only been done with trichoid and basiconic sensilla, the most abundant and accessible types on the antenna. There are, however, two more antennal sensillum types with an olfactory function: auricillic and coeloconic sensilla (Shields and Hildebrand, 1999a, 1999b). The response spectrum of auricillic sensilla in other moth species is diverse, including plant volatiles and sex pheromone components (Ansebo et al., 2005; Pophof et al., 2005), and is therefore difficult to match with our results. Neurons housed in coeloconic sensilla, in contrast, are stereotypically tuned to acids, aldehydes, and amines in moths (Pophof, 1997; Pophof et al., 2005), as well as in other insect species like vinegar flies (Yao et al., 2005), dragonflies (Piersanti et al., 2014), and cockroaches (Altner et al., 1977). Thus, glomeruli #22 and #23, which were activated by acids, aldehydes, and, in the case of #22, also by an amine, are presumably targeted by olfactory sensory neurons housed in coeloconic sensilla. These mutual findings, together with the correlation of neural representations of the odorants and their physicochemical properties, support the validity of the glomeruli identified in our study.

Testing the Valence of Odorants in Two-Choice Experiments in the Wind Tunnel

Knowing the activity patterns odorants evoked in the antennal lobe of the moths, we next addressed how these odorants might affect the behavior of the animals in a wind tunnel (Figure 2A). During the 3 min after taking flight, we recorded the duration of contacts individual moths made at each of two targets: a filter paper with the diluted test odorant and a filter paper with the solvent only. We tested 20 moths per odorant and observed 12 ± 4 moths (mean ± SD) contacting at least one of the filter papers in each of the 80 experimental series. Contacts were made either with the tip of the unrolled proboscis (10 ± 4 moths/experimental series), or with the tarsi (3 ± 4 moths/experimental series), respectively. Both types of behavior occurred while the moth was hovering in front of the upright filter paper. Subsets of females contacted the targets exclusively with their tarsi when tested with a given odorant (22% ± 16% of the overall number of contacts/experimental series) or showed both behaviors alternately (16% ± 13%). Most frequently, however, moths made proboscis contacts only (62% ± 20%).

Unrolling the proboscis and contacting a target indicates a feeding attempt (i.e., the moth mistakes the filter paper for a rewarding flower). To reveal the effect of a visual, unscented stimulus, we provided both filter papers with solvent. In this control experiment, 13 out of 20 moths contacted at least one of the filter papers with their proboscis, showing that the visual stimulus alone, a small white circular object, is sufficient to attract foraging moths from a distance (Haverkamp et al., 2016a). However, the median duration of those proboscis contacts at an unscented target was only 1 s (Figure S2A). Adding an attractive odorant to a visual stimulus extends the contact duration (Raguso and Willis, 2002). Accordingly, we calculated for each moth the net duration of proboscis contacts with the odorant (contact duration at the odorant source minus contact duration at the solvent control) as a measure of the odorant's

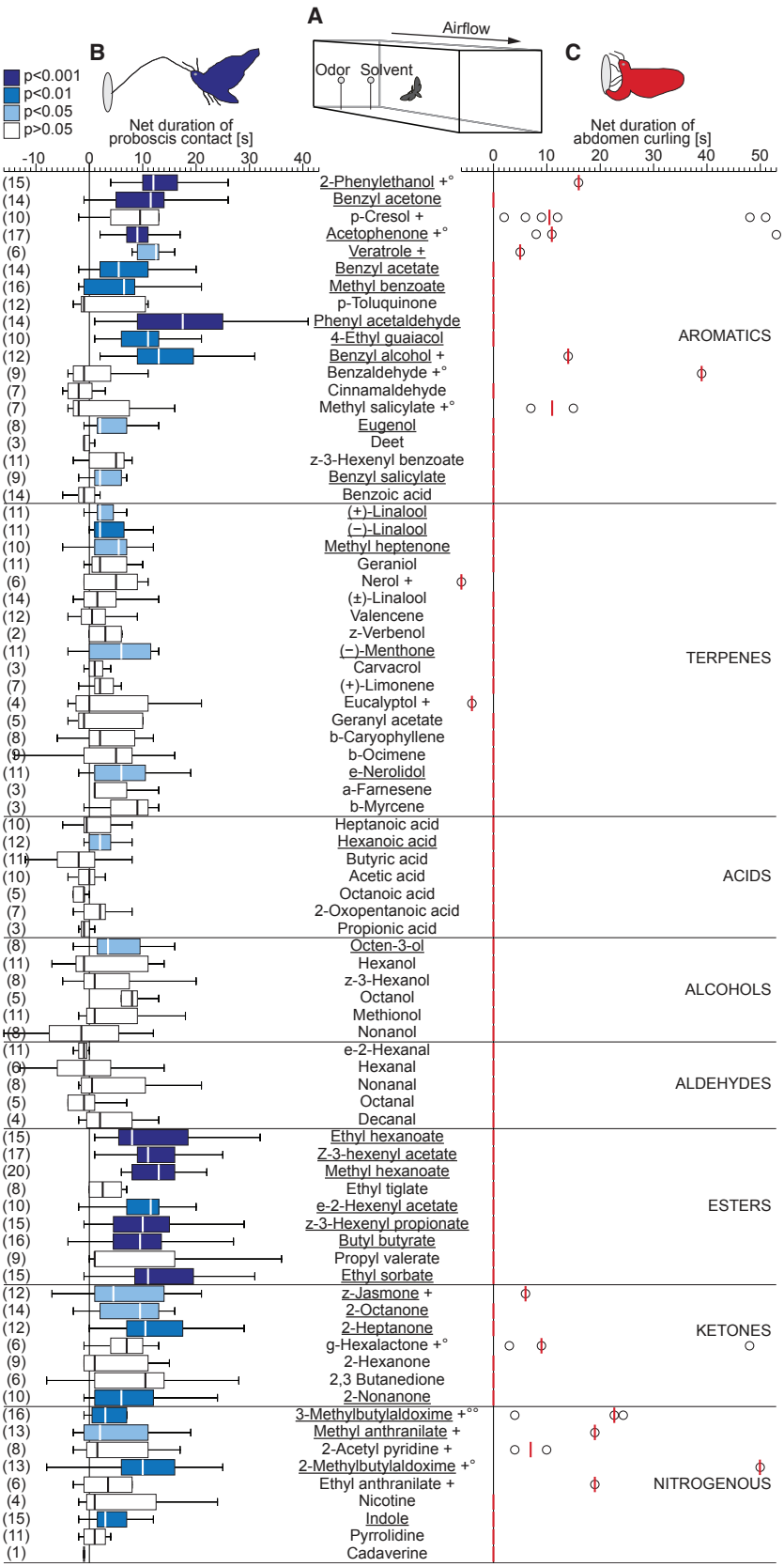


Figure 2. Odor-Mediated Behavior of *M. sexta* in a Two-Choice Experiment

(A) Schematic of the wind tunnel (2.5 m × 0.9 m × 0.9 m). Naive, starved, 3-day-old females were released individually, and their behavior at two filter papers (6 μL of diluted odorant [1:10^2 in solvent] versus 6 μL of solvent) was recorded for 3 min after takeoff; n = 20 moths/odorant.

(B) Proboscis contacts. Box plots depict the median net proboscis contact duration for each experimental series (vertical line in the box) and the 25th and 75th percentiles (left and right margins of the box) together with minimum and maximum values (whiskers) (outliers not shown). Filled boxes, net contact durations different from zero (Wilcoxon matched-pairs test). Numbers in brackets, number of moths contacting at least one of the two filter papers with the proboscis.

(C) Tarsal contacts with a curled abdomen. Open circles, net duration of abdomen curling contacts in individual moths; vertical red lines, median net values for each odorant.

Net durations of contacts were calculated as contact time at the odorant minus contact time at the solvent. Underlined odorant names, significant proboscis contacts (B); +, tarsal contact with a curled abdomen (C); °, °°, one or two moths laid at least one egg on the scented filter paper. See also Figure S2.

attractiveness (Figure 2B). Odorants were regarded attractive if they had positive net contact durations significantly different from zero.

Using this criterion, 41% of the tested odorants (33/80) had an innate positive valence for foraging moths (Figure 2B, odorant names underlined). The most attractive odorants were esters and several aromatic odorants. The median net contact duration at attractive odorants from these two chemical classes was 11 s. Some of the ketones, nitrogenous compounds, and terpenes were also attractive. However, these odorants evoked a median net contact duration of only 4.5 s.

To compare our results obtained with monomolecular odorants with the behavior a moth would show when confronted with a more natural (i.e., complex) olfactory stimulus, we tested behaviorally active subsets of the complete flower scents of two important nectar sources: *D. wrightii* (three components; 90% benzyl alcohol) and *A. palmeri* (six components; 91% ethyl sorbate). It has been shown that foraging *M. sexta* do not discriminate between these reduced blends and the respective full flower bouquets, which contain more than 60 components each (Riffell et al., 2009a). In our wind tunnel assay, both flower mimics were attractive for female moths. Notably, there was no difference in attractiveness between a flower mimic and its major component (Figure S2B). Furthermore, the observed contact duration at filter papers scented with these attractive odorants corresponds to the probing time of *M. sexta* at real flowers (Haverkamp et al., 2016b; Riffell et al., 2008). Taken together, these findings indicate that hungry moths evaluate single odorants as promising as a complete flower bouquet.

Consistent with this result, monomolecular odorants were described to attract both sexes of numerous moth species belonging mainly to the noctuid family (Dötterl et al., 2006; Haynes et al., 1991; Landolt et al., 2014; Meagher, 2001), but also hawkmoths, including *M. sexta*, are strongly attracted by single compounds in laboratory and field studies (Kessler and Baldwin, 2007; Morgan and Lyon, 1928; Scott and Milam, 1943). Male *M. sexta*, in contrast to our findings with females, were not attracted by single odorants but only by odorant mixtures when tested in a wind tunnel (Riffell et al., 2009a). This differing observation might be based on methodical constraints or depicts another example of sex specificity in hawkmoth foraging strategies (Alarcon et al., 2010; White et al., 1994).

The most attractive chemicals in our behavioral tests were aliphatic esters. These compounds (e.g., ethyl sorbate and butyl butyrate) are rare floral volatiles (Knudsen et al., 2006) and are not present in typical hawkmoth-pollinated flowers like *Datura* and *Nicotiana* (Knudsen and Tollsten, 1993). Esters, however, are characteristic for *Agave* flowers (Riffell et al., 2008), which offer a prolonged and nectar-rich resource in the semi-arid grassland habitat of *M. sexta* in southern Arizona (US). There, *Agave* pollen was the most abundant proboscis pollen load of wild male and female *M. sexta* across different years, showing that moths heavily exploit these flowers (Alarcon et al., 2008; Riffell et al., 2013). Furthermore, two-choice experiments in the laboratory revealed that this attraction to *Agave* is innate, as more than 90% of naive moths preferred paper flowers emitting the floral scent of *A. palmeri* to unscented paper flowers (Riffell et al., 2008), which is in line with our results using ester-scented filter papers.

Long proboscis contacts also occurred in tests with aromatic compounds like benzyl alcohol, benzyl acetone, and methyl benzoate, as well as with nitrogenous aldoximes. These odorants are signature chemicals for the scent of many hawkmoth-pollinated flowers (Knudsen and Tollsten, 1993), making them obvious candidates for attractive stimuli. Like the scent of *Agave*, the scent of *Datura* is innately attractive for foraging *M. sexta* (Riffell et al., 2008). However, not every floral volatile proved to be attractive, which was especially apparent in the case of terpenes. Although two-thirds of the most common floral volatiles belong to this chemical class (Knudsen et al., 2006), moths were significantly attracted by only 5 out of 18 tested single terpene compounds with much shorter proboscis contacts than in experiments with esters or aromatics. A higher impact of aromatics compared with terpenes was also reported for butterflies, as none of 14 tested terpenes but 8 out of 16 aromatic compounds elicited strong proboscis extension reflexes across all three investigated nymphalid species (Ômura and Honda, 2009). Terpenes might, however, be important constituents of attractive floral mixtures (Riffell et al., 2009a) considering the robust activity some of the terpenes evoked in the antennal lobe of *M. sexta* (Figure 1D).

In experiments with 17 odorants (some acids, alcohols, aldehydes, and aromatics), moths spent slightly more time at the control target than at the scented target. However, none of the odorants had a negative median net contact duration that was significantly different from zero (i.e., none of the tested odorants could be classified as aversive). This result indicates that our behavioral paradigm allowed the identification of attractive odorants but was not suitable for the detection of repellent odorants, as the baseline set by the control target was too low (Figure S2A).

Odorants Eliciting Oviposition Behavior

Whenever moths made tarsal contacts with the filter paper, they kept their proboscis rolled up, indicating that the moths did not try to feed. This behavior, therefore, has to be seen in a different context. In the control experiment with two unscented filter papers, 6 out of 20 moths contacted at least one of the targets with their tarsi. Thus, the visual stimulus alone could evoke tarsal contacts, but with a lower frequency than proboscis contacts (13/20 moths). Furthermore, tarsal contacts occurred in experiments with almost all odorants at least once, but were less frequently observed than proboscis contacts (4 ± 3 moths with tarsal contacts/experimental series versus 10 ± 4 moths with proboscis contacts/experimental series). Usually, moths had a straight abdomen while grabbing the filter paper with their tarsi. However, some moths markedly curled their abdomen (Figure 2C, odorant names marked with +), most of them solely at the odorant side. Tarsal contacts with a curled abdomen lasted much longer than tarsal contacts with a straight abdomen (median net duration/experimental series: 13 s versus 1 s, $p < 0.0001$, Mann-Whitney *U* test). Curling of the abdomen usually precedes oviposition, but virgin moths as well show this behavior whenever they are able to contact the leaves of a suitable host plant (Mechaber et al., 2002). In our behavioral assay, several of the moths, although virgin, even laid eggs at the scented target (Figure 2, odorant names marked with °). We therefore assume that the observed behavior was related to

oviposition. Abdomen curling at the scented filter paper did not occur randomly across odorants but was observed only in experiments with odorants belonging to three chemical classes (aromatics, ketones, and nitrogenous compounds), indicating that these monomolecular compounds are innately associated with oviposition.

Some plant species are used as a nectar source and others as an egg-laying substrate by female moths (Gabel, 1992) or butterflies (Stanton, 1984). Because post-mating nectar feeding doubles the number of fertile eggs in hawkmoths (Schmidt-Busser et al., 2011), plants that simultaneously provide floral nectar and an appropriate oviposition substrate, like *Datura* or *Nicotiana*, are especially beneficial (Alarcon et al., 2010) and might emit volatiles that are innately attractive in both contexts. Accordingly, we found that key odorants of *Datura* and *Nicotiana* (aromatics and aldoximes) were attractive feeding cues and that 6 out of 13 attractive aromatics and aldoximes also evoked abdomen curling, emphasizing the significance of these odorants in the behavioral ecology of *M. sexta*. *Agave* flowers, on the other hand, are an even more rewarding nectar source than *Datura* flowers (Riffell et al., 2008), yet hawkmoths do not oviposit on *Agave* plants (Alarcon et al., 2008). Consequently, *Agave*-specific esters were very attractive feeding cues, but abdomen curling never occurred at ester-scented filter papers. A similar although reversed phenomenon has been described in the vinegar fly: a repulsive feeding cue is preferred by female flies for oviposition (Joseph et al., 2009). The odorants that elicited abdomen curling were not as effective as shown for the complete host plant bouquet (Mechaber et al., 2002) because abdomen curling occurred only at low frequencies (maximal, 30%). In line with this finding, a recent study showed that the unaltered bouquet of a host plant is required for the choice of oviposition sites in *M. sexta* (Späthe et al., 2013a), indicating that oviposition, in contrast to foraging, might depend more on complex blends than on single compounds.

Linking Brain Activity to Odor-Directed Behavior

Moths showed clear odor-guided behavior when tested with monomolecular odorants in the wind tunnel and contacted a scented filter paper with proboscis or with tarsi and a curled abdomen. Although a small subset of attractive feeding odorants also elicited abdomen curling, most odorants were effective in only one behavioral context. Therefore, feeding-related and oviposition-related behaviors might be represented in different subsets of glomeruli in the antennal lobe. We first tested this hypothesis by comparing odor-evoked activation levels of individual glomeruli (Figure 1D) with the median net duration of proboscis contacts moths made at the same odorants (Figure 2B). In most of the cases, there was no correlation between the two datasets. For four glomeruli, however, we found a highly significant positive correlation; that is, the more these glomeruli were activated, the longer the moth contacted a scented filter paper with its proboscis (Figure 3A). Three of these "feeding glomeruli" were located at the entrance and the lateral region of the antennal lobe, and the fourth glomerulus was in a more medial position (Figure 3B). Next, we examined the coding of oviposition behavior, and found that the activation levels of six glomeruli were positively correlated with the median net duration of abdomen curling contacts (Figure 3C). These "oviposition glomeruli" were mostly located in medial parts of the antennal lobe (Figure 3D). Strikingly, we found only glomeruli whose activity was significantly correlated with feeding or with oviposition, but never with both behaviors. Moreover, we found this link between brain activity patterns and behavior, although we monitored only 33% of all glomeruli present in the antennal lobe of *M. sexta*, which is in line with studies in honeybees (24% of all glomeruli; Guerrieri et al., 2005) or vinegar flies (38%; Knaden et al., 2012).

Studies dealing with the coding of olfactory valence in insects are usually based on the comparison of attractive and aversive feeding cues (Knaden et al., 2012; Kreher et al., 2008). Our study revealed that another essential behavior, oviposition, could also be correlated to the activation of a distinct group of olfactory glomeruli. The lateral LFG and neighboring glomeruli have been suggested to be involved in the detection of oviposition sites in *M. sexta* (King et al., 2000; Reisenman et al., 2004), whereas another study using single sensillum recordings with subsequent retrograde staining in female cabbage looper moths (Todd and Baker, 1996) suggested that this region of the antennal lobe processes attractive feeding odorants. In line with the latter findings, our results favor a role of glomeruli located in the entrance area of the antennal lobe in feeding but not in oviposition behaviors (Figure 3).

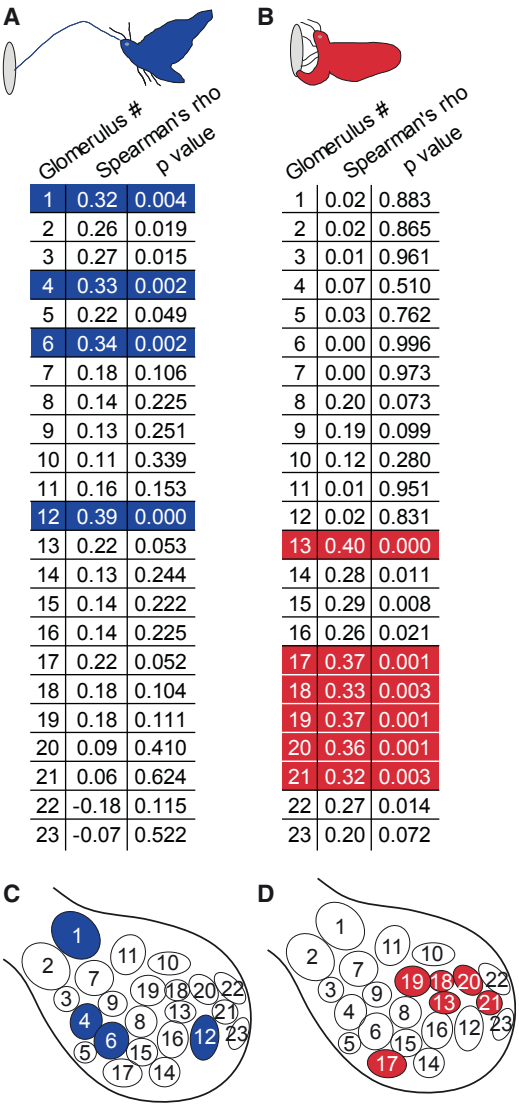
In vinegar flies, a segregated representation of attractive and repellent foraging odorants exists (Knaden et al., 2012). Different from our findings in moths, the medial part of the fly's antennal lobe codes for attractive foraging odorants, whereas the lateral part processes aversive olfactory stimuli. Thus, the position of glomeruli responding to positive feeding cues seems not to be conserved in insects. Furthermore, the representation of olfactory valence in flies is weak at the level of olfactory sensory neurons and becomes significant only at the level of output neurons (Knaden et al., 2012) in contrast to our results in *M. sexta*, as we monitored mainly the activity of input neurons. However, representation of behavioral meaning already at the first olfactory processing level is not restricted to insects but was found also in mice, where, like in *M. sexta*, olfactory valence was already coded at the input level of the olfactory glomeruli (Kobayakawa et al., 2007).

Our study provides two comprehensive resources: a functional atlas of an insect's antennal lobe based on calcium imaging experiments with 80 odorants and the innate behavioral significance of each of these odorants. Furthermore, we found two distinct sets of glomeruli whose activation levels were either correlated with feeding or with oviposition behavior.

EXPERIMENTAL PROCEDURES

Study Species

M. sexta (Sphingidae) larvae were reared in the laboratory on an artificial diet (Grosse-Wilde et al., 2011). Female pupae were kept in a climate chamber (25°C, 70% relative humidity [RH]) with a reversed light cycle (8 hr dark: 16 hr light), and moths were tested up to 3 days after hatching (calcium imaging experiments) or at day 3 after hatching (wind tunnel experiments), respectively. Moths were starved, virgin, and had no experience with plant-derived volatiles.



Glomerulus #	Spearman's rho	p value
1	0.32	0.004
2	0.26	0.019
3	0.27	0.015
4	0.33	0.002
5	0.22	0.049
6	0.34	0.002
7	0.18	0.106
8	0.14	0.225
9	0.13	0.251
10	0.11	0.339
11	0.16	0.153
12	0.39	0.000
13	0.22	0.053
14	0.13	0.244
15	0.14	0.222
16	0.14	0.225
17	0.22	0.052
18	0.18	0.104
19	0.18	0.111
20	0.09	0.410
21	0.06	0.624
22	-0.18	0.115
23	-0.07	0.522

Glomerulus #	Spearman's rho	p value
1	0.02	0.883
2	0.02	0.865
3	0.01	0.961
4	0.07	0.510
5	0.03	0.762
6	0.00	0.996
7	0.00	0.973
8	0.20	0.073
9	0.19	0.099
10	0.12	0.280
11	0.01	0.951
12	0.02	0.831
13	0.40	0.000
14	0.28	0.011
15	0.29	0.008
16	0.26	0.021
17	0.37	0.001
18	0.33	0.003
19	0.37	0.001
20	0.36	0.001
21	0.32	0.003
22	0.27	0.014
23	0.20	0.072

Figure 3. Linking Brain Activity Patterns to Behavior

(A and B) Correlation of each glomerulus' activity (see Figure 1D) and (A) feeding-related behavior (net duration of proboscis contacts; see Figure 2B) and (B) oviposition-related behavior (net durations of tarsal contacts with a curled abdomen; see Figure 2C). Correlation coefficient (Spearman's rho) and p value are given for each glomerulus; rows with a blue (A) or red (B) background highlight glomeruli with significant correlations after adjusting the significance level for multiple comparisons (modified after Bonferroni-Holms).

(C) Schematic of the antennal lobe showing the location of glomeruli whose activation level was positively correlated with feeding behavior (blue glomeruli).

(D) Schematic of the antennal lobe showing the location of glomeruli whose activation level was positively correlated oviposition behavior (red glomeruli).

Preparation for Calcium Imaging Experiments

Moths were pushed in a 15-mL plastic tube with the tip cut open. The head was protruding at the narrow end and was fixed in this position with dental wax. Labial palps and proboscis were also fixed with wax to reduce movement artifacts during the experiments. A window was cut in the head capsule between the compound eyes, and the tissue covering the brain was removed until the antennal lobes were visible. The membrane-permeant form of a fluorescent calcium indicator (Calcium Green-1 AM, Invitrogen) was dissolved in physiological saline solution (Christensen and Hildebrand, 1987) with 6% Pluronic F-127 (Invitrogen) to a concentration of 30 μmol. Twenty μL of this dye solution was applied to the exposed brain, and the preparation was incubated in a wet chamber for 45 min at room temperature. Then, the brain was rinsed several times with physiological saline solution to remove excess dye, and the moths were stored at 4°C overnight. Imaging experiments were performed the following day.

Calcium Imaging

The imaging setup consisted of a CCD camera (Olympus U-CMAD3) mounted to an upright microscope (Olympus BX51WI) equipped with a water immersion objective (Olympus, 10×/0.30). Calcium green-1 acetoxymethyl (AM) was excited at 475 nm (500 nm shortpass (SP) optical filter; xenon arc lamp, Polychrome V, Till Photonics), and fluorescence was detected at 490/515 nm (dichroic longpass/longpass [DCLP/LP]). The set-up was controlled by the software Tillvision version 4.6 (Till Photonics). Four-fold symmetrical binning resulted in image sizes of 344 × 260 pixels,with one pixel corresponding to an area of 4 μm × 4 μm.

Odorant Stimulation

We tested 80 monomolecular odorants (Table S1). Six microliters of the diluted odorants were applied onto a circular piece of filter paper (diameter: 12 mm, Whatman); 6 μl of solvent served as a control stimulus. Filter papers were inserted into glass pipettes and were renewed every day. The immobilized moth was placed upright under the microscope. A glass tube was directed to one antenna (diameter: 5 mm; ending 10–15 mm from the tip of the antenna) to deliver a constant stream of clean, moistened air (0.1 l/min). Two glass pipettes were inserted through small holes in the tube. One pipette (inserted 5.5 cm from end of tube) was empty and added clean air to the continuous airstream (0.5 L/min). This airstream could automatically be switched (Syntech Stimulus Controller CS-55) to the second pipette (inserted 3.5 cm from the end of tube) that contained an odorant-laden filter paper. By this procedure, the airstream reaching the antenna was not altered during odorant stimulation, thus reducing mechanical disturbances.

One odorant stimulation experiment lasted 10 s and was recorded with a sampling rate of 4 Hz corresponding to 40 frames. The time course of an odorant stimulation experiment was as follows: 2-s clean airstream (frame 1–8), 2-s odorant airstream (frame 9–16), and 6-s clean airstream (frame 17–40). Odorants were presented with at least 1-min interstimulus interval to avoid adaptation. Nineteen diagnostic odorants (Table S1) were tested in each animal plus ~20 other odorants. The sequence of stimulations changed from animal to animal. Stimulations with the solvent were presented at the beginning, in the middle, and at the end of an experimental series.

Processing of Calcium Imaging Data

Stimulation experiments resulted in a series of 40 consecutive frames that were analyzed with custom written software (IDL, ITT Visual Informations Solutions). Several processing steps were applied to enhance the signal-to-noise ratio: (1) background correction: background activity was defined as the average fluorescence (F) of frames 3–7 (i.e., before stimulus onset) and was subtracted from the fluorescence of each frame. This background-corrected value (deltaF) was divided by the background fluorescence to get the relative changes of fluorescence over background fluorescence for each frame (deltaF/F); (2) bleaching correction: the fluorescent bleached slowly during the exposure to light, and therefore, we subtracted from each frame an exponential decay curve that was estimated from the bleaching course of frame 3–7 and frame 26–40 (i.e., just before and after stimulus and response); (3) median filtering: a spatial median filter with a width of 7 pixels was applied to remove outliers; and (4) movement correction: possible shifts of the antennal lobe from one stimulation experiment to the next one were corrected by aligning frame 20 of each experiment to frame 20 of the median experiment in a given animal. The outline of the antennal lobe and remains of tracheae served as guides for this movement correction procedure.

Increased neural activity, indicated as an increase of the intracellular calcium concentration on odorant stimulation, was leading to spatially restricted spots of increased fluorescence in the antennal lobe. In the center of each

activity spot, the average deltaF/F was recorded in an area the size of a small to medium-sized glomerulus (60 μm × 60 μm). Time traces of deltaF/F were averaged over three successive frames for each activity spot. In these smoothed time traces, the maximum deltaF/F after stimulus onset was determined. The average of the maximum value and the value before and after the maximum was calculated and was defined as the response of the animal to the odorant stimulation at the given activity spot.

Analysis of Activity Patterns in the Antennal Lobe

For each animal, an individual schematic of activity spots (Figure 1C) was established by analyzing the activation patterns evoked by diagnostic odorants, leading to 23 spots that could consistently be identified. Responses in these 23 putative glomeruli were calculated for all stimulations in a given animal, and the average responses evoked by the solvent stimulations were subtracted. In this way, the median net response evoked by an odorant in each glomerulus could be calculated across different animals (Figure 1D). The median sample size was n = 15 moths per odorant; n = 66 animals were tested in total.

Behavioral Experiments in the Wind Tunnel

To investigate the behavioral significance of odorants, we performed two-choice tests in a wind tunnel (Figure 2A; 2.5 m × 0.9 × 0.9 m, 25°C, 70% RH, 0.3 lux, wind speed: 40 cm/s). Before the experiment, individual moths were transferred in cylindrical mesh cages (13 cm × 14 cm) from their rearing chamber to an acclimatization chamber (25°C, 70% RH, 0.3 lux), where they stayed for at least 1 hr. At the upwind end of the wind tunnel, two upright acrylic glass poles (40 cm high) were placed with a distance of 40 cm between them. A round filter paper (diameter: 35 mm) was fixed at the top of each pole, with 6 μL of diluted odorant ($1{:}10^2$) or solvent alone pipetted to the center of each filter. To control for the effect of a visual cue alone, both filter papers were provided with 6 μL of solvent. An individual female was put on a platform (40 cm high) at the downwind end of the tunnel where it started to fan its wings for 1–2 min before taking off. Moths were allowed to fly in the wind tunnel for 3 min, and their behavior close to the two filter papers was filmed (Sony Handycam DCR-SR35E, night shot mode). We analyzed the duration and type of contacts (Figure 2) displayed at the targets. Moths were tested only once (n = 20 moths/odorant, n = 1660 moths in total). Filter papers were renewed for each trial, and the positions of odorant and solvent were swapped after every second moth.

Statistical Analysis

Statistical tests were performed with PAST (Paleontological Statistics, http://folk.uio.no/ohammer/past/), Instat (GraphPad Software, La Jolla, CA, US) and XLSTAT (Addinsoft, New York, NY, US) at a significance level of $\alpha = 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.01.082.

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AUTHOR CONTRIBUTIONS

S.B.-K., S.S., M.K., and B.S.H. designed the research. S.B.-K. performed and analyzed the calcium imaging experiments. S.B.-K. and A.D. performed and analyzed wind tunnel experiments. S.B.-K. wrote the original draft. All authors revised and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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