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Olfactory ecology and the processing of complex mixtures

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Natural olfactory stimuli typically are mixtures of which the identities, concentrations, and ratios of chemical constituents are important for many odor-mediated behaviors. Despite abundant behavioral examples, links between odor-evoked behavior and the processing and discrimination of complex olfactory stimuli remains an area of active study. Coupling electrophysiological and behavioral experiments, recent studies in a variety of different insect models have provided new insights into the perceptual and neural mechanisms about how natural olfactory stimuli are processed, and how plasticity and internal state of the insect may influence the odor representation. These studies show that complex stimuli are represented in unique percepts that are different from their individual constituents, and that the representation may be modulated by experience and influenced by other sensory modalities.

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Introduction

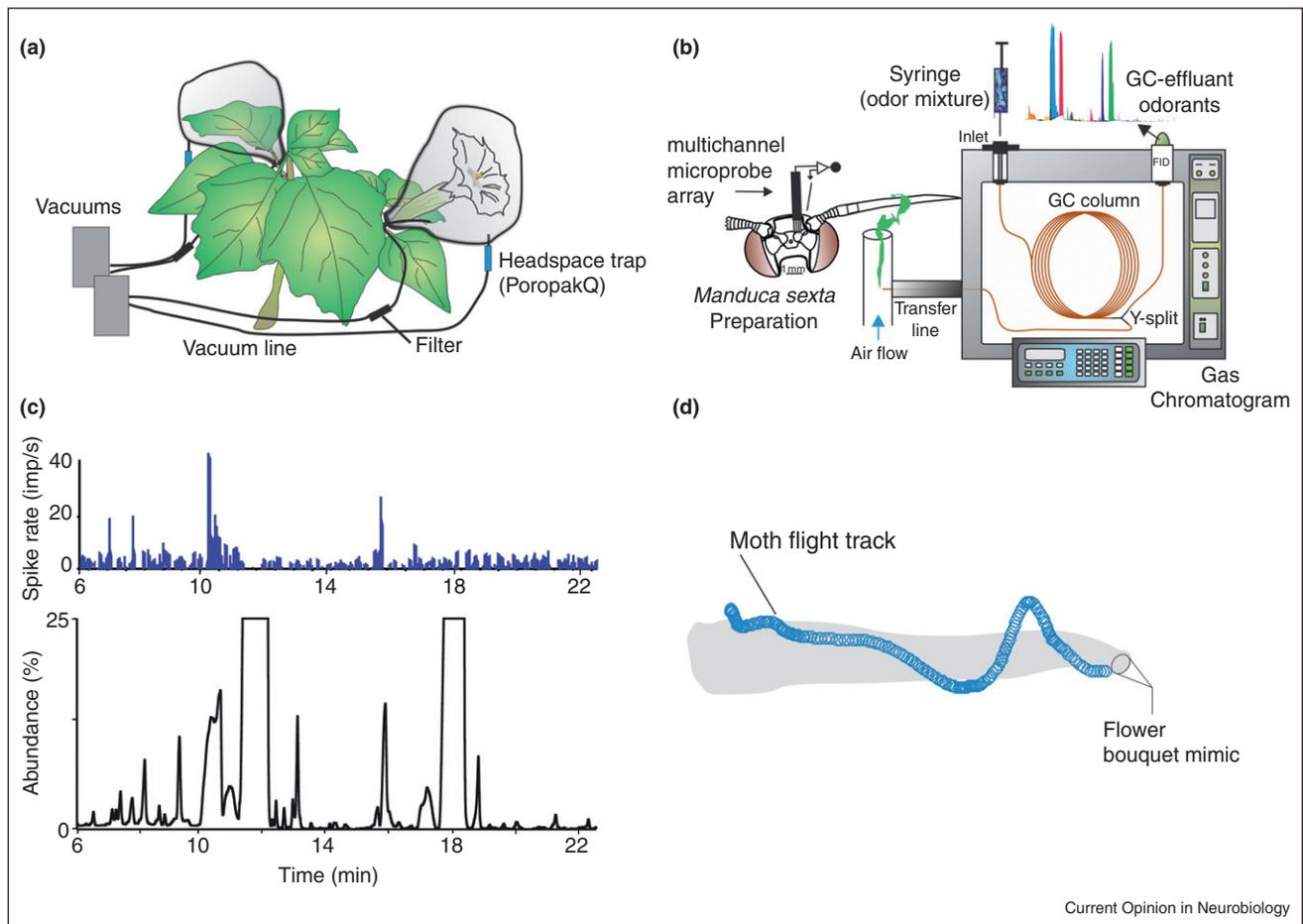
Olfaction plays a central role in the behaviors of many, if not all, animals – from humans navigating to a source of chocolate, to fruit flies flying to glass of wine. The neurobiological basis of these behaviors have benefitted from an integrated understanding of the chemical information – with reference to the identity of the chemical, and its distribution in time and space –, and the behavioral response to the odor. The behavioral strategies used in diverse organisms to locate an odor source are remarkably similar, suggesting that olfactory systems may be constrained by the physical and chemical environment. The problems faced by an animal searching for an odor are the same: How does the olfactory system quickly discriminate the signal from a noisy background and under conditions where the signal is at trace levels of intensity?

Recent advances in analytical techniques for odor identification has improved our ability to rapidly identify biologically important chemicals [1[•],2,3]. In parallel, molecular and genetic techniques have increased our understanding of the mechanisms by which chemical information is transduced and encoded by the peripheral nervous system [4–6]. Often the odors that elicit a behavioral response are mixtures of compounds that together are more behaviorally salient than their individual components [7,8^{••},9]. Unfortunately our understanding of how the olfactory system processes the odor mixtures in a unique, non-additive, manner is not well-understood, but increasing studies have begun looking at the circuit mechanisms that may be involved in mixture processing [10–14].

Insects offer many advantages for studies of the mechanisms of odor information processing, because: (i) Behavioral interactions with potential mates, appropriate sites for oviposition, and sources of food are controlled largely by natural odor mixtures that initiate innate behaviors [8^{••},9,15], and (ii) Increasing information is known about the chemical composition of such olfactory stimuli, including specific mixtures that are necessary and sufficient to mediate a particular behavior ([8^{••},9,16]; see [10,11] for reviews of the insect olfactory system) (Figure 1). Coupled to our increasing understanding of the processing of complex stimuli, is the awareness of plasticity in the olfactory system. This plasticity can operate at short timescales (seconds to minutes) or longer time scales (hours to days) and is subject to a variety of different physiological and environmental factors [17^{••},18,19]. For instance, honey bees and moths can learn the association between an odor and a reward [18,20,21], and during the acquisition phase of learning the responses in the AL become modulated. Together, innate and learned behaviors provide two olfactory channels by which information can be processed.

In this review I detail the recent progress in the behavioral and neurophysiological mechanisms of odor processing in insects. In particular, this review focuses on two aspects of olfactory neurobiology: the processing of complex stimuli, and plasticity. These two components were chosen to illustrate: (1) that natural olfactory stimuli provide outstanding opportunities to determine how neural circuits process the odor information to drive behavior; and (2) that the context by which the animal is operating can profoundly influence the behavioral output and the basic coding of information. Finally, some new directions in chemical integration are suggested as exciting new avenues of research.

Figure 1



Determination of behaviorally relevant volatiles in a complex bouquet. A combination of methods (analytical chemical, electrophysiological, and behavioral) is necessary to identify the volatiles necessary to drive behavior. **(a)** Headspace sorption methods [8**] are used to trap the volatiles from a behaviorally important odor source. In the schematic, a flower is enclosed within a Teflon bag, and using a vacuum pump, the air from the bag is sucked through a volatile trap (PoropakQ) to concentrate the emitted volatiles. The air is filtered and returned to the enclosed flower. As a control for 'background' vegetative volatiles also emitted from the plant, the headspace from the green leaf tissue is simultaneously trapped. **(b)** To identify the behaviorally important volatiles in the complex bouquet, a combined gas chromatography-multiunit recording (GC-MR) system is used. The floral odor, trapped as described above, is eluted with a solvent (hexane). The extract sample of the floral headspace is injected in the GC and the effluent from the column is split such that half of the flow enters either the GC's detector, which ionizes compounds, and the other half of the effluent is carried by a heated transfer line and arrives simultaneously at the moth's antenna. Action potentials from the AL neural ensemble are continuously recorded extracellularly during the 20 min of odor delivery via GC. **(c)** Rate histograms (bin size, 100 ms) of unit responses to the eluting compounds from the flower headspace extract (1 µl injection) (bottom trace, black). **(d)** Volatiles that elicited strong unit responses can be combined in a mixture and tested behaviorally, as shown by the flight track of a moth to the artificial mixture. Each circle of the moth flight track corresponds to a time point of 16.6 ms.

Innate behaviors and mixture processing in the antennal lobe

Many insects, including bees, flies, and moths, exhibit instinctive responses to odors that are biologically important. For example, the moth, *Manduca sexta*, is innately attracted to the floral scent of its host plant, *Datura wrightii*. Similarly, the vinegar fly, *Drosophila melanogaster*, is attracted to scents indicating appropriate food and oviposition sites [8**,9,21]. Odors that elicit innate responses provide outstanding means by which to determine the neural circuits that give rise to behavior.

The olfactory environment

The olfactory environment is one of constant flux, where olfactory stimuli are dynamic in both space and time [3,10,22,23]. In addition, natural odor stimuli are almost always mixtures of numerous single components at certain concentrations and relative proportions [21]. The constituents making up the mixture can be unique to their source, or can be common to those emitted from multiple sources and make up the 'background' of the odor environment [9,22]. Furthermore, it is unclear which of the odorant(s) comprising a mixture are utilized by an

insect when learning the association between the mixture and an appetitive or aversive stimulus. The animal must therefore discriminate and learn a complex signal from the background while processing its varying intensity through time.

Behavioral responses to mixtures

Behavioral results from diverse taxa of insects have shown that mixtures – often comprised of more than 100 individual volatiles – elicit significantly greater behavioral responses than the individual chemicals making up the mixture. Furthermore, the behavioral effectiveness of the mixture resides as a function of a few, key, volatiles in the mixture, thus suggesting a perceptual ‘binding’ mechanism in the insect’s brain. These results were initially found in the sex pheromone system of moths, where males typically responded to only two or three components in the pheromone mixture emitted by females [15,24]. Innate responses, however, are not confined to mixtures eliciting social or sexual behaviors. For example, moths also respond to three components in a complex floral bouquet comprised of approximately 70 compounds [25], and subpopulations of the apple maggot, *Rhagoletis pomonella*, can discriminate between the fruit from different host plants based on a few compounds in the fruit scent [26]. The mosquito, *Culex quinquefasciatus*, is another example of specificity towards specific compounds in a mixture: female mosquitoes innately respond to only eight aldehydes emitted from chicken feces [27]. Behaviors driven by only a few compounds may be computationally easier for the insect to process and quickly respond to. Odors such as sex pheromones, and odors indicating oviposition sites, have profound fitness consequences for the insect, and as such, the ability to accurately and quickly respond to only a few compounds may be a strong selective force in the how these mixtures are processed. This may be especially important for insects navigating in a turbulent odor plume, and experience an intermittent and spatially complex odor (see 10 for odor tracking at large spatial scales, and 11 for smaller scales).

Mixture processing in the antennal lobe

Articles in this series [10,11], and others [28], have described the organization of the insect olfactory system. Previous work has shown that many of the processing mechanisms for odor information occurs in the antennal lobe (AL) (reviewed by [10]), including combinatorial processing of complex odors causing the odors to be represented as unique patterns of spatial and temporal information. Briefly, olfactory input from the periphery (e.g. antennae, maxillary palps) converge into regions of neuropil, called glomeruli, in the AL. Glomeruli interact through a population of local interneurons (LNs), the majority of which are inhibitory [29,30]. Projection (output) neurons from each glomerulus proceed to the higher centers of the brain where the glomerular

information is integrated and processed with other modalities to ultimately drive behavior.

In parallel to the behavioral work on mixtures, recent optophysiological and electrophysiological studies have shown the antennal lobe represents the mixtures in a manner that cannot be predicted by the responses to the single odorants. For example, an elegant series of experiments showed that key odorants in the scent of the lily, *Arum palaestinum*, activates evolutionary conserved olfactory receptor proteins (*Or42b* and *Or92a*) and glomerular responses – in particular, the glomeruli DM1, DM2, and VA2 – in *D. melanogaster* [8**]. The flower mixture thereby drives an innate sensory preference in the fly that mediates the innate attraction of the flies to this flower. In addition, multi-channel recordings in the AL of *Manduca sexta* moths demonstrated that a mixture elicited a unique pattern of spatiotemporal activity by the AL ensemble compared to the single odorant responses [9,25]. Moreover, as long as certain key odorants were maintained in the mixture, the spatial (which neurons were activated) and temporal (synchrony) pattern of activity by the neuronal population remained the same as the complex scent. Importantly, innate behavioral preference also correlated with the neural representation in the *D. melanogaster* and *M. sexta* work [8**,9,25].

A coding mechanism in the AL that efficiently represents the behaviorally important mixture relative to its constituents is through the dynamics of synchronized activity. For example, in the moth, *M. sexta*, stimulation by a behaviorally important mixture elicits a unique pattern of synchronized activity in AL neurons that is different from the constituents making up the scent [9,25,31]. Moreover, the synchronized activity is time-locked to the stimulus input for short odor pulses typical to those in an odor plume (<0.5 s). By contrast, longer odor pulses (>2 s), perhaps typical to those in the headspace of the odor source, elicit strong oscillatory synchrony in AL neurons that has been shown to influence the spike-timing of downstream neurons in the insect Mushroom body (MB), the learning and memory center [32,33]. The synchronized activity of AL neurons may thus serve to increase the rapid discrimination of complex mixtures in a turbulent plume while also increasing the discrimination and subsequent memory retrieval when near the source of the odor.

What processing mechanisms take place in the AL to cause the synchronized activity and unique representation of these mixtures? Recent work has shown a variety of mechanisms that might be attributable to the unique representation, including inhibition (global and local via lateral inhibitory connections), disinhibition, and lateral excitation. Modulation of PN response is mediated by the diverse local interneurons [29,30]. Inhibition imposed by the GABAergic LNs serves to modulate the activity of PN

responses and AL activity, including enhancing the contrast in odor representation, and mediating intraglomerular and interglomerular synchrony of neural activity [31]. Inhibition has been shown to operate both pre-synaptic [13,34,35] and postsynaptic [36] to suppress local and global responses by glomeruli. In addition, new work has demonstrated the action of excitatory LNs that function as indirect, excitatory input from other activated glomeruli [37], and spread to PNs through electrical synapses [37,38]. It remains unclear how excitatory LNs may shape glomerular responses to mixtures, although they could be playing an important role in the representation of the complex odors. Excitatory input to PNs by the LNs may serve to increase the gain when the input is weak, whereas inhibitory LNs may suppress responses when the stimulus is at a high intensity. By these mechanisms, inhibitory and excitatory networks may function to shape AL processing of mixtures in a manner not predictable from responses to the single volatile constituents or from peripheral input [13,36].

Olfactory learning and plasticity

Beyond those mixtures that drive instinctive behaviors, insects can also learn associations between volatile stimuli and aversive or appetitive stimuli. For example, honey bees can learn the scent of flowers that provide a reward [18,19]. The *M. sexta* moth exhibits an innate attraction to certain host flowers but can learn to feed from others when the preferred flowers are scarce [21], and locusts have the ability to associate olfactory stimuli with the quantity and nutritional quality of food sources [39]. In the acquisition phase of appetitive learning, the neural representation in the AL changes owing to modulation by the amine, octopamine, that is released in the AL [40,41^{••}]. Pharmacological and genetic manipulations of octopamine in the brain of insects cause extreme deficits in their ability to appetitively learn, suggesting that octopamine is key neuromodulator in learning and memory [41^{••},42]. In contrast to appetitive learning, insects also have the ability to learn an association between volatiles and a harmful, or bitter, stimulus [43], and genetic dissection of the neural pathways involved in aversive learning in the flies and bees have shown that dopamine is the key neuromodulatory agent mediating the aversive behavior [44[•],45]. Although the mechanisms may be common between insects, the capacity for olfactory learning may vary between species. Nonetheless, learning has clear benefits for growth [46] and mating success [47].

Recent studies have begun coupling optical and electrophysiological approaches with classical conditioning paradigms to determine how the learning modifies the responses of odors by PNs, and downstream responses by the Kenyon cells (KC) – that make synaptic connections to PNs – in the MBs, areas and neurons suspected to be involved in learning and memory. Calcium imaging,

electrophysiological recordings, and genetic manipulations have demonstrated the importance of the MBs, and KCs, for learning and memory. In contrast to the oftentimes noisy responses by PNs, KCs in moths, bees and flies respond to odors in a sparse manner (few spikes) from a small proportion of the KCs in a population. The responses of KCs in moths [48] and bees [49] occur within a few milliseconds of the onset of a stimulus pulse, followed by an unresponsive period. Furthermore, output neurons of the bee MB respond with patterns differentiating rewarded from unrewarded odors, delayed only tens of milliseconds from the AL input to the MB [50[•]]. Together, these results suggest that odors are classified based on the responses of PNs to a brief contact of an odor stimulus, with the downstream sparse representations may be advantageous for the formation of long-term memory and new associations.

Olfactory state

The internal state of the insect can also play an important role in its response to olfactory stimuli. The age of the insect, time of day, and feeding and mating status may modify the salience of odor stimuli that are important for the survival of an individual insect. The current physiological or behavioral state of the animal will thus provide feedback to modulate the olfactory system [51]. Examples are numerous, including the circadian control of activity, and the gating of olfactory behaviors after mating [52], or feeding [17^{••}]. For example, expression of the odorant binding proteins in the *Anopheles gambiae* mosquito antenna is controlled by the light cycle, and this expression pattern correlates with the crepuscular activity of the mosquito [53[•]]. Similarly, in the moth, *Trichoplusia ni*, levels of serotonin and octopamine in the hemolymph changed in the early evening when the moth becomes active [54]. For *D. melanogaster*, appetive state is signaled by insulin that upregulates a peptide receptor on the olfactory receptor neurons that innervate the DM1 glomerulus, the activation of which is sufficient enough to drive the animal to search for food [17^{••}]. In the example with the mosquitoes, the regulation of olfactory binding proteins can influence the sensitivity of the receptor neurons on the periphery. By contrast, aminergic modulation can influence the gain of neurons at the peripheral and central levels [18,40,41^{••}]. Thus, diverse physiological and behavioral states can be achieved by modulation of different levels of the nervous system that, in turn, will influence the olfactory processing.

Chemosensory integration

Finally, increasing research has examined the molecular and neural basis of taste in mediating insect behavior [55], and new studies have begun to tease apart the relative contribution of olfaction and taste (gustation) in mediating behavior [56,57]. Are the two chemosensory channels redundant if they are involved in the same behavioral task, or do they interact in some way? Classic studies in olfactory conditioning in honeybees (*Apis mellifera*) have

demonstrated that the two pathways are linked by the ventral unpaired medial neurons (VUMmx1) that respond to sucrose stimulation. This neuron innervates the MB and the AL, and stimulation after an odor elicits the reinforcing effects of sucrose in behavioral tests [40]. Subsequent work has demonstrated that the VUMmx1 neurons are octopaminergic (OA), and that blocking OA via RNAi impairs appetitive learning in bees [58], and in flies OA conversion impairs odor learning [59].

Beyond appetitive learning, more recent studies have begun to examine how olfaction and gustation control social interactions. In a recent study using fruit flies (*D. melanogaster*), Wang *et al.* (2011) demonstrated that gustation is the dominant modality controlling behavioral interactions between male flies, and appears to 'gate' their olfactory responses to pheromones [56**]. The neural substrate and the circuits controlling the olfactory response are unknown, but future studies should help illuminate the mechanisms by which the chemosensory integration is occurring.

Conclusions

The study of olfaction requires an integrative approach, coupling sensory ecology and behavior with neurophysiology. The central goal of this review was to highlight this interdisciplinary field while demonstrating the importance of natural stimuli and context for determining how olfactory circuits operate to drive behavior. Together, this integrative research – coupling genetic and molecular tools, and behavioral assays, combined with new methods for studying olfactory circuits – has led to a dramatic increase in olfactory research over the past decade. Future work that incorporates olfactory ecology with neurobiology and physiology should lead to improved understanding of the coding and circuit properties that drive natural behaviors.

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