Mixture processing and odor object segregation in the insect olfactory system

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CHAPTER 1

General Introduction

The olfactory world is the most arcane and least understood of our sensory worlds. Natural odor stimuli are hard to capture and to predict, and their processing is not as intuitively comprehensible as compared to other modalities. We only have a faint idea of what we are smelling, and we often completely fail to recognize common odors (For a review on these issues, see Yeshurun and Sobel (2010)). Humans obviously are not very gifted smellers - not surprisingly, the composition, temporal structure, and processing of natural odors is understudied compared to other modalities. Most natural odors are actually mixtures of different substances, but when smelling mixtures of two or more odorants, most of us will agree to perceive just one odor instead of individual odorants.

1.1 Odor mixtures - a blessing and a curse for olfactory systems

We have to distinguish between different kind of odor mixtures: The first category are compound odors that emanate from an odor source (object), be it a flower, Roquefort cheese or conspecific member. Most of these odors occur frequently, and can have an acquired (=learned) or innate meaning with a hedonic value, for example pheromones, or many host plant odors. The composition, i.e. the concentration ratios of the components in these odor mixture needs to be relatively fixed to be recognized (Visser and Avé, 1978; Natale et al., 2003). Thus, mixing odorants in a specific recipe enables many species to communicate in a reliable way, and helps many animals to find the right food. Obviously, by using mixtures and not pure substances as communication cues, the number of possible cues or the perceptual distance between a given number of odor stimuli maximizes, thereby minimizing the probability that meaningful odors will be mistaken for something else.

The second category of odor mixtures comes into existence when odorants from different sources (objects) mix. These mixtures have a rather random composition, which depends on the objects that are present, and can be less meaningful. Actually, quite the contrary is given: In those mixtures, the components (which can in turn be mixtures) bear the meanings. Thus, meaningful olfactory stimuli have to be discerned and detected from these mixtures. Examples of those odors are floral odors from different species that mix on a meadow and need to be recognized by pollinators like the honeybee.

Thus, mixtures can comprise the same components, but the quality of the mixtures can be changed by the concentration ratio or the timing of the constituents. This thesis deals with questions regarding the neuronal processing of both kinds of mixtures. Before I will go deeper into how these mixtures differ conceptually and what questions arise from that (section 1.6), I will give an overview how the brain processes odors.

1.2 The processing of odors in neuronal networks

Although vertebrates and invertebrates are only remotely related, the neural architecture and odor processing mechanisms are surprisingly similar in many regards (Strausfeld and Hildebrand, 1999; Kaupp, 2010), which empowers insects as a model organism for olfaction. Odor perception starts at the olfactory sensory organs, i.e. the antennae of insects, which is the analogue of the olfactory epithelium in vertebrates, where the odors activate olfactory receptor neurons (ORNs). Insects generally have dozens of different ORN types, of which every type expresses a distinct receptor protein (Clyne et al., 1999; Vosshall et al., 2000). Every odorant has a specific potential to activate a certain receptor. This results in a specific spectrum of olfactory receptor neuron activity for every odorant. In reverse, every receptor neuron type has a specific spectrum of odorants from which it can be activated (Hallem et al., 2004). The ORNs project into the antennal lobe, the first olfactory relay center. As a rule, ORNs that express the same receptor protein innervate the same glomerulus (Gao et al., 2000; Vosshall et al., 2000; Fishilevich and Vosshall, 2005). Thus, the number of glomeruli in the antennal lobe corresponds to the number of different ORN types. As a consequence of this sorting, the ORN activity spectrum is translated into a local code in the antennal lobe: every odor elicits a distinct glomerular activity pattern in the antennal lobe or olfactory bulb (Joerges et al., 1997; Galizia et al., 2000; Wilson and Mainen, 2006; Hansson et al., 2003). Within the glomeruli, the ORNs make synapses with two other neuron types, projection neurons (PNs) and local interneurons (LNs). LNs are restricted to the antennal lobe and interconnect the glomeruli, thus providing a sort of horizontal information flow. They exhibit various branching patterns in different glomeruli and are

often highly ramified (Chou et al., 2010). This antennal lobe network has an important role in olfactory coding: It shapes responses of the projection neurons in a way that enhances sparseness, contrast and dissimilarity (odor specificity) of the odor responses of the ORNs, before the information is projected to higher brain areas, the mushroom bodies and the lateral horn (Sachse and Galizia, 2002, 2003; Olsen and Wilson, 2008; Deisig et al., 2010). PNs thus convey the output of the antennal lobe after processing.

1.3 The neuronal mechanisms for odor mixture coding

How can olfactory mixtures gain a perceptive quality that is different from their components? What happens in the brain when an animal smells an odor mixture? Several studies deal with the question how mixtures are processed: They describe so far unpredictable differences of antennal lobe neuron responses between odorant mixtures and their components (Galizia et al., 2000; Silbering and Galizia, 2007; Deisig et al., 2006). Although it is shown in moth that mixture interactions also occur on the level of ORNs (Rospars et al., 2008), in the fruit fly PNs display more mixture interactions than ORNs, separating the perceptual similarity of the mixture and their components (Silbering and Galizia, 2007) and thus awarding the antennal lobe a significant role in odor mixture processing. These results are also supported by electrophysiological studies (Sun et al., 1993; Riffell et al., 2009a) and odor mixture interactions are also described for the olfactory bulb in vertebrates (Grossman et al., 2008; Tabor et al., 2004). Many more of the mixture interactions in single neurons or glomeruli are inhibitory than synergistic, i.e. the response of a measured unit (neuron or glomerulus) to a mixture is weaker than expected from the responses to the single components. Furthermore, interactions are not only identified by the response strength of single neurons or glomeruli, but also in the alteration of the population response (Riffell et al., 2009a).

Mixture processing that qualitatively alters the neuronal response pattern or the perception is considered “synthetic” (also called “configural”) processing (Chandra and Smith, 1998; Deisig et al., 2002; Coureaud et al., 2009). In contrast, “analytic” (also called “elemental”) odor coding describes mixture processing in which the information of the components is preserved. These two categories are not exclusive (Kay et al., 2005; Lei and Vickers, 2008). Consequently, a mixture can be processed with more or less features of both synthetic and analytic processing.

Mixture interactions also become evident in the behavior of animals: Many semiochemicals like pheromones or kairomones consist of odor mixtures that can trigger innate behaviors, which are only weakly if at all triggered by their components, for example host plant odors to which insect herbivores have an innate appetence. Moth that selectively feed or oviposit on distinct plants are often attracted to the natural mixture

or a mimic containing key components, but not to the components alone (Riffell et al., 2009a). That the changes in antennal lobe response pattern evoke the different behavioral responses, or in other words, that the similarity of neuronal response patterns correlate with the perceptual similarity, has already been suggested earlier (Guerrieri et al., 2005).

1.4 Processing of an odor mixture with different component concentrations

Components in semiochemicals are subject to changes of their concentration ratios. The herbivore moth *Cydia molesta* (Oriental fruit moth) is naturally attracted to the odors of its host - the peach tree *Prunus persica*. What happens when the composition of a naturally attractive odor mixture changes? Are the moths still attracted, and to which degree are they tolerant to composition or component ratio changes? Are the glomerular responses reflecting these behavioral responses? Are there distinct glomeruli that might drive the innate preference to an odor, which should then be inhibited or disinhibited when an odor mixture is applied with a ratio that is not attractive? These questions are addressed in chapter 2 of this thesis. Since these mixtures are odor stimuli from one plant or derivatives of it, thus emanating from one object, they belong to the first category as stated above (section 1.1). An important property of these mixtures is the constant and fixed concentration ratio (generally speaking, the fluctuation of the concentrations over time is the same for every component). Hence, mixtures of this sort are also referred to as *synchronous mixtures* in further parts of this thesis.

In the other category of mixtures, odorants need to be discerned and detected from mixtures, that means that the original information of the components needs to be preserved. These two scenarios at first seem mutually exclusive. How do these two categories of odor mixtures differ? When does a mixture need to be considered as a perceptual entity, and when is it necessary to extract the components? A view on how stimuli are segregated from each other in other modalities might help to understand the problem.

1.5 Object recognition

Stimulus segregation is tightly linked to "object recognition", which is mainly used in terms of vision: the term "object" generally refers to something that can be visually observed. It describes a visible and meaningful stimulus which can be recognized under

changed circumstances that may lead to a totally different visual input, for example by rotation, change of color and size, or partial occlusion. An object might comprise several features, but to be detected as an object, the features of a (moving) object such as size, angle of illumination, or relative position of a subcompartment of the object, have to stay in a certain relationship to each other. This synchronous moving is known as "common fate" (for a review, see Wagemans et al. (2012)). A crucial precondition for object recognition is stimulus segregation. Given that many stimuli are present at the same time in the same space, objects can only be recognized if stimuli can be grouped and segregated from each other. From sensory modalities other than olfaction, different mechanisms are known to achieve stimulus grouping and segregation. One of the most common is temporal asynchrony of the onset of the different stimuli: Usher and Donnelly (1998) showed that a 16 ms time interval, which is below the integration time of the human eye, is enough to group visual stimuli into different perceptual elements. Another study showed that 20 ms asynchrony between a target stimulus and a similar background facilitates their separation (Hancock et al., 2008). Whenever we detect an otherwise perfectly camouflaged animal only because it is moving, our brain makes use of this principle. A similar phenomenon is known from audition: two sounds can be better segregated if the onsets of the sounds are temporally separated (Lipp et al., 2010). This segregation ability allows us to distinguish sounds from different sources, also known as "auditory streaming", which is of relevance for example in speech discrimination in the famous "cocktail party phenomenon" (Bronkhorst, 2000).

1.6 Segregating odors from mixtures - steps towards olfactory object recognition?

It is as yet unknown to what degree the olfactory system also performs stimulus segregation. There are substantial differences between stimuli from light or sound on the one hand, and odors on the other hand: light and sound travel quickly and are almost unaffected by air and wind, as compared to odors, which are highly prone to diffusion and wind perturbation. The result are chaotic, fluctuating odor stimuli that are unstable in time and space. As a consequence, odors from different sources mix in a complex way that is less predictable than the mixing of light or sound. It is this chaotic intermingling of odor filaments which results in an inhomogeneous mixture with temporal differences between the components (Fig. 1.1). In this thesis, I call this sort of mixtures *asynchronous mixtures*.

In the laboratory, we simulated these mixtures by applying overlapping pulses of different odorants (=components), where the offset between the components was in the range of milliseconds. The question whether honeybees perform stimulus segregation

Figure 1.1: **The intermingling of odor filaments from different objects produces inhomogeneous, *asynchronous mixtures*.** For this illustration, the filamentous structure of airborne odors from two flowers, and how they mix by turbulences, was visualized with titanium dioxide. It is produced when titanium tetrachloride and water from the air or plant cells react.

by exploiting temporal differences between the onsets is addressed in chapter 3. For historical reasons, in this chapter synchronous mixtures are called *coherent* mixtures and asynchronous mixtures are called *incoherent* mixtures.

But what neuronal mechanisms underlie stimulus segregation of odors from olfactory mixtures? If the antennal lobe plays a part in it, we should be able to see the effects of it in the antennal lobe output, i.e. projection neurons. Stimulus segregation implies that information about the odorant components is preserved in the mixture. Thus, the population response of projection neurons to asynchronous mixtures should comprise more features about the components than the response to synchronous mixtures. Moreover, asynchronous mixtures would be processed in a more analytic fashion (section 1.3). These changes could come with more or less inhibitory mixture interactions compared with synchronous mixtures. These hypotheses are addressed in chapter 4.

Asynchronous odor mixtures are generated when odors from different sources intermingle in a turbulent environment. This results in intermittent stimuli that are indeed used by insects (Murlis and Murtis, 1992; Riffell et al., 2008). As intermittent pulses are behaviorally more relevant, induce stronger responses than sustained odor stimuli (Murlis and Murtis, 1992; Justus et al., 2002; Geffen et al., 2009), and occur rather in turbulent odor environments, odor intermittency could be particularly important for the processing of asynchronous mixtures. I was therefore interested if the application of repetitive pulses is relevant for odor segregation. Repeated stimulation could lead to experience dependent short-term plasticity in the antennal lobe network, which adjusts odor-specific contrasts quickly to changing odor situations (Faber et al., 1999; Stopfer

and Laurent, 1999; Fernandez et al., 2009; Rath et al., 2011; Das et al., 2011; Locatelli et al., 2013). Moreover, an inhibitory network could also change the response latencies to one or both mixture components. If this results in a temporal separation of the two components in the antennal lobe output neurons, downstream neurons would have more time to extract the identity of the components. On the other hand, a precise and stable timing of the component responses would lead to higher reproducibility of spatiotemporal ensemble responses. Which of these two coding strategies happens in the antennal lobe? We addressed these hypotheses and questions in chapter 5.

1.7 Channel splitting versus redundancy in the antennal lobe output neurons

The reproducibility of odor responses is crucial for olfactory systems to reliably learn or recognize odor responses. On the other hand, ensemble responses of sensory systems are often subject to plasticity. Moreover, a natural odor stimulus seldom occurs in exactly the same way twice, due to its random turbulences. Some features of an odor stimulus change (temporal features), whereas others do not (odor identity, i.e. the olfactory response spectra). In most insects, more than one projection neuron conveys the information to higher brain areas. In honeybees, these are around 5, given by the numbers of glomeruli (160) and projection neurons (800). Why is the information from the antennal lobe to higher brain areas split into many neurons? Is the same information conveyed to different upstream neurons, or do different projection neurons convey different pieces of information? In order to give answer to those question, a method is required that allows to simultaneously record from multiple projection neurons of the same glomerulus. Electrophysiological recordings have their limitations in number of neurons that can be recorded simultaneously, and the drawback that cell types other than projection neurons are recorded as well. Calcium imaging in insects antennal lobe was until now limited by the spatial resolution, that did not suffice to resolve single neurites. In chapter 6, we show that simultaneous recording of calcium signals from distinct neurites is possible using a two photon microscope. This technique is potentially useful to address the question whether in antennal lobe output neurons, different features of an odor response is split into different neurons, or whether the different projection neurons convey the same information, thereby enhancing the redundancy of the antennal lobe output.

1.8 Aims and prospects of the study

The main subject of this thesis is to reveal the role of the insect antennal lobe in the processing and discrimination of similar odor mixtures. Odor mixtures that comprise the same components can have different qualities. The concentration ratio or the timing of the components contains information about the mixture quality and components. We used the oriental fruit moth *Cydia molesta* and the honeybee *Apis mellifera* as model organisms. In particular, we pursue the following questions:

- How does the oriental fruit moth respond behaviorally to changes in the ratio of the two components of the host odor derivative, and how do the females perceive these fluctuations at the neurophysiological level? Answers to these questions shall be found by behavioral assays to assess female preference for volatile mixtures differing in the ratios of their constituents using a Y-tube olfactometer, and by physiological investigation of glomerular responses to these mixtures. This is the content of chapter 2.

- Are honeybees able to extract the components of a binary mixture by using millisecond temporal asynchronies between the components? We performed behavioral experiments and describe the results chapter 3.

- What are the neuronal correlates for odor segregation from binary mixtures? Are the representations of synchronous and asynchronous odor mixtures different already on the level of antennal lobe output neurons (projection neurons), and do these neuronal responses contain more information about the components in asynchronous mixtures? We provide answers to these questions in chapter 4.

- Asynchronous odor mixtures particularly occur as intermittent, pulsed stimuli. Does an odor pulse influence the strength or latency of the responses to subsequent odor pulses? Is this dependent on the mixture quality? Answers could reveal if short-term plasticity plays a role in asynchronous mixture processing. We addressed these questions in chapter 5.

- We asked whether different projection neurons of the same glomerulus convey different features of an odor stimulus, or whether they are redundant. To answer this question, simultaneous recording of these neurons with a high spatial resolution in all three space dimensions is necessary. A capable technique - high resolution two-photon calcium imaging - is described in chapter 6.

CHAPTER 2

Behavioral and neurophysiological responses of an insect to changing ratios of constituents in host plant-derived volatile mixtures

2.1 Abstract

Ratios of compounds in host plant odors fluctuate with the phenological stage of the plant. In the present study, we investigated the effect of changing ratios of host plant volatile constituents on herbivore insect attraction and olfactory information processing. We tested a synthetic mixture of bioactive peach shoot volatiles with different concentrations of one of the mixture constituents, benzonitrile, on oriental fruit moth *Cydia (=Grapholita) molesta* females. Y-tube olfactometer bioassays showed that female attraction to the mixture was maintained while increasing the benzonitrile level up to 100 times. Further increases led to behaviorally ineffective mixtures. Then, we recorded odor-evoked neural activity patterns in the antennal lobes, the main olfactory center of the brain, using calcium imaging. Benzonitrile-containing mixtures elicited strong activation in two glomeruli, which were found to process mixture-related information in specific ways. Activation in one glomerulus directly paralleled behavioral effects of the different ratios tested whereas a deviating pattern was noted in the other glomerulus. Our results indicate that the ratio of constituents in a volatile mixture can be varied to a certain degree without reducing female attraction. Thus, volatile blends in nature might vary quantitatively within a certain range without affecting odor-guided host location. Neurophysiological results showed that the processing of mixture-related information inside the antennal lobes is not uniform across glomeruli. Thus, final processing of this information probably takes place in higher-order brain centers.

2.2 Introduction

Insect herbivores use plant volatiles to recognize and efficiently locate their host plants. Adult females perceive these odors via specialized olfactory receptor neurons, and use the volatiles as chemical cues to identify suitable plants for feeding and/or oviposition (Anton et al., 2007; Cardé and Willis, 2008; Mustaparta, 2002). Volatile blends differ between plant species both qualitatively and quantitatively (Baldwin et al., 2006; Bruce et al., 2005; Dötterl et al., 2005). The specific combination of compounds in these blends, many of which are ubiquitous, as well as their ratios, are assumed to drive host plant location in insects (Bruce et al., 2005; De Moraes et al., 1998; Tasin et al., 2006a; Visser, 1986). Even minor constituents in a blend might contribute to the attraction of an insect species to its host plant (Birkett et al., 2004; D'Alessandro et al., 2009; Piñero and Dorn, 2007; Tasin et al., 2007), and they may interact synergistically with major constituents at the behavioral and neurophysiological level, as recently demonstrated for a fruit moth (Piñero and Dorn, 2007; Piñero et al., 2008).

Affixed natural ratio between different constituents of a blend is considered crucial in chemical communication between organisms including insect–mammal (Takken et al., 1997), insect–human (Silva et al., 2005), predator–prey (Steullet et al., 2002), male–female insect (Cardé and Minks, 1995; Linn et al., 1988; Witzgall et al., 2008) and insect–plant interactions (Bruce et al., 2005; Visser, 1986). For male–female interactions in insects, empirical evidence substantiates that the affixed natural ratio of compounds in female-released pheromone blends determines the specificity of this chemical signal to males (Anton et al., 1997; Christensen et al., 1991; Jarriault et al., 2009; Linn et al., 1988; Linn Jr. et al., 1991; Löfstedt et al., 1991; Minks and Cardé, 1988). In insect–plant interactions, a similar specificity might originate from the maintenance of a specific ratio in the plant-released volatile blends (Bruce et al., 2005). Studies on insect attraction to plants have largely focused on the use of affixed natural ratio of compounds in synthetic mixtures to mimic a given host plant blend (Natale et al., 2003; Tasin et al., 2006b; Webster et al., 2008). Insect attraction disappeared when the ratios of the key compounds, as identified in the headspace of the host plant, were replaced by the ratios of the same compounds emitted by a non-host plant (Tasin et al., 2006a). Similarly, responses of olfactory receptor neurons seem to indicate that host plant discrimination by herbivore insects must be mediated by the ratio of the compounds in the volatile blend (Bichäo et al., 2003).

Such strict ratio specificity, however, would question successful chemically mediated host-location behavior by insects active across extended phenological stages of their host plants. In fruit orchards, quantitative composition of volatile blends emitted from trees varies with progressing plant development (Bengtsson et al., 2001; Dötterl et al., 2005; Vallat et al., 2005), while attraction of fruit moths is maintained over several

weeks (Vallat and Dorn, 2005). Given the variable nature of the chemical signal emitted by the same plant species, the question arises whether insect herbivores have evolved a certain degree of olfactory plasticity to locate their hosts within distinct threshold ratios of volatile blend constituents.

Among the fruit insect herbivores of worldwide distribution, the oriental fruit moth, *Cydia (=Grapholita)* molesta (Busck) (Lepidoptera: Tortricidae), belongs to the most damaging species (Hughes and Dorn, 2002; Il'ichev et al., 2003; Rothschild and Vickers, 1991). Attraction of mated females to the primary host, peach (*Prunus persica L.*), is guided mainly by olfactory cues (Natale et al., 2003). A synthetic mixture comprising two constituents at a fixed ratio, determined from the headspace of peach shoots, is as attractive to the females as the peach shoots themselves (Piñero and Dorn, 2007). The first and minor constituent of this mixture is benzonitrile, and the second constituent comprises three green leaf volatiles [(Z)-3- hexen-1-ol, (E)-2-hexenal, (Z)-3-hexenyl acetate] and the aromatic compound benzaldehyde. Moth attraction is only achieved when the two constituents are mixed but not when offered singly (Piñero and Dorn, 2007), and this behavioral effect is mirrored at the neurophysiological level (Piñero et al., 2008). Hence, this model blend offers the opportunity to test different ratios of the two constituents at the behavioral and neurophysiological level.

Calcium imaging allows quantifying changes in intracellular calcium concentrations as a measure of odor-evoked activity in the insect antennal lobes (ALs), the first center for the processing of olfactory information (Galizia and Menzel, 2001). This technique has been used increasingly to study coding of individual odors and odor mixtures in the brain of honeybees (Galizia et al., 1999b; Sachse and Galizia, 2002), fruit flies (Silbering et al., 2008) and moths (Carlsson et al., 2007; Skiri et al., 2004). In the case of the oriental fruit moth, calcium imaging has been applied to understand the neural processing of the model blend mentioned above and its constituents, alone and in combination (Piñero et al., 2008).

Hence, the system comprised of oriental fruit moth and synthetic peach shoot volatiles appears to be highly suitable to investigate the effects of different ratios of mixture constituents on odor-guided insect behavior and olfactory processing. The aim of this study was to quantify how the oriental fruit moth responds behaviorally to changes in the ratios of the two constituents in the model blend, and how the females perceive these fluctuations at the neurophysiological level. Behavioral assays were conducted to assess female preference for volatile mixtures differing in the ratios of their constituents using Y-tube olfactometry. Neurophysiological responses to the same mixtures were recorded using calcium imaging. Because in a natural environment insects encounter odor pockets as turbulent plumes (Cardé, 1996; Murlis and Murtis, 1992; Riffell et al., 2008), efficient odor-guided behavior should rely on the ability to resolve intermittent odor pulses. Therefore, we also tested the ability of the female's olfactory system to resolve such pulses.

Odor	Abbreviation	Ratio m:B	Benzonitrile concentration (ng/ µl)		
			10^{-4}	10^{-3}	10^{-2}
Mixture with no benzonitrile	mB0	100 : 0	0.0	0.0	0.0
Mixture with benzonitrile decreased 100x	mB0.01	99.99 : 0.01	0.002	0.022	0.22
Standard mixture (derived from peach shoots)	mB1	99.85 : 0.15	0.22	2.2	22.0
Mixture with benzonitrile increased 100x	mB100	86.69 : 13.31	22.0	220.0	2 200.0
Mixture with benzonitrile increased 1000x	mB1000	39.42 : 60.58	220.0	2 200.0	22 000.0
Mixture with benzonitrile increased 5000x	mB5000	12.53 : 87.47	1 100.0	11 000.0	110 000.0
Benzonitrile alone	B	0 : 100	100.4	1004.0	10 040.0

Table 2.1: Odors used in the trials with mated female oriental fruit moths. Abbreviations and ratios of the first constituent in the mixture (m) to benzonitrile (B) shown for each odor. Absolute concentrations of benzonitrile (ng/ µl) are given for three serial dilutions of each odor, with 10^{-4} vol./vol. used for the behavioral and 10^{-4} to 10^{-2} for the physiological trials.

2.3 Materials and Methods

Insects

The mated female oriental fruit moths used in this study were obtained from a laboratory colony, reared at the ETH Applied Entomology on an artificial diet (Ivaldi-Sender, 1974) for over 20–30 generations. The colony originated from individuals collected in the Emilia-Romagna region (Italy) two years prior to the start of the experiments. Newly emerged adult moths (males and females) were held in plastic containers in controlled climate chambers (Conviron Ltd, Winnipeg, MB, Canada), with L:D 16 h : 8 h, 60% relative humidity (RH) and 24°C, in groups of 40, at proportions of 3 males : 1 female. Dissection of the female bursa copulatrix for the presence/absence of spermatophores indicated that nearly all of the females tested (>98.2%, N = 250) were successfully mated. Water was provided *ad libitum*. Females were 2 – 4 days old at the time of the experiments and had never been exposed to any host plant odor prior to the experiments, so they were considered to be naive.

Chemicals and mixtures

The chemical compounds used for all mixtures tested were the green leaf volatiles (Z)-3-hexenyl acetate (Sigma-Aldrich, St Louis, MO, USA, purity >99%), (Z)-3-hexen-1-ol (Sigma-Aldrich, purity >99%) and (E)-2-hexenal (Fluka, Buchs, Switzerland, purity >99%), and the two aromatic compounds, benzonitrile and benzaldehyde (Fluka, purity >99%). Furthermore, linalool (Fluka, purity >95%) was included in the physiological experiments as a reference odor allowing normalization of the magnitude of the calcium responses across tested females. In all experiments, mineral oil from the same batch (Fluka, purity >95%) was used as the solvent.

The standard mixture mimicking bioactive peach shoot volatiles (Piñero and Dorn, 2007; Piñero et al., 2008) was composed of (Z)- 3-hexenyl acetate, (Z)-3-hexen-1-ol, (E)-2-hexenal, benzaldehyde and benzonitrile, at ratios of 69.74 : 14.62 : 2.25 : 13.24 : 0.15 vol./vol., respectively. Additional mixtures were derived from this mixture as follows: they contained the first constituent (m) comprising the first four chemicals listed above at the same concentration as in the standard mixture, and benzonitrile (B) as the second constituent (i) decreased 100-fold (mB0.01) or (ii) increased 100-, 1000- or 5000- fold (mB100, mB1000, mB5000, respectively) (Table 2.1). The first constituent alone (mB0) and benzonitrile alone (B) were also included as reference odors. After preparation of each single odor/mixture at the highest concentration of 10^{-2} vol./vol., 10-fold serial dilutions were made with mineral oil as the solvent, in order to obtain two additional concentrations of 10^{-3} and 10^{-4} vol./vol. (from here onwards

in the text the indication vol./vol. is omitted). In the physiological bioassays, all three concentrations were tested whereas only the lowest concentration (10^{-4}) was used for the behavioral bioassays (Table 2.1).

Behavioral effects of changing ratios of mixture constituents

The behavioral responses of the mated female oriental fruit moths to manipulations of the concentration of benzonitrile in the mixture were tested in dual-choice Y-tube olfactometer bioassays. Olfactometer trials were carried out as described in Piñero and Dorn (2007); Piñero et al. (2008). Briefly, the Y-tube olfactometer consisted of a Y-shaped glass tube (2.5 cm diameter, 23 cm arm length and 23 cm common arm length) connected to two tubular glass chambers (38 cm long and 6 cm in diameter), where the odor sources were placed (one on each arm). Charcoal-filtered and moistened air was drawn into each of the two glass chambers and Y-tube arms at a rate of 740±10 ml /min at the entrance. Air-flow rates were always calibrated before the initiation of and during experiments with an electronic flow meter (Agilent flow meter ADM 1000, Agilent Technologies, Centerville, DE, USA).

Bioassays were always conducted in a darkened room during the 2.5 h preceding scotophase (Natale et al., 2003), at 24–25°C and 60–70% RH. Groups of 10 female moths each were brought into the experimental room 30 min before the start of the experiments to allow acclimatization to the room conditions. A single female was released at the entrance of the common arm of the Y-tube and exposed to a particular odor combination, consisting of (a) 1 µl of the solvent mineral oil (blank), and (b) 1 µl of one of the different odors listed in Table 2.1 (all at 10^{-4}. Each odor was loaded into a silicon/Teflon septum (13 mm in diameter) (Supelco, Bellefonte, PA, USA), and the septum was placed inside one of the two chambers that connected one of the two arms of the Y-tube olfactometer. Once inside the Y-tube, the behavior of each female was observed for 10 min. A 60 W red light bulb was placed above the olfactometer to allow observation of female behavior during the 10 min. A female was considered to have made a choice if it entered either arm and crossed a score line drawn 3 cm from the intersection of the tube. By contrast, a female was considered not having made a choice if it remained in the common arm of the Y-tube by the end of the observation period (Bertschy et al., 1997). A new pair of septa was used for each individual female tested, and the position of the chambers containing the septa, as well as the position of the two arms of the olfactometer, was systematically changed after testing 3–4 moths in order to avoid positional bias. For each odor combination the sample size consisted of 60 females and each combination was tested for a minimum of three observation days. All odors tested were prepared 1–2 h prior to the bioassays, and a new olfactometer was used whenever a different odor was tested. After each day, all parts of the olfactometer

in contact with the moths were washed in a detergent solution, rinsed with acetone and hexane, and finally oven dried for at least 12 h at 150°C.

Results of behavioral bioassays were analyzed for preference (percentage of adults that made a choice between an odor or the solvent) and responsiveness (proportion of adults that made a choice) (Bertschy et al., 1997). First, chi-square tests were carried out to test the null hypothesis of no preference for a particular odor/mixture. Then, paired-sample t-tests were carried out to compare responsiveness across odor combinations (SPSS, 16.0, Chicago, IL, USA). The alpha value for each comparison was adjusted downward using the Benjamini and Hochberg procedure to correct for false discovery rates (type I errors) (Verhoeven et al., 2005). Individuals that did not make a choice were excluded from the statistical analysis. Lastly, we evaluated whether the relationship between preference values and benzonitrile concentration in the different mixtures tested was significant using a linear regression analysis (SPSS, 16.0). The mB1000 and mB5000 mixtures were excluded from this analysis, as they were behaviorally ineffective.

Physiological effects of changing ratios of mixture constituents

The odor-evoked activities in the ALs of oriental fruit moth females, in response to each of the mixtures evaluated at the behavioral level, were recorded using calcium imaging. For optical recordings, individual female moths were dissected as described elsewhere (Galizia and Vetter, 2005; Piñero et al., 2008). Briefly, an individual female was mounted on a custom-made Plexiglas®stage and fixed with soft wax to allow full exposure of the brain cavity, including the ALs. The orientation of the insects was tilted to better visualize the ventro-lateral aspects of the AL in comparison with the preceding first study with this species (Piñero et al., 2008). 10 µl of Calcium Green 5N AM [(Molecular Probes, Invitrogen, Carlsbad, CA, USA) dissolved in saline with Pluronic and DMSO (dimethylsulfoxide)] was then used to stain the brain cavity for 60 min. This procedure leads to signals that combine the activities of several different types of neurons and possibly also glial cells. In honeybees (Apis mellifera), it has been shown that the prevailing signals represent primarily sensory neuron responses (Galizia and Vetter, 2005). After removing the excess dye, the preparation was placed under an upright microscope (Olympus BX50WI, Hamburg, Germany) with a x20 water-immersion physiology objective (numerical aperture = 0.95, Olympus XLUM Plan FI). The preparation was also kept in a wind tunnel of humidified air and temperature-controlled air (wind speed: 1.58 ± 0.27 m/s, 19.3 ± 0.56°C) to avoid contamination from external odors in the room. The brain was kept in buffer (in mmol/l 130 NaCl, 6 KCl, 4 MgCl2, 5 CaCl2, 160 sucrose, 25 D-glucose, 10 Hepes free acid, pH 6.7, 500 mOsmol) at all times.

All serial dilutions comprising 10^{-4} (low concentration), 10^{-3} (intermediate concentration) and 10^{-2} (high concentration) were tested. 5 ml of the resulting solutions was kept in a 20 ml glass vial filled with gaseous nitrogen to avoid chemical oxidation and sealed with aluminium ring caps fitted with a silicon/Teflon septum (Axel Semrau, Sprockhövel, Germany) as described and detailed elsewhere (Pelz et al., 2006).

Stimuli (i.e. 2 ml of odor-loaded headspace from the 20 ml vials) were applied at 1 ml/s in succession, always starting with the lowest concentration, with a computer-controlled autosampler (Combi PAL, CTC Analytics AG, Zwingen, Switzerland). Each stimulus was presented as double pulse of 1 s each, at 1.5 and 6 s. Our stimulation protocol was designed to simulate the encounter to a second odor filament in a moth flight – a situation that allows for sensory priming (at low concentrations) and for measuring adaptation effects (at high concentrations). In a natural situation, consecutive odor filaments can occur at high or at low frequency. Our simulation covers the latter case, as we could not use shorter intervals because recordings were performed with a temporal resolution of 4 Hz. Glomerular responses to the reference odor linalool (at 10^{-2}), the solvent mineral oil and air served as controls and were always recorded at least three times (i.e. start, middle and end) during an experiment. The order of the remaining odors was randomly changed every time a new moth was tested in order to minimize odor position bias. Images were acquired with a CCD camera (Imago QE, T.I.L.L. Photonics, Lochhamer Schlag, Germany) attached to the microscope. An 8x8 binning on chip was applied to a spatial resolution of 1.57 µm * 1.57 µm / pixel. Optical recordings consisted of 80 frames taken for each stimulus at a frequency of 4 Hz with 30–70 ms exposure time per image, depending on the basal fluorescence values of the individual females. Excitation light was 470 nm and emission was filtered by a LP505 (Carl Zeiss GmbH, Hamburg, Germany).

Female moths with clearly visible calcium responses throughout the odor stimulations and no or only negligible responses to the solvent mineral oil and air were selected for data analysis with custom-made programs in IDL (Research Systems, Inc., Boulder, CO, USA). Raw data were first corrected for lateral movement artifacts using anatomical landmarks. Then, data were logarithmically corrected for fluorescence intensity decay due to bleaching (Galizia and Vetter, 2005) and filtered using spatial and time median filters with a size of 3 pixels each, to reduce noise. An unsharp mask filter set to 2 pixels was applied to reduce scattered light produced by strongly activated glomeruli on neighboring non- responding areas within the ALs (Galizia and Vetter, 2005). The relative calcium change was then calculated for each frame as relative changes in fluorescence ($\Delta F/F$). For the false color images, the background fluorescence (F) was defined as the mean fluorescence of frames 4–6 for every pixel and ΔF was defined as the difference between the mean fluorescence of frames 34–36 and F for the same pixels. Thus, each pixel was assigned a value that was then translated into a color. For the time traces, F was defined as the mean fluorescence of frames 4–6 and ΔF

was calculated for every single frame as the difference between that frame and F. Time frames for glomeruli were calculated from squares with a side length of 11 pixels and always well within each identified glomerulus. For statistical comparison, each female was normalized to the linalool responses in the linalool-sensitive glomerulus (LIS). The response to the second odor stimulus was calculated with reference to the minimum signal between the two odor stimuli. All analyses were done on individual recordings, not on averaged repeated stimuli.

Normalized calcium signals were analyzed using general linear model (GLM) to test the null hypothesis of no differences in the responses induced by the different odors tested. Odors and concentration (i.e. 10^{-2}, 10^{-3}, 10^{-4}) were used as fixed factors. Fisher's least-squares difference (LSD) tests were then used, when appropriate, to separate means. As for behavior, a linear regression analysis was carried out to test for a relationship between glomerular responses and benzonitrile concentration across all mixtures (at the three odor concentrations). An independent analysis was carried out for each identified glomerulus.

Mixture interactions for the different identified glomeruli were calculated based on the assumption that the response to a mixture is at least as strong as the response to the stronger constituent of the mixture (i.e. the lower bound), if there is no network activity (Silbering and Galizia, 2007). This assumption would only be violated in cases of negative odor responses. As we never observed negative odor responses, they are not considered here. Therefore, we analyzed each benzonitrile-containing mixture as a binary mixture of two constituents: (1) the four compounds included in the standard mixture without benzonitrile (mB0), and (2) benzonitrile alone (B), at different concentrations (Table 2.1). As the relative concentrations of benzonitrile within the mixtures containing this compound were not equivalent to those measured for benzonitrile alone, we inter- or extrapolated all relevant odor responses accordingly, using a linear model (R Development Core Team, 2011). A response to the mixture that is lower than the lower bound indicates the existence of inhibitory mixture interactions. We did not find any synergistic effect (Silbering and Galizia, 2007), and therefore did not consider them here. To test for differences between the responses evoked by the different mixtures and the lower bounds at the three odor concentrations, we carried out a multivariate GLM analysis for every glomerulus. Lower bounds and responses were included in the analyses as dependent variables. Mixture type and odor concentration, however, were treated as independent and fixed factors. Post-hoc comparisons were made, when appropriate, using Fisher's LSD tests (SPSS 16.0). Furthermore, a linear regression analysis was carried out to test for a relationship between relative benzonitrile concentrations and mean differences between the responses and the lower bounds induced by each mixture in each identified glomerulus (SPSS 16.0). Data from the analyses described above (shown in Figs. 2.3-2.5) were plotted using R (R Development Core Team, 2011).

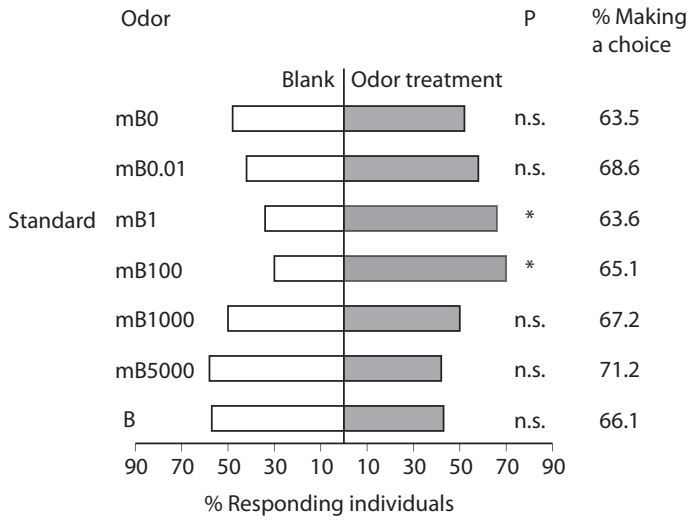

Figure 2.1: Behavioral responses of mated female oriental fruit moths in a Y-tube olfactometer to synthetic odors derived from peach volatiles: five mixtures differing only in the proportion of benzonitrile (mB0.01 to mB5000), a mixture with no benzonitrile (mB0) and benzonitrile alone (B). Preference for odors dissolved in mineral oil at 10–4 vol./vol. versus blank (solvent control). N = 60 females for each dual choice. P-values based on chi-square tests: $*<0.05$; n.s. >0.05

2.4 Results

Behavioral effects of changing ratios of mixture constituents

The attraction of oriental fruit moth females to the odors increased gradually and almost linearly with increasing proportion of benzonitrile in the mixture from zero (mB0) to low (mB0.01) and standard levels (mB1) and even to 100 times the standard level (mB100) ($R = 0.98$, $P<0.05$, $n = 4$ odors) (Fig. 2.1). Preference for the odor treatment was significant over two orders of magnitude in benzonitrile concentration (mB1: $\chi^2 = 8.82$, $P<0.003$ and mB100: $\chi^2 = 11.67$, $P<0.001$, Table 2.1). Further increases to 1000 and 5000 times the standard level of benzonitrile rendered the resulting mixtures mB1000 and mB5000 behaviorally ineffective. The ratios of the two mixture constituents (m:B) encompassed in the attractant mixtures ranged from 99.85 : 0.15 to 86.69 : 13.31 (Table 2.1). Female responsiveness, i.e. the percentage of females that made a choice, by contrast, was not significantly affected by changing the proportions of benzonitrile in the mixtures ($P>0.0001$ after the Benjamini–Hochberg procedure) (Fig. 2.1).

Physiological effects of changing ratios of mixture constituents

Each of the odors tested induced strong calcium signals in the ALs of oriental fruit moth females. These signals were spatially structured and corresponded to patterns consisting of individual olfactory glomeruli. We identified homologous glomeruli across individuals based on their responses and position rather than on morphology. For example, linalool elicited responses in a single glomerulus, the linalool-sensitive glomerulus 'LIS' (Fig. 2.2 B). This glomerulus was thus considered as the reference glomerulus

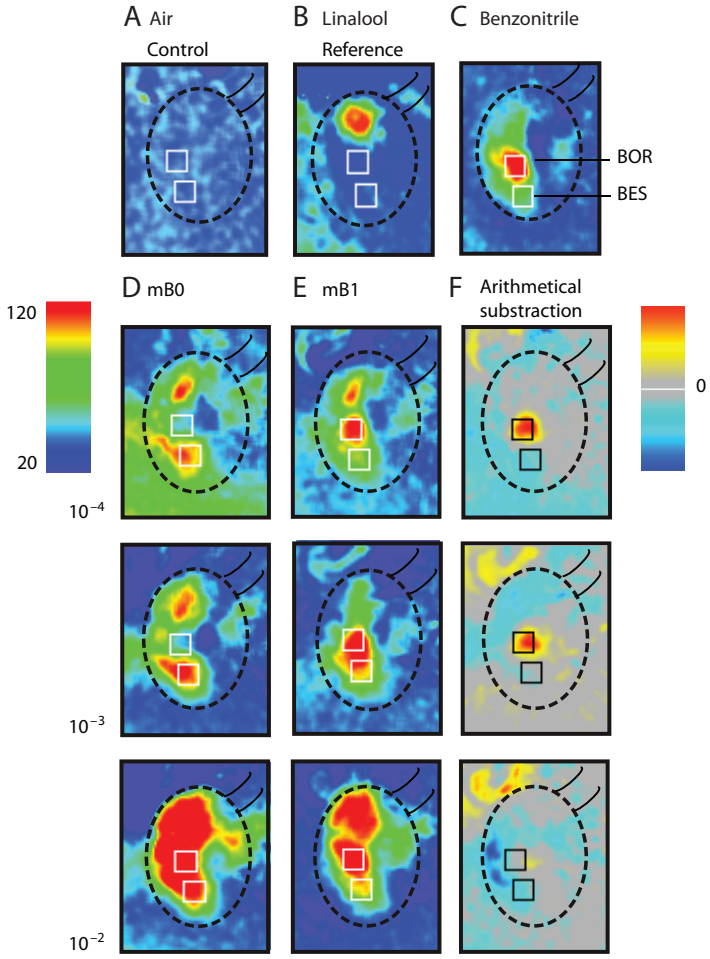

Figure 2.2: Spatial odor response patterns in the right antennal lobe (AL) of a representative mated female oriental fruit moth using calcium imaging. Differential odor response profiles measured as relative changes in fluorescence ($\Delta F/F$) and in false-color coding shown for two selected glomeruli (BOR and BES). (A–E) Responses during stimulation with (A) air (control), (B) linalool (odor reference at 10^{-2}), (C) benzonitrile alone (at 10^{-3}), (D) the mixture without benzonitrile (mB0) (10^{-4} to 10^{-2}) and (E) the standard mixture (mB1) (10^{-4} to 10^{-2}) – see scale bar to the left of D. (F) Arithmetical subtraction of the glomerular response to the mB0 mixture from the response to the mB1 mixture – see scale bar to the right.

and was used to normalize responses across females. The different mixtures tested elicited strong activation of two additional glomeruli, the benzonitrile optimum response glomerulus 'BOR', and the benzonitrile sensitive glomerulus 'BES' (for details see below) (Fig. 2.2 C,D). Odors at the high concentration (10^{-2}) elicited mostly high responses, often exceeding significantly responses to the same odors at the low concentration (10^{-4}) (Fig. 2.3).

To evaluate whether odor-evoked responses in the BOR and BES glomeruli are subjected to mixture interactions, we made an arithmetical subtraction of responses to mB1 (Fig. 2.2 E) and mB0 (Fig. 2.2 D), and compared the calculated result (Fig. 2.2 F) with the response to benzonitrile alone (Fig. 2.2 C). In the BOR glomerulus, responses coincided for the low (10^{-4}) and intermediate concentrations (10^{-3}) but not for the high concentration (10^{-2}), indicating inhibitory interactions or response saturation (compare with Fig. 2.3 B). In the BES glomerulus, responses to the mixture without benzonitrile (mB0) (Fig. 2.2 D) were stronger than those evoked by the mixtures containing benzonitrile (Fig. 2.2 E), even at low concentrations of benzonitrile (compare with Fig. 2.3 E). Thus, saturation cannot explain the behavior of this glomerulus but inhibitory interactions can do so (see below). Glomerulus 'A', previously described to be activated by the standard mixture mB1 (Piñero et al., 2008), is located dorso-medially in the AL and was not visible in our recordings, which focused on the ventro-lateral aspects of the AL.

The time courses of calcium signals induced by the different odors recorded across the AL of the female moths were qualitatively similar (Fig. 2.3 A,D). They were characterized by a first upward stroke reaching its maximum intensity within 1 s following the first odor stimulation, decreasing soon after stimulus offset. This was followed by a weaker but yet distinguishable second upstroke elicited by the second odor stimulation given 4.5 s after the first one. Therefore, we analyzed the patterns of response to both the first (Fig. 2.3 B,E) and the second (Fig. 2.3 C,F) odor stimuli in order to gain more insights into the physiological properties of the receptor neurons involved in odor recognition and representation as well as into the network interactions taking place within the ALs of the females. Almost negligible calcium signals were induced by the two controls, the solvent mineral oil (C in Fig. 2.3 A–F) and the air control (Fig. 2.2 A).

Odor-evoked activity in the two mixture-sensitive glomeruli

In order to further understand the specific patterns of odor-evoked responses and mixture interactions elicited in the AL of the female moths by the different odors tested here, each of the two identified mixture-sensitive glomeruli (BOR and BES) was analyzed independently, as described below.

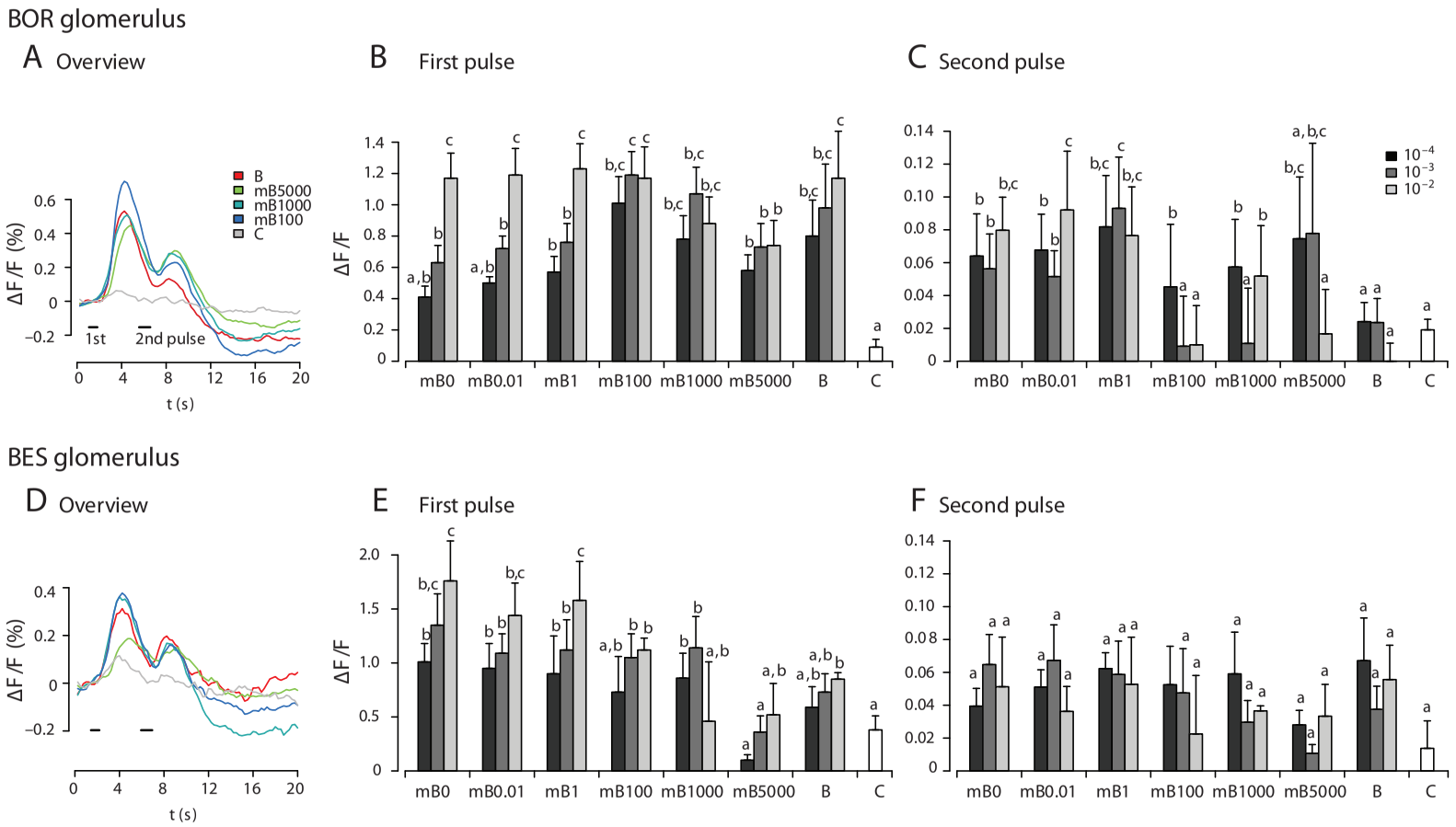

Figure 2.3: Time traces and dose-response relationships elicited by two consecutive pulses of the same odor in two selected glomeruli inside the antennal lobes (ALs) of mated female oriental fruit moths. Tested were five different peach-derived synthetic mixtures (mB0.01, mB1 standard mixture, mB100, mB1000 and mB5000), a mixture without benzonitrile (mB0), and benzonitrile alone (B), all at absolute concentrations of 10^{-2}, 10^{-3} and 10^{-4}. The solvent mineral oil was included as a control (C). Overview of calcium signal traces evoked by representative mixtures in (A) BOR and (D) BES glomerulus. The times of stimulations (1 s each) are indicated as black lines. The delay in the response is due to the distance the odor travels before it reaches the insect antennae. Represented in bars are the dose–response relationships recorded in BOR (B,C, N = 7) and BES (E,F, N = 4) to the first and second odor pulse. Values represent averaged responses (±s.e.m.). Bars followed by the same letters are not significantly different from one another (Fisher's LSD post-hoc test).

Benzonitrile optimum response (BOR) glomerulus

The BOR glomerulus responded to changing the proportion of benzonitrile in the mixtures in a manner mimicking the behavioral results. Responses to the first pulse of odors at 10^{-4} increased gradually and almost linearly with an increasing benzonitrile proportion in the mixture from zero (mB0) to low (mB0.01) and standard levels (mB1) and even to 100 times the standard level (mB100) (R^2=0.885, n=4 odors, P=0.05). Similar results were found for odors at 10^{-3} (R^2=0.969, n=4 odors, P=0.016). Further increases in benzonitrile proportion (mB1000 and mB5000) led to lower mean response values (Fig. 2.3 B). Responses to odors at the highest concentration (10^{-2}), however, did not follow this linear relationship (R^2=0.201, n=4, P=0.551) (Fig. 2.3 B).

Responses to the second odor pulse were lower and less dose dependent in comparison with the first pulse (Fig. 2.3 C). At 10^{-4}, they first increased gradually and almost linearly with increasing benzonitrile proportion in the mixture from zero (mB0) to low (mB0.01) and to standard (mB1) ($R^2 = 0.953$, $n = 3$ odors, $P = 0.022$). Beyond this proportion, benzonitrile dependency disappeared, and response values were generally lower except for the mixture mB5000. Responses to the second pulse were not directly dependent on the odor concentration, either. Indeed, mixtures at 10^{-3} led to weaker, but in most cases not statistically significant, mean response values ($F = 0.4365$, d.f. $= 1$, $P>0.05$), compared with mixtures at 10^{-4}, except for the standard mixture mB1 and the mixture mB5000.

Marked mixture interactions are indicated by the responses of the BOR glomerulus to the two constituents of the mixture, mB0 and B (Fig. 2.4 A,B). Responses to the mixture were significantly lower than responses to the stronger constituent of the mixture, i.e. the lower bound (Pillai's trace: $F = 3.609$, d.f. $= 8$, $P = 0.001$), indicating inhibitory mixture interactions. Focusing on the behaviorally relevant mixtures mB1 and mB100, the measured responses and lower bounds, in pairwise comparisons, each reached similar values across all concentrations (10^{-4} to 10^{-2}) (Fig. 2.4 A), as is also reflected in the synopsis of responses shown in Fig. 2.4 B. Significant inhibitions were only noted for the mixture mB1000 at 10^{-3} and 10^{-2} and for mB5000 at 10^{-2} (Fig. 2.4 A,B). As no inhibitory responses to benzonitrile alone (B) and to the mixture without benzonitrile (mB0) were observed in BOR across the three concentrations tested (Fig. 2.3 B), inhibitions could only be the result of network activity (Fig. 2.4 B).

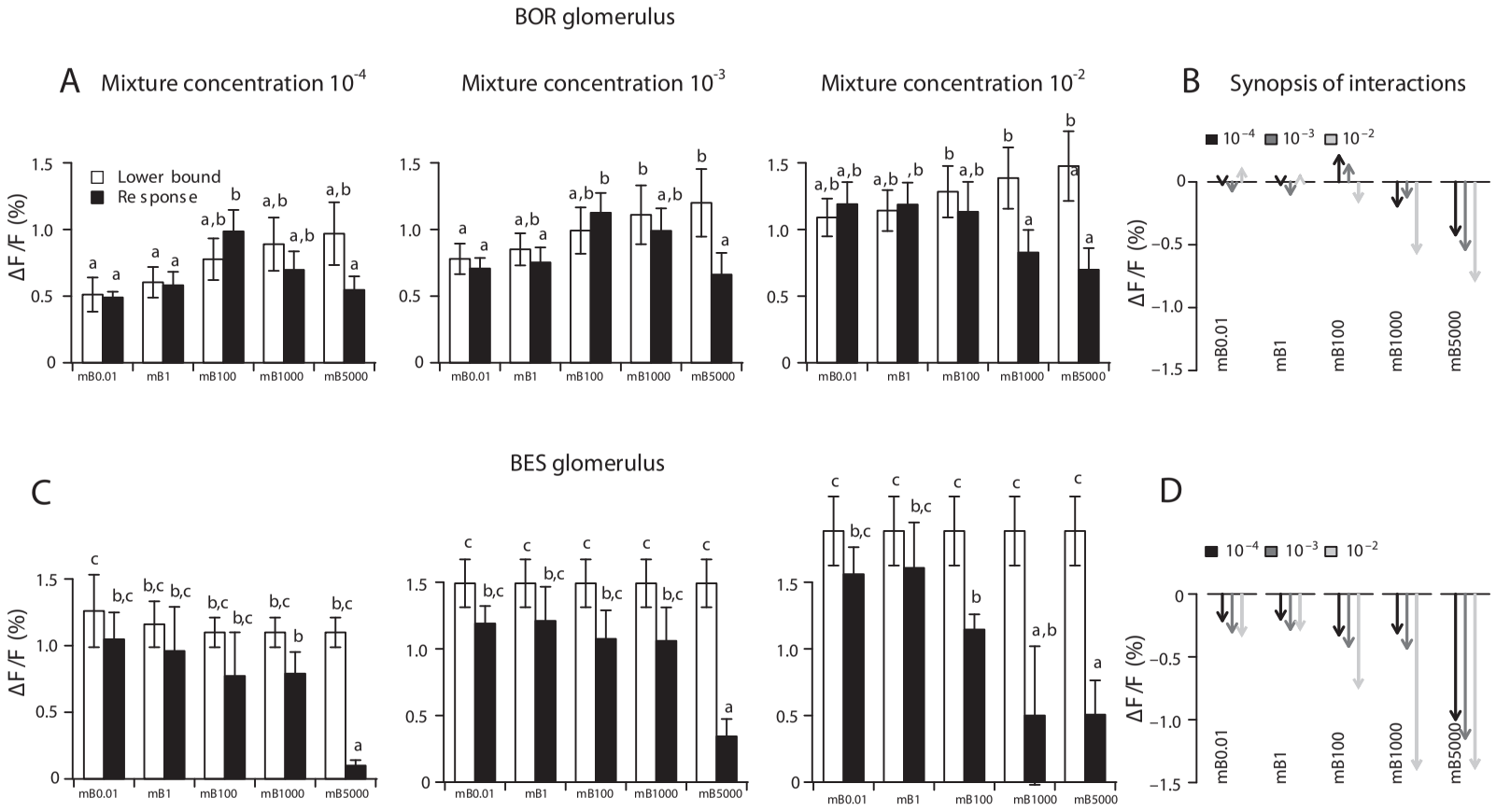

Figure 2.4: Mixture interactions calculated for two selected glomeruli (BOR and BES, same individuals as in Fig. 2.3) identified inside the antennal lobes (ALs) of mated female oriental fruit moths (absolute concentrations of 10^{-2}, 10^{-3} and 10^{-4}). Responses to a given odor (black bars) and its calculated lower bound (white bars) are shown for (A) BOR and (C) BES glomeruli. Bars followed by the same letters are not significantly different from one another (Fisher's LSD post-hoc test). (B) and (D) show a synopsis of the mixture interactions displayed in (A) and (C), respectively. The length of the arrow indicates the difference between a given response and its lower bound.

Benzonitrile sensitive (BES) glomerulus

The BES glomerulus responded to changing proportions of benzonitrile in the mixtures in a manner that did not correspond to the behavioral results. Highest mean values of response were achieved in the absence of benzonitrile (Fig. 2.3 E). The response to the first odor pulse decreased gradually and almost linearly with increasing benzonitrile proportion in the mixture, at each of the three different odor concentrations tested ($R^2 = 0.728$, $n = 6$, $P = 0.019$ for odors at 10^{-4}, $R^2 = 0.827$, $n = 6$, $P = 0.012$ for odors at 10^{-3}, and $R^2 = 0.925$, $n = 6$, $P = 0.002$ for odors at 10^{-2}) (Fig. 2.3 E). Benzonitrile alone elicited weaker mean responses compared with the mixtures, with the exception of mB5000. Responses to the second pulse in BES were smaller than to the first pulse and were independent of odor concentration ($F = 0.365$, d.f. $= 2$, $P > 0.05$) and mixture tested ($F = 0.681$, d.f. $= 10$, $P > 0.05$) (Fig. 2.3 F).

A different pattern of mixture interactions was observed in BES compared with BOR. In BES, the responses to the different mixtures at the three odor concentrations tested decreased almost linearly with increasing benzonitrile proportion, indicating a strong inhibitory effect of benzonitrile per se in the mixtures (Fig. 2.4 C,D). Likewise, the differences between the responses and the lower bounds increased significantly (Pillai's trace: $F = 3.072$, d.f. $= 8$, $P = 0.004$) and almost linearly ($R^2 = 0.724$, $n = 15$, $P = 0.001$) with increasing benzonitrile proportion. This effect became more dramatic when the mixture containing the highest proportion of benzonitrile (mB5000) was tested (Fig. 2.4 C,D).

Differences in odor responses and mixture interactions across individuals

Females varied in their abilities to respond to the two consecutive pulses of odors and in the strength of the mixture interactions taking place inside their ALs. Calcium responses in the glomeruli of some females clearly followed both odor pulses, whereas others only responded to the first pulse (Fig. 2.5 A), indicating a variability in temporal resolution to pulsed stimuli. Further, mixture interactions were variable across individuals. In some females (e.g. females F and G in Fig. 2.5 B) the differences between lower bounds and actual responses to the first stimulus were higher than zero across all mixtures measured, indicating no or very low mixture interactions, whereas for other females (e.g. females B and C in Fig. 2.5 B) the responses to mixtures were lower than the calculated lower bounds, indicating the prevalent presence of strong inhibitory mixture interactions. Analysis of the responses to the reference odor (linalool) indicated that variability between stimulations in the same individual was minimal (data not shown).

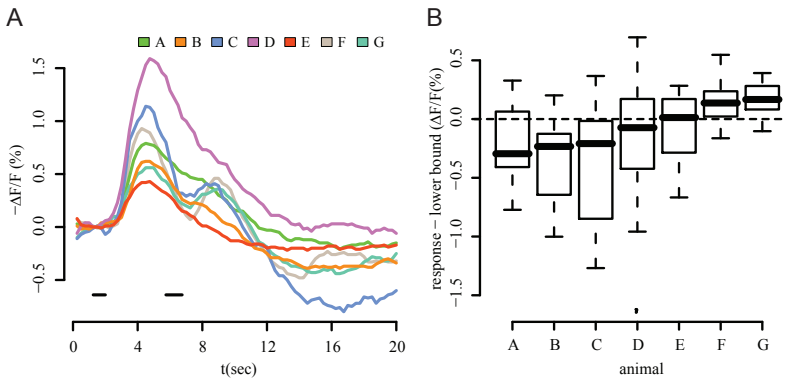

Figure 2.5: (A) Individual time traces recorded inside the BOR glomerulus in response to a representative mixture (mB100, 10–3). Each color represents a different female (N = 7, same individuals as in Fig. 2.3). The stimulations (1 s each) are indicated as black lines. Note the variability across individuals in following repeated stimulations. (B) Inhibitory mixture interactions across mixtures and concentrations (n = 15 stimuli) in glomerulus BOR for each of the seven females tested (A–G). Each box plot shows the difference between the mixture response and the calculated lower bound (median, quartiles and data range. Circles indicate outliers outside 1.5x inter-quartile range). Negative values indicate inhibitory mixture interactions, positive values indicate the lack of inhibitory effects.

2.5 Discussion

In this study we started from a standard synthetic mixture that recreates the natural ratio of peach shoot volatiles as a model blend to quantify the effects of changing ratios of mixture constituents on insect behavior and neurophysiology. We present for the first time empirical evidence that the ratio of constituents in a mixture can be changed to a certain threshold, without affecting behavioral discrimination and olfactory recognition by oriental fruit moths. Calcium imaging revealed that two distinct glomeruli within the AL of the moths process mixture-related information in specific ways and that the responses in one glomerulus resemble the behavioral pattern. Furthermore, we noted individual variations in the behavioral and glomerular responses elicited by the mixtures. The implications of these findings are discussed below.

Behavioral effects of changing ratios of constituents in mixtures

Behavioral bioassays showed that the benzonitrile level in the peach- derived mixture can vary by two orders of magnitude without losing bioactivity. Above and below the odor preference range, attraction to the mixture was no longer significant as most females could not discriminate between this mixture and the blank (solvent). These findings indicate that not only one single ratio of mixture constituents sustains insect

attraction at a single point in time but that the ratio of constituents can vary within a certain range. As volatiles mediating insect attraction to host plants are subjected to seasonal fluctuations in their ratios of release (Dötterl et al., 2005; Johnson et al., 2004; Vallat and Dorn, 2005; Vallat et al., 2005), a relatively broad tolerance to the constituents' ratio could be to the animal's advantage. It could represent an adaptation enabling insects to find suitable host plants for the completion of their life cycle, despite fluctuations in the host plant signal. Indeed, in a closely related species, the codling moth *Cydia pomonella*, attraction of female moths to apple shoots is maintained over several weeks, despite quantitative differences in the composition of the volatile blends over extended periods (e.g. fruiting season) (Vallat and Dorn, 2005). Seasonal periods without attraction were also documented but the underlying factors for this behavior have not yet been identified. Ratio effects, as shown in the present study, might have contributed to the documented variation in seasonal attraction. Seasonality in moth responses to plant tissues have also been recently documented for *Cydia molesta* (Piñero and Dorn, 2009), and ratio effects might also explain this behavioral pattern. A recent paper showed that attraction of female grapevine moths (*Lobesia botrana*) to a host plant-based synthetic blend containing disparate ratios of some compounds did not differ from that to a blend containing the same compounds but at natural ratios (Tasin et al., 2010). The reasons underlying these findings were not investigated. A broad tolerance to ratio constituents as empirically shown in the present study could well explain the similar attraction to the blends. A broad tolerance was also found to mere dilutions of host plant-derived synthetic mixtures tested in wind tunnel bioassays with the sphinx moth *Manduca sexta* (Riffell et al., 2009a). Behavioral responses of the moth did not change over the 1000-fold dilution range tested. In the Caribbean spiny lobster *Panulirus argus*, a model organism in chemically-mediated predator–prey interaction studies, prey-based mixtures sharing all the same components but differing markedly in blend ratios are more attractive than mixtures that have unique components but whose common components have relatively similar blend ratios (Steullet et al., 2002). The authors argue that because the quality and quantity of any prey-related odor stimulus varies over time and space, an animal would benefit from being able to filter out small differences in odor composition. Honeybees perceived odor differences among cultivars of snapdragon (*Antirrhinum majus*) differing only in volatile ratios but not among flowers of the same cultivar (Wright et al., 2005). The authors postulated that the perceptual qualities that arise from the ratios of volatiles might be a function of the magnitude of the ratio, such that greater differences in the ratios from one cultivar to another would be easier to perceive than small differences in ratios across conspecific flowers. Thus, olfactory systems of insects and crustaceans, both invertebrate taxa, might have a similar broad tolerance to constituent ratios. For vertebrates, particularly humans, current evidence seems to point to very precise ratios of constituents needed for odor recognition (Le Berre et al., 2008). It would be interesting to test if broad

tolerance to blend constituent ratios also exists in vertebrates.

Odor representation of plant-derived mixtures varying in constituent ratios

Odor-evoked responses in the female moths ALs mirrored behavioral responses to manipulations of the benzonitrile ratio in the mixture in a particular glomerulus (BOR). This finding supports previous evidence based on a different glomerulus, that representation of olfactory information in the AL of this moth species correlates with behavior (Piñero et al., 2008). Similar correlates of neural activity and behavior have also been reported for M. sexta female moths in response to plant-derived olfactory stimuli (Riffell et al., 2009b), and for *Heliothis virescens* and *Helicoverpa zea* males in response to the female pheromone (Vickers et al., 1998).

The two most active mixtures at the behavioral level, i.e. mB1 and mB100, were similarly represented in the BOR glomerulus. Hence, despite some quantitative changes in fruit tree odors with progressing season, along with changes in ratios between constituents (Vallat and Dorn, 2005), behaviorally active natural odors might elicit similar perception correlates in insects along the olfactory pathway. A second glomerulus (BES) also responded to manipulations of benzonitrile ratios in the mixture but in a manner that did not reflect behavior. Even minute concentrations of benzonitrile in the mixture led to inhibitory interactions. Thus, this glomerulus seems to be highly sensitive to benzonitrile, suggesting that the corresponding receptor is similarly sensitive. Benzonitrile also evoked inhibitory mixture interactions in BOR but only when present beyond behavioral threshold levels (mB1000 and mB5000). Hence, the inhibitory effect of benzonitrile was more striking in BES glomerulus than in BOR. The finding that two different glomeruli process mixture-related information in specific ways indicates that odor processing inside the ALs of oriental fruit moth females is not uniform. Further, it suggests that interactions across these two glomeruli, and perhaps other glomeruli not yet identified, and between local and projection neurons might take place in the ALs prior to final odor processing and integration in higher-order brain centers (e.g. mushroom bodies). Experimental evidence of AL neuronal circuitry and synaptic interactions in the AL has been already provided for other insect species, including moths (Christensen et al., 1989, 1993; Vickers et al., 1998) and locusts (Geffen et al., 2009; Bazhenov et al., 2005; Laurent et al., 2001). Response properties rather than spatial position seem to determine connectivity between glomeruli (Linster et al., 2005; Reisenman et al., 2008), and local AL circuitry seems to play an important role in shaping projection neuron responses in *Drosophila sp.* (Olsen and Wilson, 2008; Olsen et al., 2007; Silbering and Galizia, 2007; Silbering et al., 2008). Because BOR and BES glomeruli have overlapping odor response profiles despite being spatially distant, it is likely that they are interconnected via local interneurons and that the activity in

the BOR glomerulus contributes to the response profile of the BES glomerulus, and vice versa. It would be interesting to further investigate (a) network effects inside the ALs of the moths to test for interconnectivity between the BOR and BES glomeruli, and (b) the type of information conveyed by projection neurons into higher-order brain centers.

In their natural environment, moths are exposed to temporally complex odor stimuli while flying through turbulent odor plumes. Our stimulus protocol – two intermittent odor pulses at low frequencies – was designed to mimic the encounter to a second odor filament in such a flight. Therefore, we analyzed glomerular responses to the second pulse of our stimulus to investigate how the olfactory system of female oriental fruit moths can resolve consecutive pulses of the same odors. For both the BOR and BES glomeruli, the responses to the first and second odor pulse differed, particularly at high benzonitrile proportions. For most animals, a very strong response to a first pulse is followed by a very weak (and in some cases non-existing) response to a second pulse. Furthermore, while responses to the first pulse showed a clear dose–response relationship, response amplitude to the second pulse depended much less on odor concentration, particularly at high benzonitrile ratios, and in at least some females. Low or no responses to the second pulse of odors were probably the result of receptor adaptation (Kaupp, 2010) and/or other processes such as interglomerular inhibition (Reisenman et al., 2008), and need additional studies.

Individual variations in behavioral responses and odor representation in the ALs

We found substantial differences in terms of behaviour (i.e. benzonitrile ratio tolerance) and neurophysiology (i.e. ability to resolve consecutive odor pulses and mixture interaction patterns inside the AL) across individual females despite their similarity in age, mating and feeding status. They were all derived from a colony kept in the laboratory for two years, and were devoid of any previous host plant odor experiences. The origin of variability across individuals is not due to variation between stimulations, as indicated by analysis of the responses to the reference odor (linalool). Thus, the observed variability could only derive from variability between individuals. Although variability is a common feature known to all experimentalists, individual variability might also be an adaptive trait, and advantageous for the species (Keil et al., 2001). In the case of female moths, variations in response to host plant-derived odors across individuals could (a) help dealing with qualitative and quantitative fluctuations in plant odor signals, and (b) prevent plants evading herbivore attack. Such variation is likely genetically based. Our experiments were not designed to explore this postulate, but results are consistent with published findings. In the hymenopteran parasitoid

Cotesia glomerata, individuals from different full-sib families differed strongly in their response to odors from host-infested plants (Gu and Dorn, 2000). This difference in olfactory responses has a genetically basis (Wang et al., 2003), and it influences both host recognition and parasitism (Wang et al., 2004).

2.6 Acknowledgments

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CHAPTER 3

The Speed of Smell: Odor-Object Segregation within Milliseconds

3.1 Abstract

Segregating objects from background, and determining which of many concurrent stimuli belong to the same object, remains one of the most challenging unsolved problems both in neuroscience and in technical application. While this phenomenon has been investigated in depth in vision and audition it has hardly been investigated in olfaction. We found that for honeybees a 6-ms temporal difference in stimulus coherence is sufficient for odor-object segregation, showing that the temporal resolution of the olfactory system is much faster than previously thought.

3.2 Introduction

Most natural odors consist of many components, though they are perceived as unitary odor-objects (Jinks and Laing, 2001). Because airborne odorants intermingle and fluctuate at fast timescales (Murlis and Murtis, 1992; Riffell et al., 2008), the olfactory system needs to segregate concurrent odors from independent sources in order to recognize them as different odor-objects (Stevenson and Wilson, 2007). This problem is analogous to figure-ground segregation in vision (Treisman, 1996) and concurrent sound segregation in audition (Carlyon, 2004). Both, the visual and auditory system analyze temporal coherence between stimuli for object segregation (Carlyon, 2004; Blake and Lee, 2005). It is not known whether odor-object segregation is also based on temporal stimulus coherence. Studies on mixture processing in honeybees and other species demonstrated that mixtures have a perceptive quality that is different from

		Channel1	Channel1	Difference (Ch.1 - Ch.2)
Time to 10 %	EAG 1	75.9 ± 1.6	75.8 ± 1.3	0.1 ± 2.0
	EAG 2	74.8 ± 1.5	74.5 ± 1.9	0.3 ± 2.4
Time to 30 %	EAG 1	87.2 ± 1.2	87.0 ± 1.0	0.2 ± 1.5
	EAG 2	84.7 ± 1.4	84.4 ± 1.3	-0.4 ± 2.0
Time to 63 %	EAG 1	101.9 ± 1.6	102.4 ± 1.7	-0.5 ± 2.3
	EAG 2	97.5 ± 1.8	97.6 ± 1.6	-0.1 ± 2.3
Rise time 10-90 %	EAG 1	44.2 ± 2.9	46.0 ± 4.0	-1.5 ± 5.9
	EAG 2	38.5 ± 1.7	39.7 ± 3.0	-1.2 ± 3.3

Table 3.1: Temporal characteristics of EAG responses. Time intervals between channel openings and reaching 10, 30 or 63 % of amplitude maxima, and rise time, measured as time required for the EAG to rise from 10 to 90 % (means and standard deviation, all data in ms). EAG 1 and EAG 2 are two EAG recordings (same as in Fig. 3.1). The differences are calculated for all possible pairs of channel 1and 2 (EAG 1: 26 recordings per channel, 676 pairs; EAG 2: 28 recordings per channel, 784 pairs).

their components (Chandra and Smith, 1998; Linster and Smith, 1999; Smith, 1998; Deisig et al., 2003; Lachnit et al., 2004; Eschbach et al., 2011), thus making it difficult to recognize odor-objects from mixtures. These studies only considered static step like stimuli. Rapid odorant fluctuations, however, contain information that can be used for odor-source tracking (Vickers, 2000; Justus et al., 2002; Cardé and Willis, 2008; Andersson et al., 2011). Accordingly, information contained in the fast temporal structure of odorant stimuli might be used to segregate an odor-object from a mixture (Stevenson and Wilson, 2007; Hopfield and Gelperin, 1989).

To address this idea, we asked whether honeybees can use short temporal differences between two components of a binary odorant mixture to extract information about its components. We first trained honeybees to respond to an odorant A by pairing A with a sugar reward (Bitterman et al., 1983). Then, we tested memory retrieval with a mixture of A and a novel odorant B. We found that a 6 ms asynchrony in the onset of A and B is sufficient to enhance the salience of the component odor information, and that it is not necessary that the component in question was presented alone at anytime during the stimulus.

3.3 Results

Studying the effect of millisecond time-differences in stimulus coherence on the perception of odorant mixtures requires temporally precise odorant stimuli. In our experiments, we mixed two odorants with an onset or offset delay of 6 ms. We therefore tested the temporal precision of odorant delivery in this time range using electroantennogram (EAG) recordings. Odorant stimuli evoked EAG responses with fast and reproducible response dynamics (Fig. 3.1 A). The rise time (10 to 90 %) was less than 50 ms, and

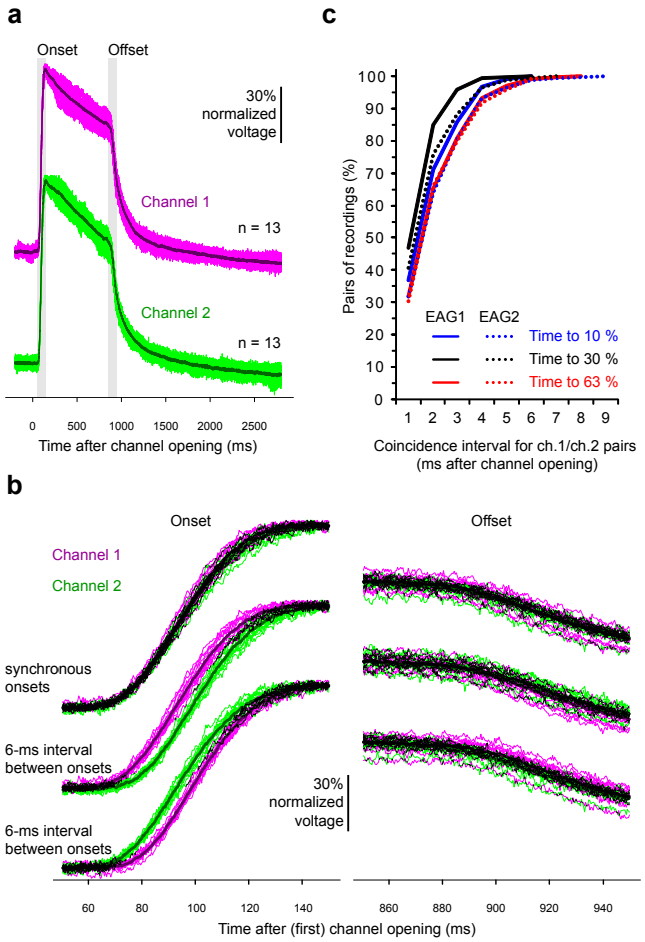

Figure 3.1: Temporal characteristics of the odorant stimuli. (a) Electroantennogram (EAG) response to odorant stimuli delivered by channel 1 (magenta, shifted up for clarity) and channel 2 (green) of the olfactometer. 13 single measurements and superimposed mean (dark trace). Stimulus duration was 800 ms. Channel 1 and 2 were measured sequentially. (b) Blow-up of the stimulus onset and offset (shaded period in (a)), shifted vertically for clarity. Top: Channel 1 and 2 opened and closed simultaneously (data from (a)). Middle: Channel 2 opened and closed 6 ms after channel 1. Bottom: Channel 1 opened and closed 6 ms after channel 2. N= 13 measurements each. To detect possible mechanical effects of opening two channels in the incoherent mixture, a blank channel was opened 6 ms before or after the opening of the tracer channel (middle: blank opened 6 ms after channel 1 or 6 ms before channel 2, bottom: vice versa). All traces were normalized to the amplitude maximum. (c) Percentage of EAG recordings for pairs of channel 1 and 2 that reached either 10, 30 or 63 % of the amplitude maximum within a given coincidence interval. EAG 1 (26 recordings per channel, 676 pairs, same data as in (a) and (b)) and EAG 2 (28 recordings per channel, 784 pairs) show data from two independent EAG recordings.

the difference in reaching 30 % of amplitude maxima between two odor channels was 0.4 $\pm$ 2 ms (mean $\pm$ standard deviation) (Table 3.1). The 6-ms interval between the opening of channel 1 and 2 used for our mixture experiments was clearly visible in the

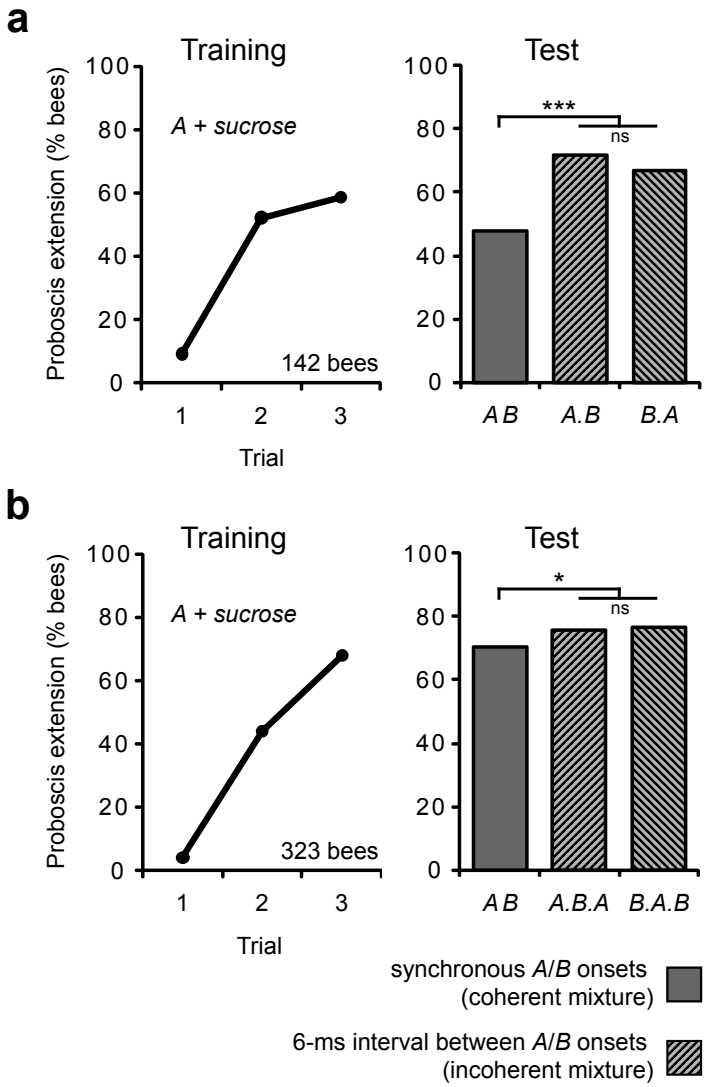

Figure 3.2: A 6-ms temporal difference in stimulus coherence is sufficient for odor-object segregation. (a) Each bee received 3 rewarded training trials with A, and the percentage of bees showing odor-evoked proboscis extension is shown. Odorant stimulus duration was 800 ms. During the memory test, odorants A and B were presented simultaneously (coherent mixture, AB) or with a 6-ms interval between their onsets (incoherent mixture). One incoherent mixture started with A (A.B), the other with B (B.A). Test stimulus sequence was randomized. The proboscis extension rate for the incoherent mixtures was higher than for the coherent mixture (one-way RM ANOVA; $F_{(2,\ 425)} = 17.1$, $p < 0.001$, Holm-Sidak posthoc test; $N = 142$). (b) Same experimental protocol as in (a) but odorant A was presented against the background of odorant B (B.A.B) and odorant B against odorant A (A.B.A). Background-odorant lasted 806 ms, starting 6 ms before and stopping 6 ms after the 794-ms long foreground-odorant. The proboscis extension rate for the incoherent mixtures was higher than for the coherent mixture ($F_{(2,\ 968)} = 4.7$, $p < 0.01$; $N = 323$). Experiments in (a) and (b) were done at different times of the year, and the response difference during training and testing to AB might reflect seasonal differences in learning and memory performance. $***$, $p<0.001$; $*$, $p<0.02$.

onset of the EAG responses (Fig. 3.1 B). The offset, however, was less precise and the 6-ms interval could not reliably be reproduced. When opening channel 1 and 2 simultaneously more than 40 % of the EAG signals coincided within 1 ms and more than 75 % coincided within 2 ms in reaching 30 % of the maximum (Fig. 3.1 C).

Bees were trained to associate an odorant A with a sugar reward, learning to extend their mouthparts (proboscis) in response to the odorant and in anticipation of the reward (3 trial classical conditioning, Fig. 3.2). Thirty minutes after training, odorant A was presented in temporally coherent (synchronous odorant onset and offset) or incoherent (asynchronous odorant onset and offset, 6 ms delay) mixtures with a new odorant B. How much a bee "recognized" A in the mixture was assessed by its proboscis extension response. We first tested whether a 6-ms interval between the on- and offsets of A and B would facilitate their segregation from the mixture (Fig. 3.2 A). Bees' response rates to the incoherent mixtures A.B (odorant A first) and B.A (odorant B first) were significantly higher than to the coherent mixture AB. Interestingly, there was no statistically significant difference between A.B and B.A. This data suggests that bees either use temporal incoherence or the 6-ms presence of a pure odorant, or both to segregate a component odorant from a mixture. To distinguish these alternative explanations, we modified the test and presented either A against the background of B (B.A.B; B onset 6 ms before A onset, A offset 6 ms before B offset) or B against the background of A (A.B.A) (Fig. 3.2 B). Most bees which learned during the training did not discriminate between the coherent mixture AB and the incoherent mixtures A.B.A and B.A.B, and 79 % responded equally to the three mixtures. However, the response rates for the incoherent mixtures were higher than for the coherent mixture, and it did not matter whether A or B was used as background. Again, we found no difference between A.B.A (a situation where, for 6 ms, A could be smelled alone), and B.A.B (a situation where A is never presented alone). These results indicate that bees use temporal incoherence rather than the 6-ms presence of a pure odorant for odor-object segregation.

3.4 Discussion

One of the most intriguing capacities of our brain is the so-called cocktail party effect: the possibility to extract the voice of our conversation partner amidst a cacophony of different voices and sounds. This is particularly impressive given the strong overlap in the frequency range, and hence the receptor neuron activation, of the different sound sources that the brain is able to segragate. It is believed that this capacity of the brain is based on an analysis of the fine-scale temporal structure and coherence of the different sources (Carlyon, 2004). Similar effects have been shown for the visual

modality, in particular for object segregation in dynamical visual fields (Blake and Lee, 2005). In the acoustic system of humans, delays of 30 ms are sufficient to hear that two different sources are causing a sound (Bregman and Pinker, 1978), while the human visual system requires delays of 6 ms (Sekuler and Bennett, 2001). Even though physiological responses to odor-mixtures with asynchronous onset has been studied to some extent (Broome et al., 2006), and the dynamical response properties of olfactory receptor neurons are known for some species (de Bruyne et al., 2001; Justus et al., 2005; Schuckel et al., 2008; Spors et al., 2006), only one behavioral study about dynamical odor-object segregation is known to us (Hopfield and Gelperin, 1989).

After conditioning to respond to an odorant A, honeybees were more likely to respond to a mixture of A and a novel odorant B if the onsets of A and B were shifted by 6 ms. From this result we conclude that the short time difference between the onsets of two overlapping odorant stimuli facilitates their segregation. An alternative conclusion would be that the 6-ms time difference between odorant stimuli increases the mixtures‘ saliency due to mechanical interference between the channels of the olfactometer. We therefore took great care in designing an olfactometer that produces odorant pulses free of mechanical interferences (Szyszka et al., 2011). The opening of an empty channel 6 ms before or after an odor channel did not produce any visible disturbance in EAG recordings (Fig. 3.1 B).

We conclude that honeybees can detect temporal incoherence between odorant stimuli in the millisecond range and use this information to extract odorants‘ identity. This seems a remarkable performance considering that the sense of smell is regarded to be a relatively slow sense as compared to the auditory or visual senses. Odor discrimination tasks in different species showed that 200 to 600 milliseconds are required for odor recognition (Ditzen, 2003; Uchida and Mainen, 2003; Abraham et al., 2004). Thus, the insect olfactory system reveals a hitherto unknown fast-processing property. Our findings open new perspectives for the study of odor-object perception, and suggest mechanisms that allow us to recognize a whiff of perfume in a mall full of other odorants.

It will be interesting to examine the physiological mechanisms underlying odor-object segregation. In Drosophila olfactory receptor neurons can encode the dynamics of odorants that fluctuate as fast as 100 Hz (Schuckel et al., 2008, 2009; French et al., 2011), and in locust neural representations of mixtures partly match those evoked by the individual components if their onsets differ by 100 ms (Broome et al., 2006). It remains to be shown whether this also holds true for the bee and for onset-differences of just a few milliseconds. Olfactory coding follows similar rules across animal species from mammals to insects (Ache and Young, 2005). Therefore, these mechanisms might be generalizable to mammalian olfaction, another hypothesis that remains to be tested. Moreover, they could be used to develop control algorithms for autonomous odor-source tracking robots.

3.5 Materials and Methods

We used 1-hexanol and 1-nonanol (diluted 1:100 in mineral oil; all from Sigma-Aldrich) as odorant stimuli. 1-hexanol and 1-nonanol were equally often used as odorant A and odorant B. As a reward during training we presented a 3-s long sucrose stimulus (1 M in water) which started 1.2 s after odorant offset. The intertrial interval was 10 minutes. Thirty minutes after the end of training odorant A was presented in temporal coherent and incoherent mixtures with a new odorant B. The sequence of the mixture stimulation was balanced across bees to exclude sequence-effects, and the experimenter was blind for the stimulus identity.

The olfactometer consisted of three channels. Through each channel air (300 ml/min) was injected into a carrier air stream (2100 ml/min). During the conditioning experiments, channel 1 was used for 1-nonanol and channel 2 for 1-hexanol. The exit diameter of the olfactometer was 6.8 mm, resulting in airspeed of 138 cm/s. Bees were placed 2 cm in front of the olfactometer. A more detailed description of the olfactometer and conditioning procedure is given in [27].

The temporal characteristics of the odor stimuli were measured with electroantennogram (EAG) recordings 2 cm in front of the olfactometer (7 cm away from where the channels are injected into the carrier airstream). Two EAG recordings were done, each with a single bee antenna (EAG1, EAG2). 10 µl of pure 2-heptanone was used as tracer odorant. The 4 different stimuli (channel 1 and channel 2; 0 and 6 ms delays) were presented in an alternating sequence and the interstimulus interval was 30 s. For EAG recordings a single antenna was cut in the middle of the scapus and was mounted with conductive gel (GEL+, Ritex) between the two poles of a stainless steel electrode (Kombi PROBE, Syntech). The signal band-pass filtered for the 0.1 Hz to 3 kHz range (AM 502, Tektronix) and digitized at a sampling rate of 2500 Hz (Digidata 1200, Axon Instruments). EAG signals were normalized to the amplitude maximum to correct for changes in response strength and the baseline was shifted to zero to correct for baseline drifts. Data was analyzed with R (R Development Core Team, 2011). Similar measurements were done with a photoionization device (Vetter et al., 2006) to exclude biological influences, with comparable results (data not shown).

3.6 Acknowledgments

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CHAPTER 4

Millisecond Stimulus Onset-Asynchrony Enhances Information about Components in an Odor Mixture

4.1 Abstract

Airborne odorants rarely occur as pure, isolated stimuli. In a natural environment, odorants that intermingle from multiple sources create mixtures where on- and offset of odor components are asynchronous. Odor mixtures are known to elicit interactions in both behavioral and physiological responses, changing the perceptive quality of mixtures as compared to the components. However, relevant odors need to be segregated from a distractive background. Honeybees can use stimulus onset asynchrony of as little as 6 ms to segregate learned odor components within a mixture. Using in vivo calcium imaging of projection neurons in the honeybee, we studied neuronal mechanisms of odor-background segregation based on stimulus onset asynchrony in the antennal lobe. We found that asynchronous mixtures elicit response patterns that are different from their synchronous counterpart: The responses to asynchronous mixtures contain more information about the constituent components. With longer onset shifts, more features of the components were present in the mixture response patterns. Moreover, we found that the processing of asynchronous mixtures activated more inhibitory interactions than the processing of synchronous mixtures. This study provides evidence of neuronal mechanisms that underly odor-object segregation, on a timescale much faster than found for mammals.

4.2 Introduction

Many tasks in an animal's life involve the detection of meaningful stimuli in a distractive environment. When stimuli occur together, object recognition requires grouping and segregation of stimuli. Sensory systems use stimulus asynchrony for segregation in vision (Usher and Donnelly, 1998; Hancock et al., 2008) and audition (Zera and Green, 1993; Bronkhorst, 2000; Lipp et al., 2010). It is unknown how the olfactory system segregates stimuli on the basis of asynchrony. The temporal relationship between components of a mixture contains information about the number of odor sources, potentially decisive for the ability to segregate the components from it (Stevenson and Wilson, 2007) and theoretically sufficient for a neural network to perform source separation (Hopfield, 1991). We therefore distinguish between two mixture qualities: Odorants from the same source form mixtures with fixed concentration ratios and will be referred to as synchronous mixtures. Odorants from different sources mix in a complex way (Riffell et al., 2008) and comprise delays between its components, i.e. changing concentration ratios. They will be referred to as asynchronous mixtures.

Synchronous mixtures often induce inhibitory mixture interactions in the principal neurons of the insect antennal lobe (Joerges et al., 1997; Galizia et al., 2000; Deisig et al., 2006; Silbering and Galizia, 2007; Deisig et al., 2010; Najar-Rodriguez et al., 2010; Silbering et al., 2008) or the vertebrate olfactory bulb (Tabor et al., 2004; Grossman et al., 2008). Inhibitory mixture interactions have been implicated with synthetic (or configural) odor processing, which leads to a loss of component information (Chandra and Smith, 1998; Smith, 1998; Deisig et al., 2002; Coureaud et al., 2009).

Perception and processing of asynchronous mixtures has been barely studied, and it is unknown how the brain uses millisecond stimulus-asynchrony for odor-background segregation. Some studies suggest that processing of odor mixtures becomes more analytic (or elemental), which enhances information about the components, when the components are applied asynchronously (Hopfield and Gelperin, 1989; Baker et al., 1998). Honeybees can segregate components from a mixture better when they are presented with an offset of 6 ms, producing an asynchronous mixture (Szyszka et al., 2012). In locusts, overlapping odor sequences evoke spatiotemporal patterns in the antennal lobe (AL) neurons that differ from both the single components and the synchronous mixture (Broome et al., 2006).

Using the same stimuli as Szyszka et al. (2012), we investigate neuronal responses to synchronous and asynchronous odor mixtures with calcium imaging of projection neurons (PNs) to answer the following questions: Are inhibitory mixture interactions dependent on the timing of the components? And, do responses to asynchronous mixtures contain more information about the components than responses to synchronous mixtures? Our results show that PNs can resolve ms-stimulus asynchrony and we

conclude that olfactory object segregation is possible at the level of the AL.

4.3 Materials and Methods

Animals

Free flying honeybee foragers (*Apis mellifera*) were used in summer. During winter, a hive was kept in a moistened and heated flight room with 12 h visible & UV-light / 12 h dark regime supplied with sucrose and pollen. Foragers (females) were caught at the hive entrance (summer bees) or from the ceiling of the flight room (winter bees), immobilized on ice and mounted in custom-made acrylic glass stages with Deiberit adhesive wax (Dr. Böhme & Schöps, Goslar, D).

Calcium imaging

Oregon Green-dextran (Kd = 1180 nM, 10000 MW, Invitrogen, Eugene, OR, USA) was dissolved in a water droplet on a microscope slide to a viscous solution and applied to the tip of the glass needles, which were pulled on a Sutter horizontal puller (P-87, Sutter Instruments, Novato, CA, USA). Antennae were sticked to the forehead with Eicosane (Sigma-Aldrich, Steinheim, D). The head capsule was removed with a razor splint between compound eyes, antennae and the medial ocellus. Glands and tracheae were removed from the mushroom body calyces. The dye was injected into the brain at the junction of the calyces of the mushroom body into the antenno-protocerebral-tract that contains the axons of PNs, and allowed to travel along the axons overnight. The next day, glands and tracheae were removed from the ALs. In order to reduce movement, the esophagus was extended with forceps through a cut above the labium, and the abdomen was immobilized with a piece of sponge. The brain was covered by a thin layer of two-component silicone (KwikSil, World Precision Instruments, Sarasota, FL, USA). A plastic coverslip separated the antennae from the imaging area in order to keep them dry and accessible for odor stimulation. The temperature at the setup was set to 28 C.

Odors and Olfactometer

Odor stimuli generally consisted of 800 ms long square pulses of odorants. Synchronous mixtures were created by opening the valves of two odorants at the same time. Asynchronous mixtures were created by applying the odorant pulses with a time delay of different length, resulting in overlapping stimuli with either hexanol leading and nonanol

trailing, or *vice versa*. For an overview of the used stimuli, see Fig. 4.1. Linalool (CAS 78-70-6), 1-hexanol (CAS 111-27-3, 99,9 %), and 1-nonanol (CAS 143-08-8, 98 %, all Sigma-Aldrich, Steinheim, D) were diluted 1:100 in mineral oil (Sigma-Aldrich) and kept in glass vials with argon or nitrogen atmosphere to prevent oxidation. 100 µl odor solution were placed on a cellulose pad (SugiPad, Kettenbach GmbH, Eschenburg, D) in a 3 ml plastic syringe (NormJect, HSW GmbH, Tuttlingen, D), with the plunger set to 2.5 ml. Syringes were placed in a custom-build 4-channel olfactometer (Szyszka et al., 2011). Fresh odor syringes were used for every day of experiments. Each odor channel was set to 300 ml/min flow volume, which was injected into a carrier airstream of 1800 ml/min. The airspeed at the outlet was 1.4 m/s. Solenoids were selected in pairs in order to achieve onset accuracies of 1 ms. Solenoid switch was controlled from a different computer than the one used for data acquisition. Stimulus control software was written in LabView 8.0 (National Instruments, Austin, TX, USA), allowing control pulse application with sub-µs accuracy. The solenoid switch pulse consisted of a 1 ms long 24 V pulse followed by a 12 V hold for the time of the stimulus (spike and hold), using custom electronics. This circuitry allows for fast and temporally precise stimulus control. The olfactometer was placed 1 cm in front of the bees' antennae. We used two odor stimulation blocks. The first block consisted of the components, the synchronous mixture and the 6 ms-asynchronous mixtures. The second block consisted of the solvent control, a diagnostic stimulus (linalool), followed by the stimuli from the first block, pseudorandomized with and completed by asynchronous mixtures with 50 and 200 ms delay. Since the first odor responses of an experiment are known to be highly variable (Stopfer and Laurent, 1999) and we only gave a subset of stimuli during the first block, we only analyzed the responses of the second experiment block.

Data acquisition and analyses

We recorded 203 glomeruli in 14 bees, an average of 14.5 glomeruli per bee, with a standard deviation of 4.4 glomeruli per bee. Bees were imaged with an Olympus BX50WI Microscope, equipped with a XlumPlanFL 20x, NA = 0.95 W objective. Excitation light was set to 488 nm. Emission was filtered with a GCamp Filter Set (495 Dichroic Mirror & 505 long pass filter), recorded at 170*128 pixel (437*329 µm) and 12 bit depth with an imaging system (IMAGO QE CCD Camera and Till Vision software, Till Photonics, Munich, D). Each measurement consisted of 200 frames measured at a frame rate of 20 Hz, for a total length of 10 seconds. Time between measurements was 60 seconds. Raw data movies were processed with custom written programs in IDL (RSI, CO, USA) to execute offline movement correction, logarithmic bleaching correction and a mean time filtering with a kernel size of 3. Signals were calculated as relative fluorescent changes $\frac{\Delta F}{F}$. For visualization in color-coded images, odor re-

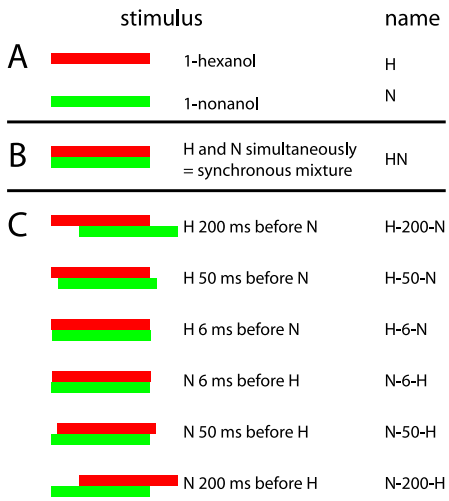

Figure 4.1: **Overview of the stimuli used in an experiment.** Odorant pulses of 800 ms were given alone (A), together (B, synchronous mixture), or with a time delay between them (C, asynchronous mixtures).

sponses were additionally median filtered in space with a kernel size of 3, and response strength was defined as the maximal signal change within 4 seconds after stimulus onset. Glomerular response traces were calculated within a square of 7*7 pixel (approx. 18 µm side length) placed on the individual glomeruli (Fig. 4.2 A). We used an algorithm to segment glomeruli (Strauch et al. (2012), Fig. 4.2 B) and identified a subset of them by means of their position, using the honeybee AL standard atlas (Galizia et al., 1999a). The constant pixel number allows for a direct comparison of spontaneous activity (standard deviation of the signal before stimulus onset) and response strength. Average response traces show the mean calculated between subjects.

Correlation Analyses

Correlation matrices show the time-resolved similarity within (autocorrelation) and between (crosscorrelation) odor response patterns (Fig. 4.5). Response patterns were represented as vectors consisting of the 203 glomeruli recorded in 14 bees. Every pixel in the matrix gives a Pearson's correlation coefficient of two response patterns during one time point (measured response frame). The diagonal represents the correlation of response patterns at the same time point. Pixelwise deviation from the diagonal shows the correlation of the two response patterns when shifted frame-wise. Correlation traces were extracted from the matrices for frame 64 (150 ms after stimulus onset) of the component response.

Principal Component Analysis

Principal components were calculated on the 203-dimensional hyper-volume spanned by the analyzed glomeruli (Fig. 4.6). Principal component analysis was performed with the "prcomp" function of the package "stats" in R (R Development Core Team, 2011). The transformation matrix was calculated on a time window within the initial 200 ms (frames 62 - 65) of the odor responses to the components, the synchronous mixture and the 6 ms-asynchronous mixtures. We deemed this time window as relevant because neurons one or two synapses downstream of PNs respond within this time (Szyszka et al., 2005; Strube-Bloss et al., 2011, 2012). The 2-dimensional $m * n$ loading matrix L was calculated as

$$L_{ij} = \frac{\sum_{k=1}^{p} R_{ik} X_{kj}}{s_j^2 * d}$$

where $p = 203$ is the number of glomeruli, which is the column number in the response matrix R. The rows of R contain the concatenated response traces (1 frame per row, for a total of 1800 rows) to the different odor stimuli. X is the transformation matrix from the PCA applied on the stimulus subset. $1 \leq i \leq m$ is the row index of R and L and $1 \leq j \leq n$ is the column index of X and L. s is a row vector containing the standard deviations of the principal components, and d is an integer value giving the degree of freedoms.

Statistical analyses

A glomerulus calcium signal was considered as a response when the mean response during 4 seconds after stimulus onset was at least 2.5 times greater than the noise level of that glomerulus. The noise level was defined as the standard deviation of the signals before stimulus onset. Response strengths of the asynchronous mixtures were compared with the response strengths of the synchronous mixture using a repeated measurements ANOVA with a posthoc test (Holm-Sidak alpha-value adjustment) with R and SigmaStat (Systat, San Jose, CA, USA). The global significance level was set to $p = 0.05$.

Additional experiment

A subset of experiments (17 bees) were performed (Data from Fig. 4.4) with the following deviations for the Methods: We used 1-octanol (CAS 111-87-5) and 2-heptanone (CAS 110-43-0, both p. a. quality, Sigma Aldrich, Germany) as odors, an olfactometer with 6 instead of 4 channels, Fura-2 (invitrogen) instead of Oregon Green, and aquired the data ratiometrically by taking double frames with excitation wavelengths of 340 and 380 nm. Excitation and emission light were separated with a 420 nm dichroic

mirror and a 490 - 530 nm emission filter. The signal was calculated as $\Delta\frac{340}{380}$. Frame rate was 5 Hz instead of 20 Hz.

4.4 Results

Odor and post-odor responses are stimulus- and glomerulus specific

We stimulated the antennae with hexanol (H), nonanol (N), and their synchronous and asynchronous mixtures (Fig. 4.1) and recorded calcium signals of the PNs in 203 glomeruli in 15 ALs of 14 animals. 57 glomeruli responded to H only, and 14 glomeruli responded to N only. 26 glomeruli responded to both components, and 106 responded only weakly (less than 2.5 standard deviatons) or did not respond (Fig. 4.2 A). Strongest excitatory responses to H were recorded in glomeruli 28, 36 and 38, whereas for N, the strongest excitatory responses were recorded in glomeruli 17 and 33 (Fig. 4.2 C, D, E). Time courses of responses were also both glomerulus- and odor-specific. For example, responses of glomerulus 17 exhibited a slow decay to stimulation with N, and a faster decay to stimulation with H. Responses of glomerulus 28 were strong to H with a weak excitatory post odor response, and weak to N without a post odor response. Responses of glomerulus 29 consisted of a short excitatory phase followed by a strong inhibition already during ongoing odor stimulation with both H or N. Furthermore, it showed the strongest spontaneous activity. Responses of glomerulus 33 to N were excitatory during stimulation, followed by a weak but persistent inhibitory period after the stimulus, which was absent when stimulated with H. These odor and post-odor responses were consistent across animals and correspond well with those described in previous studies, e. g., compare responses to N in Fig. 5 C in Szyszka et al. (2011).

Inhibitory mixture interactions in synchronous mixtures

Generally, glomeruli that responded to one component, also responded to the synchronous mixture. In most glomeruli, the response pattern to a synchronous mixture resembled the response to the stronger component (Fig. 4.2 D,E). Averaged over all glomeruli, however, the response to the mixture was weaker than the response to the stronger component (Fig. 4.3 Ai). The average glomerular response to H was stronger than the response to N. This partly reflected the fact that less glomeruli respond to N than to H (Fig. 4.2 A). The average response to the synchronous mixture was lower than the response to the stronger component ($p = 0.02$, paired student's t-test). The mean value for the stronger component is higher than the mean value for H because

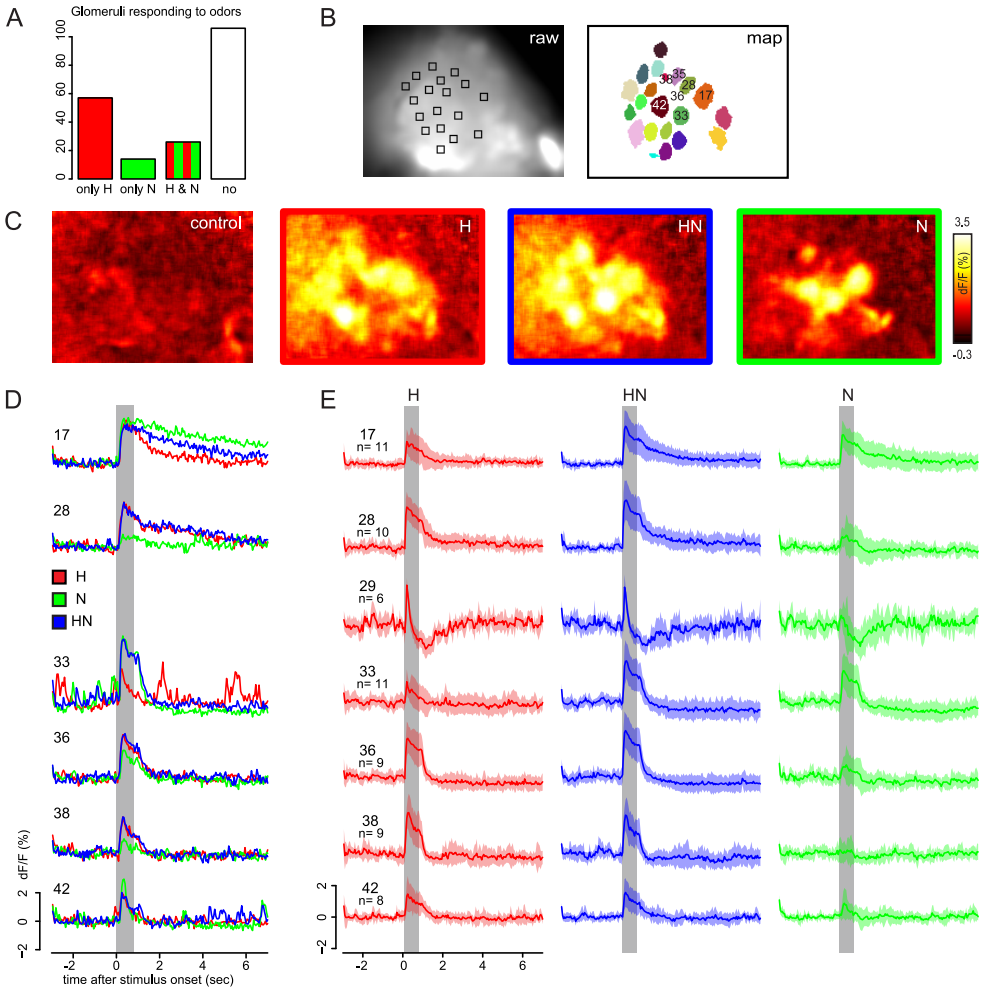

Figure 4.2: **Calcium signal from responses to hexanol (H), nonanol (N) and the synchronous mixture (HN).** A) Number of glomeruli that responded either to H only, to N only, to both odors, or to neither of them. See methods for the criteria after which a signal was considered as a response. B) Left: Example of a raw fluorescence snapshot of an AL at 488 nm excitation. Squares indicate regions of interest from which traces were extracted. Right: A glomerular map attained from the data movies by the algorithm described in Strauch et al. (2012). C) Color coded images show odor responses in an individual AL as relative calcium changes to mineral oil (control), the two components (H and N) and the synchronous mixture (HN). D) Response time courses of 6 glomeruli from the same individual AL as in B and C to the components and the synchronous mixture. Numbers indicate the identity of T1-glomeruli as described in Galizia et al. (1999a). E) Response time courses of identified glomeruli averaged across animals. Traces show mean ± standard deviation. Grey bars indicate the odor stimulus. We did not identify glomerulus T1-29 in the specimen shown in B-D.

in some glomeruli, N was stronger than H. The inhibitory mixture interaction for synchronous mixtures could not be related to specific identified glomeruli, indicating that the response strength of the entire AL is weaker than expected by the response of the individual components, or that inhibitory mixture interactions occur in different glomeruli in different animals. The latter interpretation is consistent with the finding that the interglomerular inhibitory network is variable across animals (Girardin and Galizia, 2012).

Temporal stimulus properties influence inhibitory interactions

Mixture interactions are partly generated by a network of inhibitory neurons (Joerges et al., 1997; Silbering and Galizia, 2007; Deisig et al., 2010). We were interested if the processing of asynchronous mixtures elicits higher or lower activity in the inhibitory network than the processing of synchronous mixtures. Higher or lower inhibitory network activity would result in smaller or larger responses across all glomeruli, respectively. Indeed, the response strength was dependent on the mixture quality (Fig. 4.3 Ai). We observed the strongest suppression at a delay of 50 ms. The response strengths to asynchronous mixtures with 6 ms delay between the components did not differ from the synchronous mixture. When N preceded H with 200 ms, the average response strength was significantly higher than to the synchronous mixture (Holm-Sidak post hoc test vs. synchronous mixture, overall significance level $p = 0.05$).

We were interested in whether the additional asynchronous-mixture interactions can be related to specific glomeruli. In many glomeruli, we found the tendency that with a delay of 50 ms between the components, the mixture response was lower than without delay or with a delay of 6 or 200 ms, for example in glomeruli 17 and 28 (Fig. 4.3 Aii, Aiii), but the effect with respect to identified glomeruli were not significant across animals. Moreover, a substantial part of the inhibition occurred in the post-odor response, whereas during the stimulus, the response to the mixture was often stronger than or equal to the stronger component (Fig. 4.3 B).

In the subset of experiments with 1-octanol and 2-heptanone and 9 different onset delays ranging from 0 to 600 ms, we found similar results as in the main experiments. Again, the response strength was dependent on the timing of the components, and we observed the strongest suppression at delays around 50 ms (Fig. 4.4): Responses of mixtures in which the octanol onset preceded the heptanone onset with 20 or 50 ms were significantly lower than the response to the synchronous mixture. Responses of mixtures in which the heptanone onset preceded the octanol onset were lower than the synchronous mixture for delays between 5 and 50 ms, and 200 ms (Holm-Sidak post hoc test vs. synchronous mixture, global significance level $p = 0.05$).

Because of the temporal complexity in many observed response traces, mixture in-

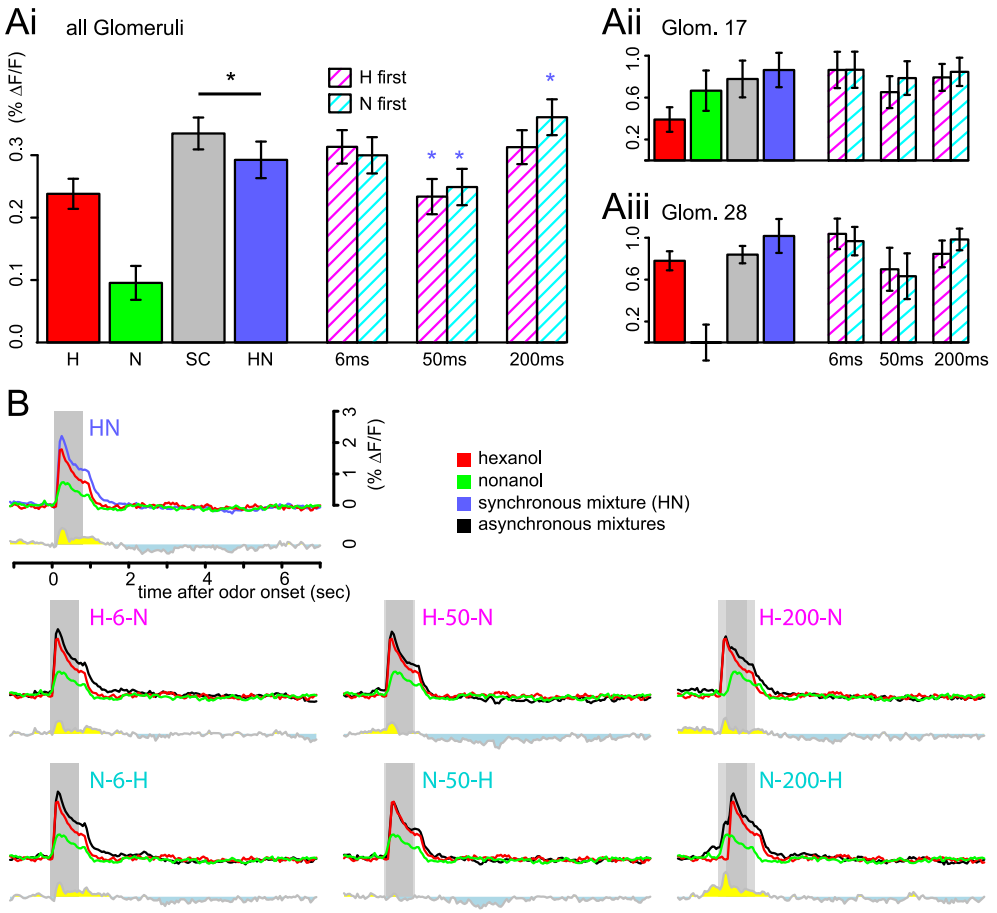

Figure 4.3: **Asynchronous mixtures induce more inhibitory interactions than synchronous mixtures.** Ai) Average PN response strengths during 4 seconds after stimulus onset pooled over all measured glomeruli (bars show mean ± SEM, n = 203 glomeruli, 14 bees). The grey bars show the average response strengths to the stronger component, which corresponds to the minimal expected response strength to the mixture in the absence of mixture interaction. RM ANOVA revealed significant differences between mixtures ($F_{6,202} = 7.43$, $p = 7.8 * 10^{-8}$). Blue asterisks denote significant differences to the synchronous mixture, (Holm-Sidak post-hoc test, global $p < 0.05$). Aii) same analysis as in Ai, performed over glomeruli T1-17, n = 11. Aiii) same analysis as in Ai, performed over glomeruli T1-28, n = 10. B) Average response traces of 203 glomeruli to the synchronous mixture (blue), the asynchronous mixtures (black) and their components hexanol (red) and octanol (green). Component traces were shifted according to their delay in the mixture. Lower, grey traces are the averaged, time resolved difference between the mixture and the stronger component, calculated on the level of subjects and then averaged. Times at which the response to the mixture is higher are marked yellow, times at which the response to the stronger component is higher are marked blue (indicating inhibitory mixture interaction).

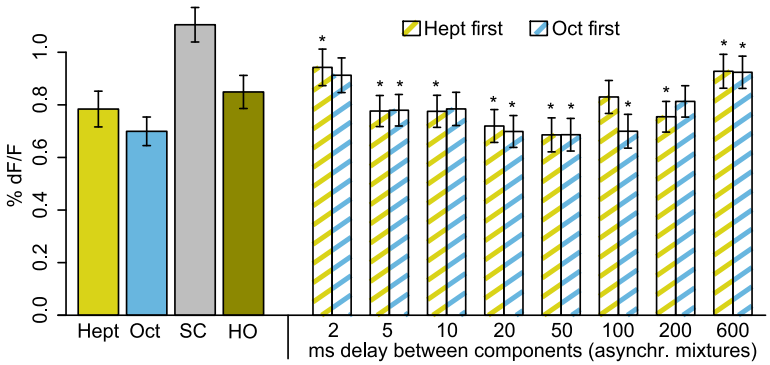

Figure 4.4: **Inhibitory mixture interactions are strongest at 50 ms asynchrony.** Average response strengths (± SEM) of 171 glomeruli (different subjects as for H and N) during 4 seconds after stimulus onset to 2-heptanone ("Hept"), 1-octanol ("Oct") and the synchronous (HO) and asynchronous mixtures. The grey bar shows the average response strengths to the stronger component. RM ANOVA revealed significant differences between mixtures ($F_{16,170} = 13.172, p < 10^{-15}$). Asterisks denote significant difference of asynchronous mixtures to synchronous mixture. All mixtures were significantly lower than the stronger component (not indicated, Holm-Sidak post-hoc test, global p < 0.05, corrected for multiple testing).

teractions might occur in other response parameters than the mean response strengths. We therefore performed multivariate analyses to describe mixture responses in comparison to the component responses on a global level, taking into account the spatiotemporal kinetics of odor responses.

Synthetic and analytic information in synchronous and asynchronous mixture responses

Honeybees recognize H and N better in an 6-ms asynchronous mixture than in a synchronous mixture of these two components (Szyszka et al., 2012). Thus, the perception of an asynchronous mixture is more analytic and less synthetic than that of a synchronous mixture. Since similarity of glomerular odor response pattern correlates well with perceived odor similarity (Guerrieri et al., 2005; Szyszka et al., 2011), we asked whether this effect is also visible in the physiological responses of PNs in the AL. If so, the representation of an asynchronous mixture should contain more information about its components than the synchronous mixture. We addressed this idea and quantified the similarity between PN responses to components and synchronous and asynchronous mixtures. We performed time-resolved correlation analyses across all responses, where the glomerular response pattern at any time frame of a stimulus was correlated to the pattern of every time frame of the same response (autocorrelation, left panels in Fig. 4.5 B-E), and of the component responses as reference (center and right panels in Fig. 4.5 B-E). Odor-responses consisted of stable odor-response phases (visible as boxes of

	correlation with H		correlation with N	
	mean	sd	mean	sd
HN	0.63	0.11	0.50	0.09
H-6-N	0.56	0.09	0.30	0.12
N-6-H	0.64	0.12	0.46	0.10
H-50-N	0.64	0.11	0.46	0.11
N-50-H	0.55	0.16	0.44	0.10
H-200-N	0.53	0.08	0.33	0.16
N-200-H	0.59	0.20	0.43	0.13

Table 4.1: **Correlation coefficients between mixtures and its components H and N**. Values are averaged (mean) values and standard deviatons (sd) of the cross-correlation analyses from Fig. 4.5 during 0.1 - 0.9 seconds after stimulus onset.

high correlation, diagrammed in Fig. 4.5 Ai). Responses to H and N had a short transient phase that lastet about 200 ms, and then a stable odor-response that lasted as long as the stimulus, while the synchronous mixture lacked the transient phase. After stimulus offset, PN activity changed into a post-odor response that was not correlated with the odor-response, but that was relatively stable within itself (corresponding regions are diagrammed in Fig. 4.5 Aiii). Both odor-response patterns and post-odor patterns were odor-specific. Cross-correlation across odors was low (0.23 ± 0.12), and intermediate for odor-component against the synchronous mixture (Table 4.1). The mixture response was more correlated to H than to N, reflecting the larger number of glomeruli responding to H than to N, i. e. a larger overlap of responding glomeruli between H and the mixture. Interestingly, the correlation between the synchronous mixture and the initial H response was high only in the beginning of the stimulus, and decayed fast, whereas the correlation with the initial N response was generally lower but persisted the entire stimulus, and decayed slowly (traces in Fig. 4.5 B-E). The sequence of odors in a 6 ms asynchronous mixture plays a role for the similarity of the mixture with the components: When H was given 6 ms before N (H-6-N), the correlation with N across all frames during the odor stimulation (diagrammed in Fig. 4.5 Ai) was lower than in the synchronous mixture and in N-6-H. Moreover, the correlation with the components was higher in N-6-H than in H-6-N (Table 1, and compare traces and matrices between Fig. 4.5 B and C).

Successive synthetic and analytic component representations in asynchronous mixtures

When the delay between the two components was increased to 50 ms and 200 ms, the overall correlation of the asynchronous mixtures (both sequences) to H was still stronger than the correlation to N (Table 1 and Fig. 4.5 D, E). Due to our sample

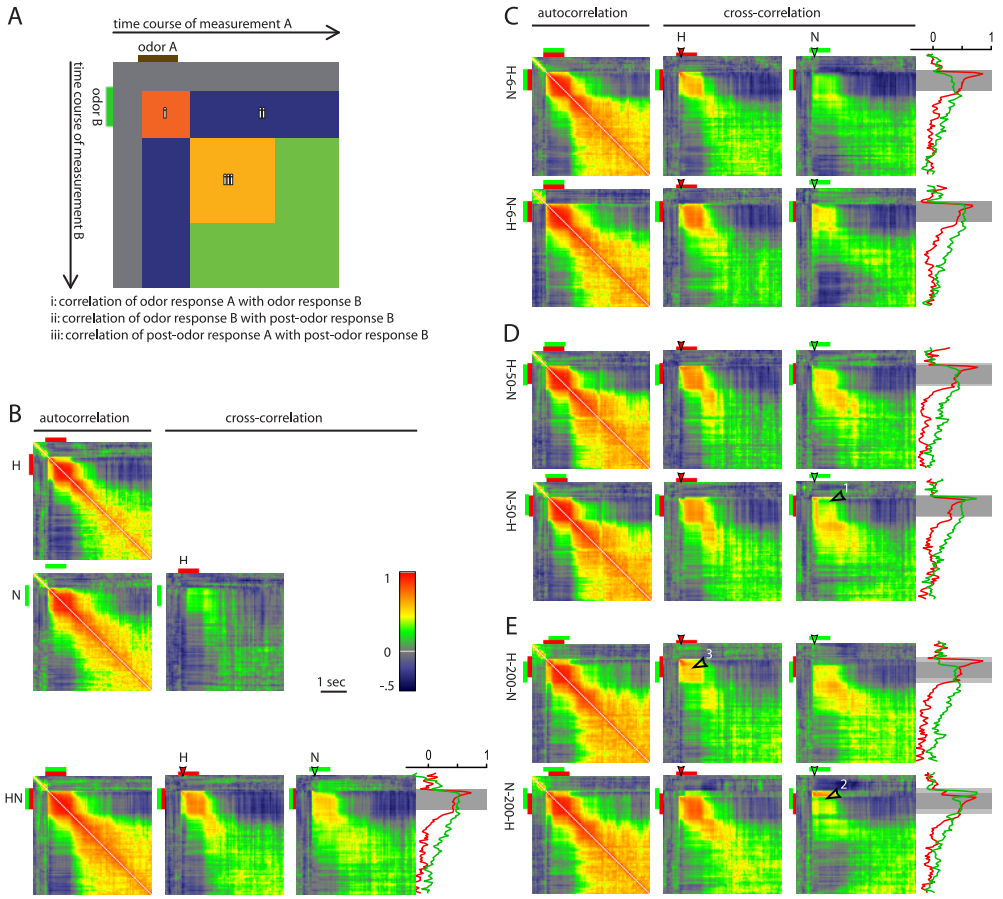

Figure 4.5: **Cross-correlation matrices of glomerular response patterns of two stimuli.** Every pixel gives a correlation value of two glomerular response patterns (vectors of length 203), for a certain time lag between two stimuli. A) Schematic of the cross-correlation between an odor A and an odor B, explaining the meaning of the components in each cross-correlation image. B) Auto- and cross-correlation of the components and the synchronous mixture. N (green): nonanol, H (red): hexanol. Time-traces of the correlation values in the vertical line indicated by the filled arrowheads are shown at the side, red: correlation between the initial hexanol response and the mixture response, green: correlation between the initial nonanol response and the mixture response. C-E) Auto- and cross-correlation of the components and the asynchronous mixtures. Numbered arrowheads refer to effects described in the text.

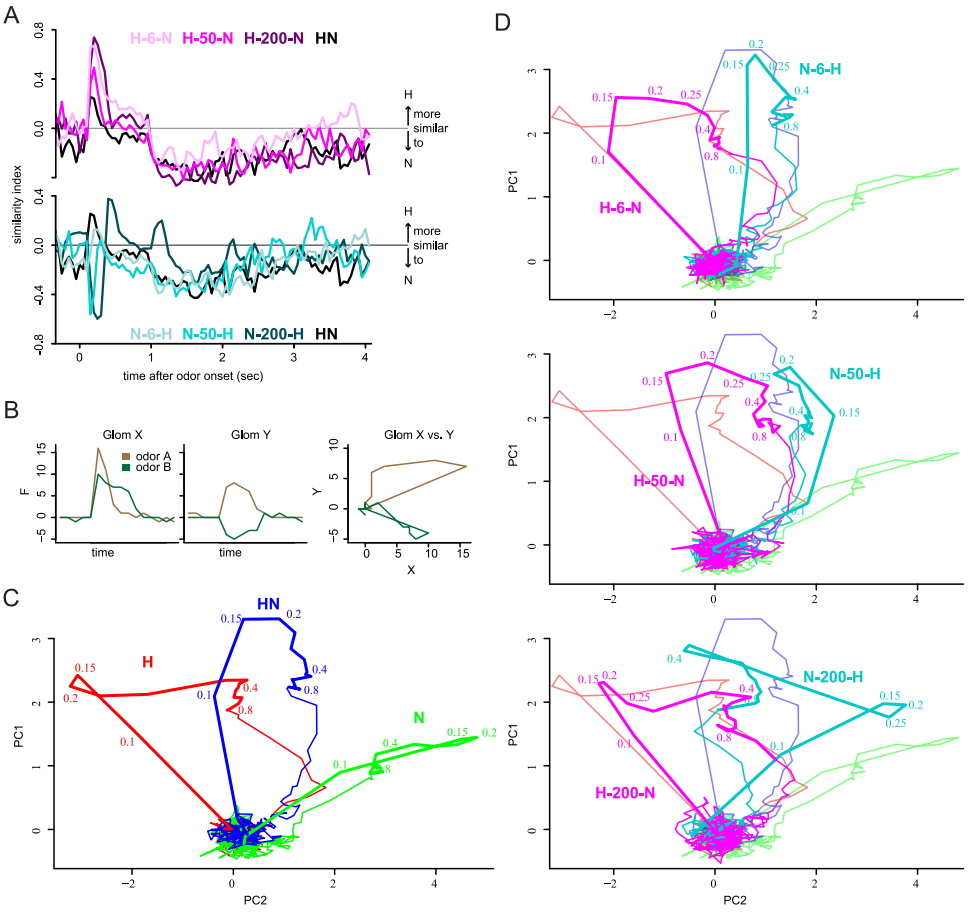

Figure 4.6: **The leading odor becomes more prominent in asynchronous mixtures.** A) The similarity index $correlation[H\,vs.\,mix] - correlation[N\,vs.\,mix]$ for synchronous and asynchronous mixtures. Positive values indicate higher correlation between H and a mixture, negative values indicate higher correlation between N and a mixture. Upper panel: Similarity index for the synchronous mixture HN and for asynchronous mixtures which start with H. Lower panel: Similarity index for HN and for asynchronous mixtures which start with N. The initial responses of asynchronous mixtures were more similar to the leading odor than to the trailing odor (Same data as traces in Fig. 4.5). B) principle of trajectory calculation for two glomeruli (fictive response time courses). C) Trajectories of the responses from 203 glomeruli for the components and the synchronous mixture, and D) for asynchronous mixtures with 6 ms (top), 50 ms (middle) und 200 ms (bottom), each with hexanol first (magenta) and nonanol first (cyan). Plots contain the trajectories from C for comparison. Numbers next to trajectories indicate the time after stimulus onset in seconds. PC1 and PC2 explain 80 % of the variance.

interval of 50 ms, the leading odor starts one measurement frame before the trailing odor for 50 ms onset (four frames for 200 ms onset shift). This resulted in high correlation values with the leading odor in the beginning of the stimulus. Beside this trivial effect, we observed other, network-generated sequence effects: The correlation of H-50-N and N was persistent from the onset of N until the end of the stimulus. The correlation of N-50-H and N (leading odor) collapsed when H (trailing odor) was added (arrowhead 1 in Fig. 4.5 D). However, the correlation of H-50-N and H (leading odor) did not collapse when N (trailing odor) was added. Thus, the representation of H and N does not follow the same logic when given in changed sequence. A similar effect occurred at a delay of 200 ms (Fig. 4.5 E): The addition of H in N-200-H led to a collapse of the N representation (arrowhead 2) - which later recovered - and to a strong correlation with H, but not *vice versa* (arrowhead 3). The representation of the initial component response in the mixture N-200-H changed several times during the stimulus (see traces), suggesting a successive and analytic representation of the stimulus rather than a synthetic representation. After N offset, the correlation of the response with the N response decreased again, but recovered after H was set off, too.

These manifold changes were also evident in the autocorrelation patterns. They exhibited 4 phases with a stable, high correlation within themselves, but different from each other: The first phase corresponded to the representation of the leading odor, the second phase corresponded to the mixture response, the third phase corresponded to the trailing odor, and the fourth phase was the post odor response.

Taken together, the correlation between the asynchronous mixture and the components was high when an odor was added to the mixture, and when it is present alone in beginning and end of the stimulus. Further, the correlation with the leading odor decreased temporarily when the new odor was added, but recovered shortly after. Thus, these stimuli created temporally complex responses, i.e. the glomerular response pattern changed from one odor identity to another. Finally, against component and asynchronous mixture responses, the responses to the synchronous mixture was less temporally complex, comprising only one stable odor response pattern and the post-odor response.

The leading component dominates the odor response to asynchronous mixtures

The correlation analysis showed that synchronous and asynchronous responses are generally more similar to the H pattern than to the N pattern (Fig. 4.5). To see whether stimulus asynchrony changes the component-to-mixture similarity we calculated a similarity index as the difference between the H-to-mixture correlation and the N-to-mixture correlation (Fig. 4.6 A). The initial responses to asynchronous mixtures

were more similar to the leading odors than to the trailing odors, and the responses to leading odors were more similar to the responses to asynchronous mixtures than to synchronous mixtures.

Although pairwise correlation analyses can describe the absolute similarity of one odor response with one certain reference trace with precise temporal resolution, it cannot display the evolution of a response pattern relative to all other stimuli at the same time. By means of PCA, we overcame these limitations and projected the multidimensional responses as trajectories into a two-dimensional space (exemplified for two glomeruli in Fig. 4.6 B), chosen to display the highest possible amount of variance - the first two principal components. The trajectories of the two components H and N were clearly separated in this plane (PC1 and PC2, Fig. 4.6 C). The synchronous mixture trajectory evolved between the components. Odor responses reached their biggest separation 0.2 s after stimulus onset and reached a steady state after 0.5 s, after which only minor changes in the pattern occurred until odor offset. Trajectories of the asynchronous mixtures deviated from the synchronous mixture towards the leading odor component (Fig. 4.6 D). After the initial deviation, trajectories moved towards the synchronous mixture (or, at a delay of 200 ms, towards the trailing odor). Generally, the longer the delay, the more often the trajectory changed its direction. Asynchronous mixtures with 200 ms delay initially followed the leading odor. After onset of the trailing odor, they turned into its direction, before they reached a steady state in the synchronous mixture area. This effect was most distinct at a delay of 200 ms. The trajectory of the asynchronous mixture N-200-H headed to the direction of H twice: first, after H was added to the mixture, and in the end of the stimulus, when N was set off but H remained on. Similarly, the trajectory of H-200-N headed to N in the end of the stimulation. Thus, in asynchronous mixtures with long delays between the components, there are time windows in which the glomerular activation pattern deviates from the mixture patterns in favor of the components. Taken together, the evolution of the responses to asynchronous mixtures are biased towards the leading odors, with 200 ms generating the most distinct deviation. Moreover, the processing of the mixtures tend to be shifted from a synthetic towards an analytic fashion.

4.5 Discussion

In the wild, insects experience both synchronous odorant mixtures (e. g. the bouquet of a flower odor, which consists of many chemicals) and asynchronous mixtures (e. g. when the odor of two flowers mix in the turbulent air). Here we analyzed how synchronous and asynchronous mixtures are processed in the honeybee AL and show that PNs are sensitive to millisecond asynchrony between the components of asynchronous mixtures.

Asynchronous mixtures contain information about their components

We found that in projection neurons, synchronous mixtures elicit odor responses that are similar to the component responses, but reduced in strength due to inhibitory interactions, confirming previously published observations in insects (Joerges et al., 1997; Galizia et al., 2000; Silbering and Galizia, 2007; Deisig et al., 2010; Najar-Rodriguez et al., 2010) and vertebrates (Tabor et al., 2004; Grossman et al., 2008). However, when mixtures were asynchronous, inhibitory interactions increased. Most importantly, we found that even an onset delay of 6 ms was sufficient to generate a response pattern that was initially biased towards the leading odor. With increasing odor-onset delays (50 ms, 200 ms) the AL showed an increasingly analytic processing mode, in that leading odor, mixture, and trailing odor were represented successively. Accordingly, the total amount of inhibitory interactions was not increased any more with time-delays of 200 ms. Local neurons in the AL might favor different coding strategies (analytic or synthetic), depending on the stimulus timing (Meyer and Galizia, 2012). The increased inhibitory interactions which occurred between 5 and 100 ms suggests a timing-dependent inhibitory network that is activated only during a distinct time window after an odor onset, e. g. a winner-takes-all network that changes the response to odor mixtures if asynchrony is detected (Nowotny et al., 2012). Whether this timing-dependent inhibitory network plays a role in enhancing the separability between two components in asynchronous mixtures remains to be tested.

Small odor-onset time delays of 6 ms helps honeybees to segregate odor components from mixtures (Szyszka et al., 2012). In the moth pheromone system, time differences of as little as 1 ms between the pheromone and an antagonist were sufficient for the animals to judge the mixture as not being a perfect blend, rendering the antagonistic effect less efficient (Baker et al., 1998). Together, the previous behavioral data and our physiological data presented here show that olfactory processing in insects is fast, though they do not indicate how fast - indeed, the fact that we could see the 6 ms delay in measurements with a sampling interval of 50 ms indicates that fast effects in the periphery might have physiological effects at a slower timescale in the brain.

Nikonov and Leal (2002) reported of mixture suppression of ORN compartmentalized in the same sensillum, which occured only when both components were delivered synchronously. This supports the idea of on-site coincident detectors realized by sensilla housing ORNs of different tunings. Moreover, lateral inhibition of neighboring ORNs has been reported (Hillier and Vickers, 2011; Su et al., 2012). In honeybees, ORNs are mainly located in sensilla placodea. These sensilla contain up to 30 ORNs that innervate the AL with highly diverse glomerular patterns (Kelber et al., 2006), thus maximizing the chance that two arbitrary odors may activate two ORNs within the same sensillum. The ORNs which respond to the leading odor could suppress the ORN responses to the trailing odor in the same sensillum. Such a mechanism could

explain both the increased inhibitory interaction and the dominance of a leading odor in asynchronous mixtures. Together, these studies give rise to the hypothesis that already within a single sensillum, the processing of one odor might be affected by the presence and the timing of another odor.

As compared to behavioral data in honeybees (Szyszka et al., 2012), our data show one discrepancy: while with 6 ms time shift we see the identity of the leading odor in masked, even though the behavioral effect is almost symmetrical. Here, either the temporal resolution of the calcium sensitive dye or of the intracellular calcium concentration was not sufficient (both have fast time-constants for calcium increases, but slower ones for calcium decreases), or the identity of the leading and the trailing odor might be extracted by other regions of the bee brain. For instance, this could be the mushroom bodies (MB), whose Kenyon cells code odor information in a highly synthetic fashion (Laurent, 2002; Perez-Orive et al., 2002; Szyszka et al., 2005; Jortner et al., 2007): Odors that activate overlapping sets of PNs in the AL activate distinct sets of Kenyon cells in the mushroom body. Moreover, Keynon cells are particularly sensitive to synchronous input and respond mainly to odor-onset. Thus, the leading and the trailing component in an asynchronous mixture might activate different Kenyon cell ensembles that resemble the single components rather than the synchronous mixture. These hypotheses remain to be tested, e. g. by measurements with higher sampling rates, and by recording Kenyon cells in the mushroom bodies.

Are there further aspects in the activity patterns that would help the animal differentiate between a synchronous and an asynchronous mixture? E.g., components could be processed sequentially, as has been shown previously for humans (Laing et al., 1994; Jinks and Laing, 1999). The multiphasic odor-response patterns that we observed for 50 and 200 ms delay would argue in favor of such a view: Unlike the fairly stable "plateau" responses of the synchronous mixtures, these patterns had a temporal complexity that by itself could be used by the brain to extract information about the stimulus quality.

Odor-dominance effects

In our recordings, H was generally dominant over N, i. e. mixtures were generelly more similar to H than to N. We took great care to develop a perfectly symmetrical olfactometer (Szyszka et al., 2012), thus we exclude that the time-difference may have been unequal. Therefore, it is possible that odors differ in their dominance within the AL network (e. g. due to a stronger connection to the inhibitory network, based either on innate or on acquired properties). However, in our case it could also be a consequence of the fact that H elicited activity in more glomeruli than N (Fig. 4.2 A), which results in H having a stronger weight both in the correlation analyses and

in the PCA analysis. Since we could only sample a subpopulation of glomeruli in the AL, it might be that if recorded across all 160 glomeruli, odors would become more symmetrical. However, this observation might also reflect another effect: Odors, which activate many glomeruli (i.e. for which there are many responsive olfactory receptors), might have a net advantage on other odors in their processing dominance in the AL.

Odor object segregation

Previous studies showed that mammals can use stimulus asynchrony for odor-background segregation, although on a slower timescale: several seconds vs. milliseconds in insects (Kadohisa and Wilson, 2006; Linster et al., 2007). The principle that onset asynchrony between stimuli can be used to enhance separability, and thus prevent the creation of concurrent stimuli as a unitary percept (object or "gestalt"), has been studied in other modalities, including vision, audition and tactile senses, and provides a basic principle for object recognition (Bronkhorst, 2000; Hancock et al., 2008; Gallace and Spence, 2011; Pressnitzer et al., 2011; Wagemans et al., 2012). These studies have shown that the synchrony of a stimulus can be used by the brain to "bind" its components, whereas asynchrony helps to segregate concurrent stimuli and thus enables the creation and segregation of objects. Our data shows that this concept may also apply to the olfactory system. Binding the activity of different glomeruli in the response pattern of a synchronous mixture may be the substrate for creating the "perfume" percept, i. e. the experience of an odor in which the unique identity of the mixture obliterates the information of the mixture components. In other words: temporal asynchrony between two components in a stimulus may inhibit the binding and result in segregation, i. e. the information of the components is preserved in the odor response (= two objects), whereas stimulus synchrony leads to binding, i. e. the processing of the mixture renders a new odor quality (= one object). We found more inhibition in responses to asynchronous mixtures (segregation of components) than to synchronous mixtures (binding of components). Thus, the mere strength of inhibition in the glomerular responses might be not correlated to the degree of creating new odor qualities, unlike suggested elsewhere (Silbering and Galizia, 2007; Deisig et al., 2010). Instead, it could reflect the activity in inhibitory local neurons, which are involed in creating spike synchrony across PN ensembles, which has been suggested to be important for odor processing (Perez-Orive et al., 2004; Giridhar et al., 2011). Further, the mushroom bodies are ideally suited to extract this synchrony (Laurent, 2002; Szyszka et al., 2005; Turner et al., 2008).

4.6 Acknowledgments

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CHAPTER 5

Short-Term Plasticity in the Insect Olfactory System Enhances Sensitivity for Stimulus-Onset Asynchrony

5.1 Abstract

Sensory systems use millisecond asynchronies between concurrent stimuli for object segregation. Honeybees are able to segregate odor-objects from asynchronous odor mixtures, when components of mixtures were given with only 6 ms time offset. How these short differences are used by the olfactory system to establish distinguished physiological and behavioral responses is not yet understood. Here we studied the effect of sensory experience on the processing of asynchronous odor mixture. We applied odorants and their synchronous and asynchronous mixtures as trains of 3 odor pulses, and recorded the projection neuron responses with high speed calcium imaging in the honeybee antennal lobe. We found inhibitory mixture interaction in the responses to asynchronous mixtures for the second and third, but not for the first odor pulse, suggesting that the processing of these stimuli involves the delayed activation of an inhibitory network. Moreover, we found that response latencies to odorants did not differ in the various mixture situations, but increased when the same odor was repeated. This study confirms that projection neurons are sensitive to millisecond short stimulus onset asynchrony and shows that processing of asynchronous mixtures might involve short-term plasticity in the antennal lobe.

5.2 Introduction

Segregation of concurrent stimuli is one of the most important tasks of sensory systems to ensure detection and recognition of objects. Mechanisms for stimulus segregation

have been studied intensively in audition and vision but less in olfaction. Sensory systems have in common that they use temporal differences in stimulus onset for stimulus segregation (Zera and Green, 1993; Baker et al., 1998; Usher and Donnelly, 1998; Bronkhorst, 2000; Hancock et al., 2008). This is also highly relevant in the turbulent olfactory world, as airborne odorants from different sources mix in a complex, chaotic way (Riffell et al., 2008), resulting in asynchronous mixtures. They contain more information about the components, as revealed by behavioral, (Baker et al., 1998; Szyszka et al., 2012; Andersson et al., 2011), theoretical (Hopfield, 1991), modeling (Nowotny and Jacob S. Stierle; C. Giovanni Galizia; Paul Szyszka, 2013) and physiological studies (Broome et al., 2006; Stierle et al., 2013). We recently showed that the processing of asynchronous mixtures involves more inhibitory mixture interactions in the honeybee antennal lobe network than the processing of synchronous mixtures (Stierle et al., 2013). Inhibitory mixture interactions are commonly known from insects (Joerges et al., 1997; Galizia et al., 2000; Deisig et al., 2006; Silbering and Galizia, 2007; Deisig et al., 2010; Najar-Rodriguez et al., 2010) and vertebrates (Tabor et al., 2004; Grossman et al., 2008), and derive from a network of inhibitory local neurons that interconnects glomeruli (processing units that receive the input from sensory neurons with the same receptor protein (Vosshall et al., 2000). The antennal lobe (AL) network plays an important role in modifying odor response patterns, as it makes similar odor response patterns more separable for higher brain areas (Sachse and Galizia, 2003; Silbering et al., 2008) and categorizes odor patterns according to their behavioral meaning (Niewalda et al., 2011; Knaden et al., 2012). Moreover, experience dependent plasticity in the antennal lobe network adjusts odor-specific contrasts quickly to changing odor situations (Faber et al., 1999; Stopfer and Laurent, 1999; Fernandez et al., 2009; Rath et al., 2011; Das et al., 2011; Locatelli et al., 2013). Accordingly, previous experience with asynchronous odor mixtures might help to segregate them into their components. Here, we used fast confocal calcium imaging to investigate strength and latency of an odor response. We show that short-term plasticity in the AL increases projection neuron (PN) sensitivity for stimulus-onset asynchronies. We found that the response strength is inhibited in consecutive pulses, and that inhibitory mixture interactions are stronger in the late pulses of asynchronous mixtures. Moreover, we show that projection neuron latencies increase within a train of pulses of the same odorant. However, latencies are not increased by processing of other odors in neighboring glomeruli.

5.3 Material and Methods

Animal preparation

Honeybee foragers (females) were caught from the entrance of different hives kept at the institute, immobilized on ice and fixed with adhesive wax (Deiberit, Dr. Böhme & Schöps). Calcium-sensitive dye (Oregon Green-dextran BAPTA1, Kd=1180 nM, 10000 MW, Invitrogen) was dissolved in a water droplet on a microscope slide to a viscous solution and applied to the tip of the glass needles, which were pulled on a Sutter horizontal puller (P-87, Sutter Instruments). Antennae were sticked to the forehead with Eicosane (Sigma-Aldrich, Steinheim, D). The head capsule was removed with a razor splint between compound eyes, antennae and the medial ocellus. Glands and tracheae were removed from the mushroom body calyces. The dye was injected into the brain at the junction of the calyces of the mushroom body into the antenno-protocerebral-tract that contains the axons of PNs, and allowed to travel along the axons overnight. The next day, glands and tracheae were removed from the ALs. In order to reduce movement, the esophagus was extended with forceps through a cut above the labium, and the abdomen was immobilized with a piece of sponge. The brain was covered by a thin layer of two-component silicone (KwikSil, World Precision Instruments). A plastic coverslip separated the antennae from the imaging area in order to keep them dry and accessible for odor stimulation. The temperature at the setup was set to 25 °C.

Odor stimuli

Odor stimuli consisted of a train of three 800 ms long pulses of odorants with an onset-to-onset interval of 1600 ms. Synchronous mixtures were created by applying two odorants at the same time, with synchronized on- and offsets. Asynchronous mixtures were created by shifting one odorant stimulus by different length (5, 50, or 200 ms), resulting in overlapping stimuli. Stimuli were abbreviated with the first letter of the components and the time of the odor offset between leading odor and trailing odor. For example, "X-50-Y" describes the asynchronous mixture in which odorant X was given 50 ms before odorant Y started. The stimuli were repeated up to four times in each animal. 2-Heptanone (CAS: 110-43-0) and 1-Octanol (CAS: 111-87-5) (all $\geq$ 99.7%, Sigma-Aldrich) were diluted 1:100 in mineral oil (Sigma-Aldrich) and kept in glass vials with argon or nitrogen atmosphere to prevent oxidation. 200 µl of odor solution were placed on a cellulose pad (SugiPad, Kettenbach) in a 3 ml plastic syringe (NormJect, HSW GmbH, Tuttlingen, D), with the plunger set to 2.5 ml. Fresh odor syringes were used for every day of experiments. Syringes were placed in a custom-build 7-channel

olfactometer, similar to the one used in (Szyszka et al., 2011). Odor channels were set to 160-190 ml flow volume in order to adjust for same stimulus dynamics, and were injected into a carrier air stream of 1600 ml/min. The airspeed at the outlet was 1.3 m/s. Solenoid valves were controlled by custom software written in LabView 8.0 (National Instruments, Austin, TX, USA), allowing control pulse application with sub-µs accuracy. The solenoid switch pulse consisted of a 1 ms long 24 V pulse followed by a 12 V hold for the time of the stimulus (spike and hold), using custom electronics.

Data acquisition and analyses

In every animal, we searched for 2 adjacent glomeruli of which each responded to only one of the two odorants before starting the actual experiment. We recorded odor responses of 34 glomeruli in 16 bees, of which 19 were suited for analysis because they responded to only one of the two odors. One recording lasted 10 seconds, then we waited 60 seconds before we gave the next stimulus. Bees were imaged with a confocal microscope (LSM 510, Zeiss), equipped with an 20 x water immersion objective with a numerical aperture of 1 (WPlan Apochromat, Zeiss). We used a shortpass 700/488 nm main dichroic mirror and an argon laser with 488 nm for excitation. The emission light was filtered by a secondary dichroic (490 nm) and a 505 nm longpass filter and measured by a photo detector. Acquisition frame rate was chosen as high as technically possible and varied between 130 Hz and 218 Hz, depending on the subject. Raw data movies were processed with custom written programs in IDL (RSI, CO, USA) to execute logarithmic bleaching correction. Time traces were calculated as fluorescence changes divided by the background fluorescence $\frac{\Delta F}{F}$ from all pixels within regions of interests in the data movies. The background fluorescence was calculated as the average of the 4th frame to the last but 2nd before stimulus onset. Further quantitative and statistical analysis was performed in R (R Development Core Team, 2011): All time traces were upsampled from at least 130 Hz to the maximal frame rate (218 Hz) by insertion of additional frames and interpolation (arithmetic mean of the two adjacent time frames), and filtered with a median filter (15 frames).

Response strengths were calculated for every pulse separately as the mean calcium signal within 0-1.6 s after the onset of each pulse, which corresponds to the pulse interval. The "stronger component" was the higher of two values for each pair of measured odorant responses. Values from repeated recordings in the same glomerulus were averaged before pooling the data with other glomeruli.

Response latencies were defined as the time from the trigger that opened the valve to the point of strongest calcium increase within 20-700 ms after the trigger. The onset was detected automatically with roughly a 95% chance, and needed to be manually corrected in single cases due to some double peak responses or strong spontaneous

activity. In order to analyze the response latencies of the trailing odor in a mixture, only glomeruli were analyzed that responded excitatory to only one of the odorants: Latencies of glomeruli that responded only to octanol were assessed in the asynchronous mixtures starting with heptanone, and vice versa. Statistical tests were done in SigmaStat (Systat).

5.4 Results

In every bee, we imaged projection neurons in 2-3 adjacent glomeruli (Fig. 5.1). In 15 animals, we imaged 34 glomeruli. 40% were identified as glomeruli A29 and A38 (nomenclature after Galizia et al. (1999a)). All glomeruli were located on the most rostral part of the AL (see Fig. 5.1 A). We found consistent responses for both heptanone and octanol. Glomeruli that responded to one of the odorants, also responded to the different mixtures, although the mixture responses were sometimes weaker than the responses to the stronger component. Generally, PNs were able to resolve the 3 odor pulses. We found different categories of temporal response time courses (in order of appearance in Fig. 5.1 D): i) phasic responses to only the pulse onset, ii) phasic responses to on- and offset of an odor pulse, iii) tonic responses without initial peak, iv) phasic-tonic responses, which consisted of a strong initial calcium peak to the onset of a pulse and a weaker response that lasted until pulse offset, v) inhibitory responses, which decreased the baseline or the spontaneous activity, and vi) complex response time courses. A majority of responses featured a distinct and steep increase to the onset of an odor pulse, which we used to measure the latency of the response. Spontaneous activity was often reduced after the stimulus.

Different pulse-repetition effects on synchronous and asynchronous mixtures

Time differences in stimulus onset presumably drive mechanisms for odor segregation. We therefore were interested if the responses to an odor in one glomerulus affected the responses in another glomerulus, and whether this is a function of the timing of the components. We applied synchronous mixtures as well as asynchronous mixtures with different time-lags. Since stimuli in nature are intermittent, especially in a turbulent environment where odors from different sources intermingle to asynchronous mixtures, we asked whether mixture effects change during repeated stimulation.

We investigated the effects of mixture quality (synchronous and asynchronous with different delays) and repetition of pulses on the response strength, and we performed a two-way repeated measures analysis of variance (two-way RM ANOVA). We found

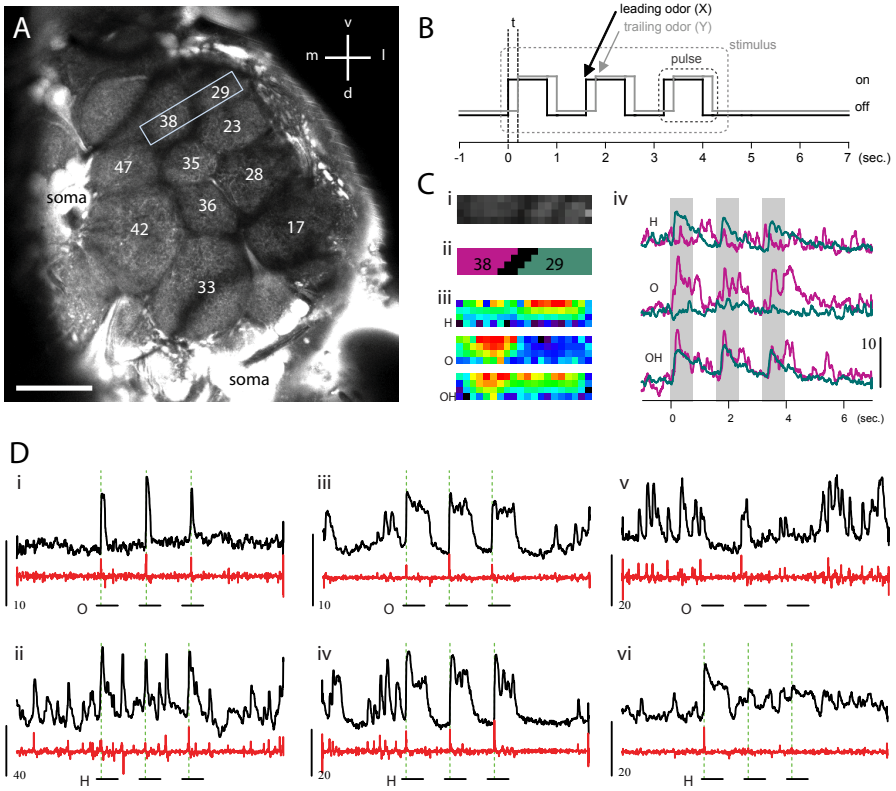

Figure 5.1: **Glomerular odor responses recorded by confocal calcium imaging.** A) Fluorescence from an antennal lobe in vivo. Projection neurons were stained retrogradely with Oregon Green. Glomeruli could be distinguished and identified by their boundaries (Nomenclature after Galizia et al. (1999a) without the prefix "A" for T1-glomeruli. White areas are soma clusters or tracheae. Tracheae cast shadows on subjacent area, which must not be taken as boundaries from glomeruli. The rectangle indicates the area used for the calcium imaging experiment. Scale bar 50 µm, *m* medial, *l* lateral, *d* dorsal, *v* ventral. B) Odor stimuli always consisted of 3 pulses of either heptanone, octanol, or different mixtures of both. This example shows "X-t-Y", with X as leading odor, Y as trailing odor, and t as time offset between the components. C) calcium imaging data from the two glomeruli indicated in A. Two adjacent glomeruli were selected in a way that one responds to heptanone and the other to octanol. i) raw fluorescence . ii) regions of interests to extract time traces . iii) Color coded pictures show maximal excitatory responses to odors heptanone (H), octanol (O) and the mixture (HO) in the two selected glomeruli. iv) time traces of the responses. Grey areas indicate the odor application to the antennae. D) Black traces: Selected responses from glomeruli of other animals to heptanone (upper panel) and octanol (lower panel). Different glomeruli show very distinct response properties: They could be phasic (i), on- and off (ii), tonic (iii), phasic-tonic (iv), inhibitory (v), or temporally complex (vi, phasic tonic response that shuts off with the stimulus but rebounds shortly after). Response onsets (dashed vertical lines) were detected within 700 ms after pulse onset at the time points with the maximal signal increase (red traces). Note that even in complex response traces, this simple algorithm reliably marks the response onset. Numbers next to scale bars indicate signal strength in Δ F/F.

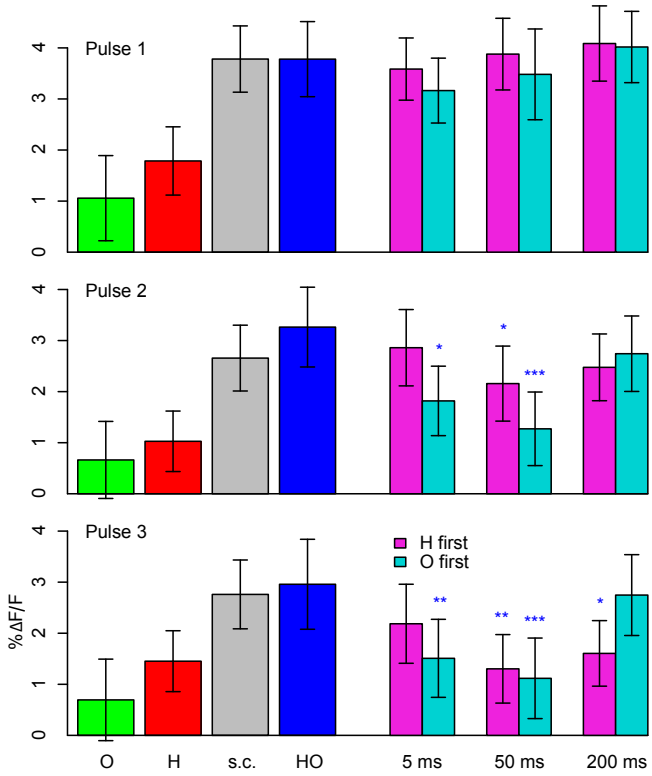

Figure 5.2: **Response strength depends on both pulse number and odor mixture quality.** Average response strength to the odorants octanol (green) and heptanone (red), their synchronous mixture (blue) and asynchronous mixtures (magenta and cyan). Shown are mean fluorescence values within 1.6 seconds after pulse onset averaged across glomeruli, ±s.e.m. The gray bars show the average response to the stronger component (s.c.) and corresponds to the minimal expected response strength to the mixture in the absence of mixture interaction. Lower values for mixtures than for s.c. indicate inhibitory mixture interactions. We found no significant differences between s.c. and HO. Asterisks indicate significant differences to synchronous mixture (HO), *** p<0.001, ** p<0.01, * p<0.5, Holm-Sidak posthoc-test, n = 34 glomeruli.

that a) the average response strength to the 3 pulses were different ($F_{2,33} = 20.724$, $p < 0.001$), b) the mixture quality had an effect on the response strength ($F_{6,33} = 2.830$, $p = 0.012$), and c) an interaction of the pulse number and the mixture quality ($F_{12,33} = 3.339$, $p < 0.001$). Responses to both the second (mean=2.4 %) and the third pulse (1.9 %) were significantly lower than the response to first pulse (3.7 %, both p< 0.001, Holm-Sidak post-hoc test). Next we asked whether processing of mixtures involves inhibitory interactions as has been reported previously for synchronous and asynchronous mixtures Deisig et al. (2006); Silbering and Galizia (2007); Stierle et al. (2013). We did not find inhibitory mixture interactions for the synchronous mixture in any of the 3 pulses (Fig. 5.2). However, response strength was considerably reduced in some of the asynchronous mixtures in the second and third pulse. Interestingly, this *asynchronous*

mixture suppression did not occur in the first pulse, and was most prominent in the last pulse. Strongest asynchronous mixture suppression was found in mixtures with 50 ms offset between the components. Taken together, we conclude that a first odor stimulus gives rise to delayed inhibitory activity which increases inhibitory interactions in asynchronous mixtures and which lasts for at least 1.6 seconds (the inter-pulse interval), but less than 70 seconds (the inter-stimulus interval).

Response latency to odorants depends on odor pulse repetition but not on mixture interactions

We were interested whether asynchronous mixture suppression also affects the latency of an odor response. Retarding the response to the trailing odor in asynchronous mixtures with small time offsets would lead to a temporal separation of the two odorants, that could help to increase their separability. To test this hypothesis, we assessed the time a glomerulus takes to respond to the trailing odorant in asynchronous mixtures, and compared it to the latencies of the plain odorant and the synchronous mixture. To this end, we selected glomeruli that only responded to one of the mixture components. Then, for O and the mixtures H-t-O (t in [0,5,50,200]), we assessed the response latencies in the glomeruli that responded only to odorant O ("O-glomeruli", n = 7). Likewise, for H and the mixtures O-t-H, we assessed the response latencies of glomeruli responding to H ("H-glomeruli", n = 12). Most of the response latencies for the first pulse were in the range of 100-150 ms (Fig. 5.3), with a second mode between 300 and 350 ms (that is, 200 ms later). This is what we expected, as 200 ms offset of the trailing odorant should also results in a 200 ms delay of the response. We saw a similar latency distribution for the other pulses, however, latencies were longer for consecutive pulses. Since we were interested in AL network effects on the latencies, we compared the latencies of the trailing odorant responses in the asynchronous mixtures after subtracting the time offset of the trailing odor, and tested the results with a two way-RM ANOVA. For latencies of the first pulses, we observed a slight but not significant increase in the latencies as a function of trailing odor offset time, for both heptanone and octanol. In contrast to the response strength, there was no interaction of mixture quality and pulse number, and mixture quality had no significant effect on the response latencies ($F_{4,6} = 0.622$, $p = 0.611$ for the O-glomeruli, and $F_{4,11} = 0.428$, $p = 0.787$ for the H-glomeruli). Interestingly, we found a considerable increase of the latencies from pulse to pulse ($F_{2,6} = 28.991$, $p < 0.001$ for the O-glomeruli (137, 187 and 220 ms for pulses 1, 2, and 3) and $F_{2,11} = 38.434$, $p < 0.001$ for the H-glomeruli (129, 177 and 200 ms). Taken together, we found that a repeated stimulation of the same odor temporary increased response latencies of projection neurons, whereas the stimulation with a second odorant, which elicits an excitatory response in a neighboring glomerulus shortly before

(5-200 ms) or at the same time, did not did not affect the response latency. Again, this was a short-term effect as it lasted for the 1.6 second long inter-pulse interval but not for the 70 second long inter-stimulus interval.

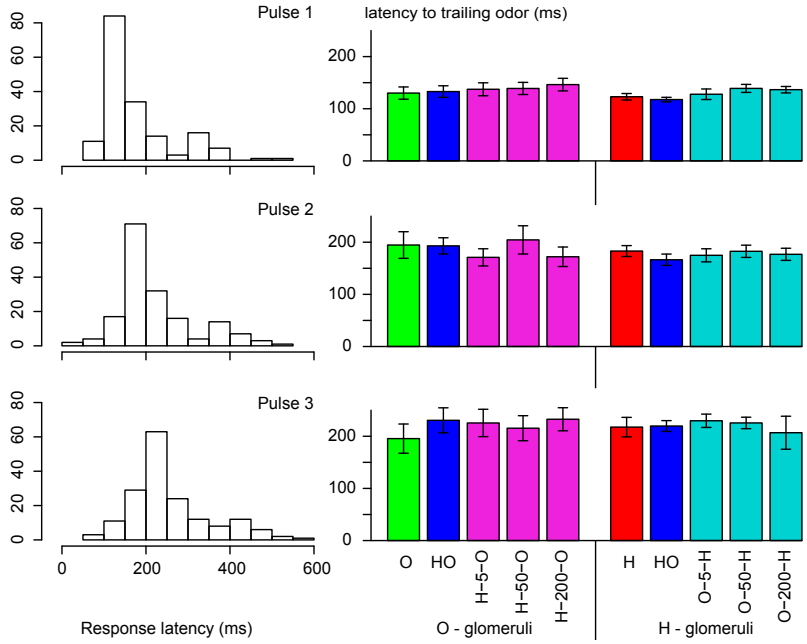

Figure 5.3: **Response latencies are longer for later pulses, but did not differ between stimuli.** Histograms show the distribution of pooled latencies from H-glomeruli (to all stimuli except O) and O-glomeruli (to all stimuli except H). Since a portion of the stimuli are asynchronous mixtures with the potent odor as trailing odor, distributions are right skewed. Bar plots show the latency to the potent component (O for O-glomeruli, H for H-glomeruli) applied alone (green, red), as synchronous mixture (blue), or as trailing odor in the asynchronous mixtures(magenta, cyan). Time onsets of the trailing odors in asynchronous mixtures were subtracted before averaging. Values are mean across glomeruli ±s.e.m., n = 7 (O-glomeruli) and n = 12 (H-glomeruli).

5.5 Discussion

Although highly relevant and ubiquitous in a natural environment, it is still understudied how olfactory systems segregate components from mixtures of odorants from different objects. In this study, we investigated different parameters of asynchronous odor mixture responses to gain insight on how the insect olfactory system detects onset asynchronies.

The relevance of pulsed odor stimuli

Many PNs responded only to the odor onset or to on- and offset, but were otherwise silent during odor stimulation. A pulsed stimulation is therefore much more effective in eliciting PN responses than a sustained odor stimulus (Geffen et al., 2009). This is not surprising, as natural odor stimuli are highly intermittent and behaviorally more relevant (Willis and Baker, 1984; Baker et al., 1985; Murlis and Murtis, 1992). For odor segregation it might be advantageous to encounter multiple asynchronous odor pulses instead of only one as this increases the temporal incoherence between the components of the mixtures. Moreover, pulsed odor stimuli offer an experimental advantage as one can investigate the effect of odor-onset asynchrony on different time scales. Odors evoke not only fast excitatory responses, but also activate inhibitory neurons. In the insect antennal lobe, projection neurons are inhibited by GABAergic local neurons, mediated by fast ionotropic $GABA_A$ receptors and by slower $GABA_B$ receptors which activate within several hundred milliseconds and that lasts for several seconds (Wilson and Laurent, 2005).

Short-term plasticity increases inhibitory mixture interactions in asynchronous mixtures.

In both our previous study (Stierle et al., 2013) and this study, we found mixture suppression in large parts in the late responses (2-3 seconds after stimulus onset). In the earlier study, we used short stimulations and thus found these inhibitions in the post-odor response, which was different from the odor response. In this study, however, our odor stimulus lasted for several seconds, thus the inhibitions during this time were still part of the odor-response. We conclude that this late inhibitory phase and the resulting mixture interactions are not specific to possible post-odor responses, but reflect the delayed onset of inhibition after stimulus onset. Nowotny and Jacob S. Stierle; C. Giovanni Galizia; Paul Szyszka (2013) suggested that millisecond short onset-asynchrony between odor stimuli could induce prolonged changes which facilitate odor segregation in downstream circuits of the olfactory pathway. The short term plasticity of mixture interaction might be the substrate for such prolonged activity change.

Before the experiments, we selected 2-3 glomeruli in every animal for their response properties to only respond to exactly one of the two odorants. Thus, the average response strength of all glomeruli to a certain odorant is much weaker than the average response to the stronger component. In datasets that contain glomeruli with broader response tuning, this difference is much less. The fact that we did not observe inhibitory mixture interactions to synchronous mixtures, as observed in the previous study, might also be due to this preselection: inhibitory local neurons rather connect glomeruli with

similar response properties than neighboring glomeruli (Linster et al., 2005). However, we were still able to see "asynchronous mixture suppression", i.e. the portion of mixture suppression that occurs only in asynchronous mixtures. This is particularly fascinating as it shows that synchronous mixture suppression and asynchronous mixture suppression affect different glomeruli and might be mediated by different inhibitory networks. As we found asynchronous mixture suppression only in the late phase of odor responses, the $GABA_B$ mediated inhibition seems as a good candidate for future investigations of odor segregation. The antennal lobe inhibitory network has an important role in odor processing and shaping responses in the way that it makes similar odors more distinguishable (Sachse and Galizia, 2002; Silbering and Galizia, 2007; Deisig et al., 2010; Girardin and Galizia, 2012). There are hardly two stimuli that are more similar than synchronous and asynchronous mixtures with only millisecond onset differences, and yet they need to be distinguished - and can be distinguished - by the animal (Baker et al., 1998; Andersson et al., 2011; Szyszka et al., 2012). The high degree of inhibition during the processing of asynchronous odor mixtures might therefore underlie the remarkable ability of the olfactory system of the honeybee to distinguish the two mixture qualities. Since lateral connectivity via interneurons is not homogeneous (Fonta et al., 1993; Girardin and Galizia, 2012), it is possible that a different subset of glomeruli are more susceptible to inhibition by processing of odor mixtures. This might also be dependent on which odorants are mixed, since every odor elicits a different glomerular response pattern (Joerges et al., 1997; Sachse et al., 1999).

Response latencies suggest that odors are processed independently

We recorded response latencies as the total time from the valve-trigger until the response in the calcium signal. This latency therefore contains the technical delay of the setup between the trigger and the odor hitting the antennae. As we used a similar olfactometer as the one from Szyszka et al. (2012), we assume a technical delay of 70 ms. Thus the real response latencies of PNs range from 30 to 80 ms. Our response latencies are considerably shorter than previously described and correspond to the fastest PN response latencies found in Strube-Bloss et al. (2012), but were 80 to 120 millisecond faster than reported in Krofczik et al. (2009) and Brill et al. (2013).

We found that odor response latencies increased by 50 ms from pulse to pulse, when stimulated with the same odor. We asked whether this increase in response latencies was mediated by interactions between different odors in the mixture. A temporal retardation of the trailing odor would give higher brain areas more time to extract information from the leading odor. Alternatively, an independent processing of the two odors in an asynchronous mixture would rather not affect the latency of the trailing odor. Indeed, we did not find any difference in response latency when an

odorant was given alone or in a mixture, or shortly before or after another odorant (i.e., in asynchronous mixtures). This suggests that the processing time of a certain odor might be stable and not prone to disturbance by the parallel processing of other odors in other glomeruli, suggesting that the odors are processed "independently" with respect to their timing.

From our results, we conclude that a delayed inhibitory network is more active in the processing of asynchronous than in the processing of synchronous odor mixtures. When an animal enters a turbulent odor cloud, the delayed inhibition saves the strong and fast responses to the first odor filament, what might enable the animal to quickly respond to a suddenly occurring, potentially important odor, and to perform a more refined analysis of the odor-scene, i.e. odor segregation, on subsequent odor stimuli when the delayed inhibition becomes effective.

5.6 Acknowledgments

We thank Christoph Kleineidam for LabView programming.

CHAPTER 6

Calcium Imaging of Single Neurites of Projection Neurons in the Honeybee Antennal Lobe

6.1 Abstract

Calcium imaging has been established as a standard method in neurobiology to record ensembles of neurons. The advantage of calcium imaging over electrophysiology is the simultaneous recording of a whole brain region in 2 or even 3 dimensions. Many important coding mechanisms could be revealed by that technique. However, conventional calcium imaging techniques were limited to superficial neuronal tissue and a low spatial and temporal resolution. We overcame these limitations by high temporal and spatial resolution and higher penetration depth by two photon- high resolution calcium imaging. We were able to record responses of single neurites within the same glomerulus in a honeybee antennal lobe, which is thought to be the smallest olfactory processing unit. Here, we address the questions whether projection neurons (PNs) from the same glomerulus indeed convey the same information, thereby enhancing the redundancy of the antennal lobe output, or whether different features of an odor response is split into different neurons, thereby enhancing the coding capacity of the antennal lobe. We recorded responses to different stimuli categories: Simple and monomolecular odors allow for the measurement of dose-response curves, whereas temporally complex stimuli and odor mixtures allow for the recording of temporal kinetics of the single neurites and to estimate network effects. At first, our results were consistent with results from previous, conventional calcium imaging studies. Moreover, we show that 1) signals from different neurites can be clearly separated at temporal resolutions of 20 Hz, 2) the penetration depth allows for imaging of glomeruli down to 100 µm below the AL surface. We show examples of dose-response relationships of individual PN neurites, and an example that responses of neurites from the same neuron are more similar than

of neurites from different neurons.

6.2 Introduction

Understanding the mechanisms of how olfactory networks encode and decode information requires techniques that enable the simultaneous recording of multiple neurons at a temporal and spatial resolution sufficient to resolve neurites and response kinetics. Imaging techniques have been developed in the last years and are able to deal with these needs. Calcium signals have been shown to be a very good monitor for actual electrical activity of neurons (Galizia and Kimmerle, 2004; Moreaux and Laurent, 2007). Calcium imaging studies contributed a lot to the understanding of olfactory coding in many different insects (Joerges et al., 1997; Sachse et al., 1999; Galizia et al., 1999b; Carlsson et al., 2002; Sachse and Galizia, 2003; Hansson et al., 2003; Carlsson et al., 2005; Silbering et al., 2008) and vertebrates (Friedrich and Korsching, 1997; Bozza and Kauer, 1998; Fuss and Korsching, 2001). Responses to odor stimuli of antennal lobe neurons are glomerulus- and odor specific, i.e. an odor elicits activity in distinct glomeruli, and glomeruli respond to a subset of odors. It is believed that glomeruli in the antennal lobe and olfactory bulb are the smallest processing unit of the antennal lobe. The output of a glomerulus is however split into several projection neurons (PN) per glomerulus, given by the numbers of PNs and glomeruli from Bicker et al. (1993) and Rybak and Eichmüller (1993). Recent studies show that output neuron responses of the same glomerulus are highly correlated (Kazama and Wilson, 2009; Chen et al., 2009), since they possess gap junctions (Miragall et al., 1996; Kosaka and Kosaka, 2004) and are electrically coupled (Kazama and Wilson, 2009). Conventional imaging studies with a CCD camera did not investigate the neuronal responses in sub-glomerular level. Reasons are the poor spatial resolution as well as the low penetration depth. Though there exist several studies applying two-photon microscopy for calcium imaging in insects (Haase et al., 2010; Seelig et al., 2010), the spatial resolution was not high enough to image from single neurites. Thus, questions regarding the calcium response properties and differences of single neurites in the antennal lobe have not been addressed. Redundant responses in different neurons suggests that the task is signal/noise reduction or conveyance of the same signal to different targets, whereas different responses in different neurons suggest a role in stimulus encoding, e.g. intensity or quality coding. To resolve the question of what the function of channel splitting is, the relevant neurons have to be recorded simultaneously. By electrophysiology, several neurons around a punctual recording site can be recorded, the type and exact location of the cells remains often ambiguous. Moreover, highly correlated signals from different cells overlay in a signal and are not possible to be separated by spike sorting mechanisms.

Two-Photon calcium imaging allows recording from multiple sites within the tissue at once and overcomes the limitations of electrophysiology. Here, we describe a calcium imaging technique to record glomerular odor responses with sub-cell resolution of neurites within the same glomerulus by means of two photon microscopy. We selectively stained uniglomerular projection neurons of the l- and m-ACT in the mushroom body and recorded calcium responses with spatial resolution of 1 µm and frequencies of up to 20 Hz from the glomeruli with a commercially available microscope. We show that calcium responses of different projection neurons can be clearly separated. Imaging on a single cell level opens up rooms to investigate principles of neural coding in small scaled networks and the mutual influence of neighboring cells.

6.3 Material and Methods

Bee staining and Preparation

Adult worker bees were cooled on ice, harnessed in plastic stages, fed, fixed and stained with fura-2-dextran 10'000 MW (Molecular probes, Eugene, OR) as described elsewhere (Szyszka et al., 2011). Next day, bees were fed again before they were prepared for imaging. Mandibles were forced apart and fixed with Deiberit wax (Dr. B"ohme & Sch"ops, Weslar, D). Antennae were directed to caudal and fixed with eicosane (Fig. 6.1 A). Glands and trachea above the brain tissue were removed. The esophagus was accessed and stretched through a cut between the labellum and the antenna bases. The brain tissue was then covered with a thin layer of silicone (KwikSil, World Precision Instruments, Sarasota, FL). These 3 steps were primarily performed to restrict movement. Then, a plastic cover slip was glued between antennae and the opened head to keep the antennae dry. A big drop of Ringer solution (in mM: 130 NaCl, 7 $CaCl_2$, 6 KCl, 2 $MgCl_2$, 160 saccharose, 25 glucose, 10 HEPES, pH 6.7, 500 mOsmol) was applied on top of the silicone, as an immersion.

Odors and Olfactometer

1-Octanol, 2-Octanol and 2-Heptanone (all Sigma-Aldrich, Germany) were obtained in *p.a.* quality. 2-octanol and 2-heptanone were used for dose-response relationships and diluted serially in 5 steps from $1 * 10^{-1}$ to $1 * 10^{-5}$ in mineral oil (Sigma-Aldrich). 1-Octanol and 2-heptanone were used for complex odor stimuli and diluted $1 * 10^{-2}$. Two cellulose pads (SugiPad, Kettenbach GmbH, Germany) were rolled and put in a 3 ml plastic syringe(NormJect, HSW GmbH, Tuttlingen, D). The upper pad was loaded with 200 µl of the odor dilution. Syringes were put in a custom built olfactometer similar

to the one described in Szyszka et al. (2011) but with 6 channels. The flow of the channels was set to values between 110-130 ml/min, adjusted for giving stimuli with equivalent odor concentration, as monitored by a Photo-Ionization Detector (Aurora Scientific Inc., Canada). The carrier air was set to 830 ml/min. The stimuli for the dose-response relationships consisted of a 2-second square odor pulse. The stimuli for the complex stimuli consisted of 3 pulses of 800 ms length, separated by 800 ms gaps. They were given 4 times each during an experiment, in pseudorandomized order. The stimuli were generated with a custom written program in LabView (National Instruments, Austin, TX) and triggered at a certain frame of the time series recording.

Microscope

Excitation light source was a mode-locked Ti:Sapphire laser (Chameleon(R), Coherent, Santa Clara, CA) at 810 nm, which has been shown as a suitable wavelength for fura2-excitation (Wokosin et al., 2004). Calcium responses were recorded with a commercial Laser Scanning Microscope (Zeiss LSM 510 Meta, Jena, Germany) equipped with an infrared-corrected 20x water immersion objective with numerical aperture of 1 (WPlan Apochromat, Zeiss). We used an SP 650 dichroic mirror to bring the laser in the beam path, an LP 685 main dichroic mirror, an HC SP 600 emission filter and the systems Non-Descanned Detector.

Data acquisition

We acquired data movies with the software Zeiss AIM 4.2 and the in the Non-Descanned mode. Time Series from optical glomerulus slices through single neurites were recorded at resolutions of ca. 1 µm per pixel and 10-20 Hz in 12 bit. High frame rates were acquired with unidirectional sampling in x-direction. Laser power and detector gain were adjusted to get absolute intensities of 1000-2000, but with not more than 100 mW of laser power to save the tissue from overheating.

Analysis

Data were processed in IDL 6.1 (Research Systems Inc., Boulder, CO): after manual movement correction, signals were calculated as negative relative fluorescent changes $\frac{-\Delta F}{F}$. Concentration series data, but not complex stimulus data were mean filtered in time in with a pixel size of 5. Traces were extracted from the movies with square regions of interest with a side length of 5 pixels (Concentration series) or 9 pixels (complex stimuli). The time traces of the 4 repetitions of the complex stimuli were averaged between the repetitions. Statistical analysis was performed in R (R Development Core

Team, 2011): by correlation analysis of the time traces to the different stimuli, a distance matrix was calculated for the ROIs, and reduced to 2 dimensions by classical multidimensional scaling (CMD, also known as principle coordinate analysis). All plots were calculated with R.

6.4 Results

We performed calcium imaging in projection neurons of the honeybee antennal lobe (Fig. 6.1). With a laser power of 50 mW, we achieved penetration depth of up to 100 µm, enough to record signals from single neurites in glomeruli of the l-ACT (Fig. 6.1 C). The spatial resolution in overview z-stacks was $\leq$ 1 µm per pixel, which was sufficient to tell apart single neurites within the same glomerulus, and at the same time record the whole width of the antennal lobe (250 µm), what enables identification of glomeruli.

Responses of neurites to odors are concentration dependent

In order to obtain images with sufficient spatial resolution to separate the signals from different neurons, and at the same time record with high temporal resolution, we zoomed into single glomeruli. We first acquired an image with high spatial resolution at a low scanning speed to orientate within the glomerulus (Fig. 6.2 A), and then reduced the spatial resolution to 64*64 pixel in order to record with 10 Hz. We stimulated the antennae with a 2-second stimulus of 2-heptanone or 2-octanol in 5 different concentrations. Single neurites responded to increasing concentrations of both odors with stronger and longer calcium responses (Fig. 6.2 B,C). The relative fluorescence change during responses to the highest concentration (10^{-1}) was 20 % $\frac{-\Delta F}{F}$. Even a low concentration of only 10^{-4} evoked a fluorescence change of 10%.

Responses to complex stimuli

We were interested in the temporal behavior of two-photon imaging to repetitive odor pulses. We stimulated the antennae with 3 consecutive pulses of 800 ms each, separated by gaps of 800 ms (referred to as "stimulus"). We used 1-heptanone, 1-octanol and the binary mixture of both odors as stimuli. We recorded calcium signals from neurite sections in different regions of the same antennal lobe (Fig. 6.3 A). In all neurite sections of the imaged glomerulus, heptanone evoked inhibitory odor responses, whereas octanol and the mixture evoked excitatory responses (Fig. 6.3 B). The odor pulses

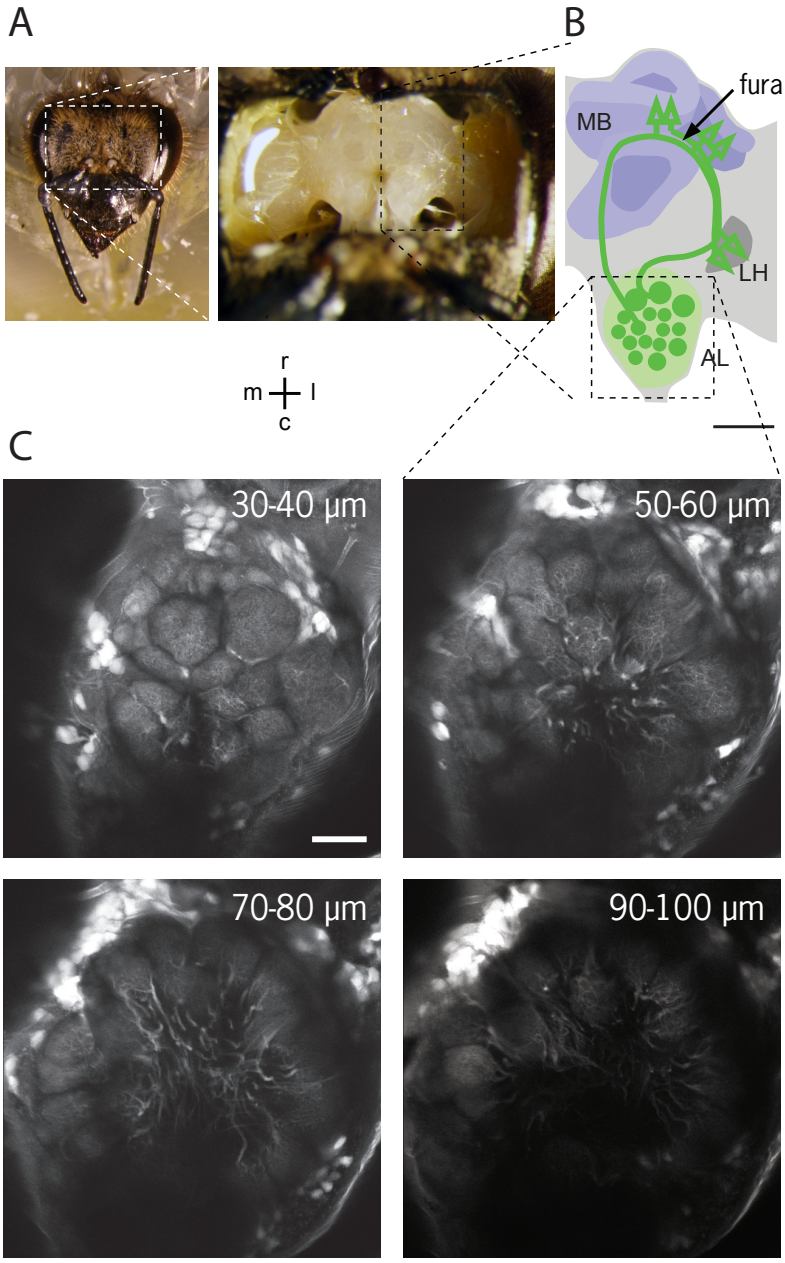

Figure 6.1: **Two photon calcium imaging in the honeybee antennal lobe.** A) photography of a honeybee head intact (left) and with opened head capsule and exposed brain as used for in situ calcium imaging (right). B) scheme of the olfactory pathway: projection neurons (PN, green) relay olfactory information from glomeruli in the antennal lobe (AL) to higher brain areas, i. e. to the calyces of the mushroom bodies (MB) and to the lateral horn (LH). PNs were retrogradely stained with Fura2-dextran between the calyces of MB (arrow). PN activity was recorded in the AL. C) background fluorescence of Fura2-stained projection neurons in different depth in the antennal lobe. Whole mount in vivo preparation, each picture consists of a projection of 10 optical slices, voxel size = 0.7*0.7*1 µm, scale bar = 50 µm.

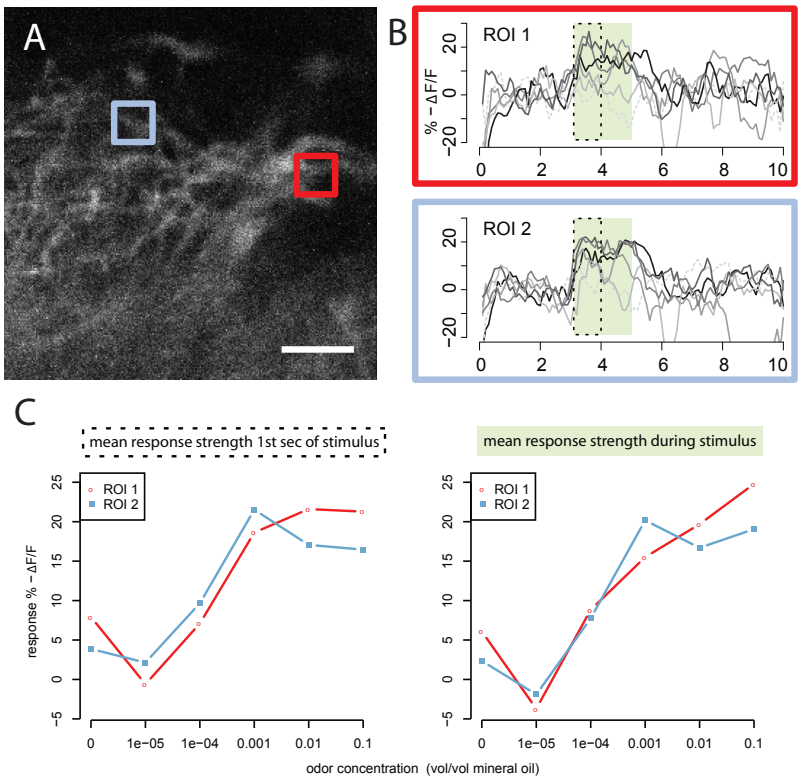

Figure 6.2: Responses of two neurite sections within a glomerulus to a 2-second long stimulus of 1-octanol at 5 concentrations. A) morphological view with indicated ROIs for response time trace and response strength extraction. Scale bar = 10 µm. B) time traces of the ROIs, darker shades are higher concentrations. Green bar shows the duration of the stimulus and corresponds to the integration time for response strength shown in C on the right (2 sec). Dotted rectangle indicates integration time for the response strength of the early response (C on the left, 1 sec). Acquisition rate was 10 Hz. C) Dose-response relationship (dilution in mineral oil vs. response strength) of the two ROIs within 1 (left) and 2 (right) seconds after stimulus onset.

evoked clearly separated response peaks. By a spatial high-resolution z-stack similar to the one in Fig. 6.1 C, we were to some degree able to tell the neurite origin. ROIs 4 and 5 in Fig. 6.3 likely belong to the same neuron whereas ROI 3 belongs to another neuron. We found that the response quantity (response strength) differed slightly between the sections (Fig. 6.3 C). Responses in ROI 3 were weaker than responses in ROI 4 and ROI 5. We then performed a principal coordinate analysis to check for similarities in the response qualities. Responses of neurite sections belonging to the same neuron clustered in the CMD plot for both tested odors and the mixture (Fig.6.3 D). This indicates that responses in different sections of a neurite might be more similar than responses from different neurons.

6.5 Discussion

In this study, we presented a method to record *in vivo* calcium signals from single projection neuron neurites in the antennal lobe of the honeybee (*Apis mellifera*) by means of two photon laser scanning microscopy. Penetration depth and spatial resolution are sufficient to resolve dendrites of different neurons within the same glomerulus. Our results show that single neurite responses can be separated. Odor responses are odor- and concentration-specific, confirming previous calcium imaging experiments with conventional techniques by means of CCD-camera acquisition (Joerges et al., 1997; Galizia et al., 1999b; Sachse and Galizia, 2002, 2003; Galizia and Kimmerle, 2004; Deisig et al., 2006, 2010), however, with considerable differences: We did not observe any photo bleaching and the relative fluorescence changes were around 4 times higher than compared to CCD-imaging with the same staining and preparation protocol (Galizia and Kimmerle, 2004; Szyszka et al., 2008; Deisig et al., 2010), as observed by other two-photon studies in the honeybee (Haase et al., 2010). However, noise level were also higher. Spatial resolution- z-stacks allows tracking of neurites only to some degree. To gain certainty in telling neurites apart, further post-experimental processing like clearing the tissue would be helpful, together with application of neuron tracers, which would be applied together with the calcium probe. Differences in signal strength and quality between neurons of the same glomerulus might either be due to actual, biological differences in response properties, or due to different staining success. Kazama and Wilson (2009) reported that the different neurites within the same glomerulus have the same response properties. The technique of manual staining of projection neurons may indeed result in inhomogeneously stained projection neurons, since the uptake of the dye depends on the rupture of the cell membrane. We experienced that in several animals treated with that technique, some glomeruli were stained whereas other were not. It is possible that even within the same glomerulus, some neurons might be

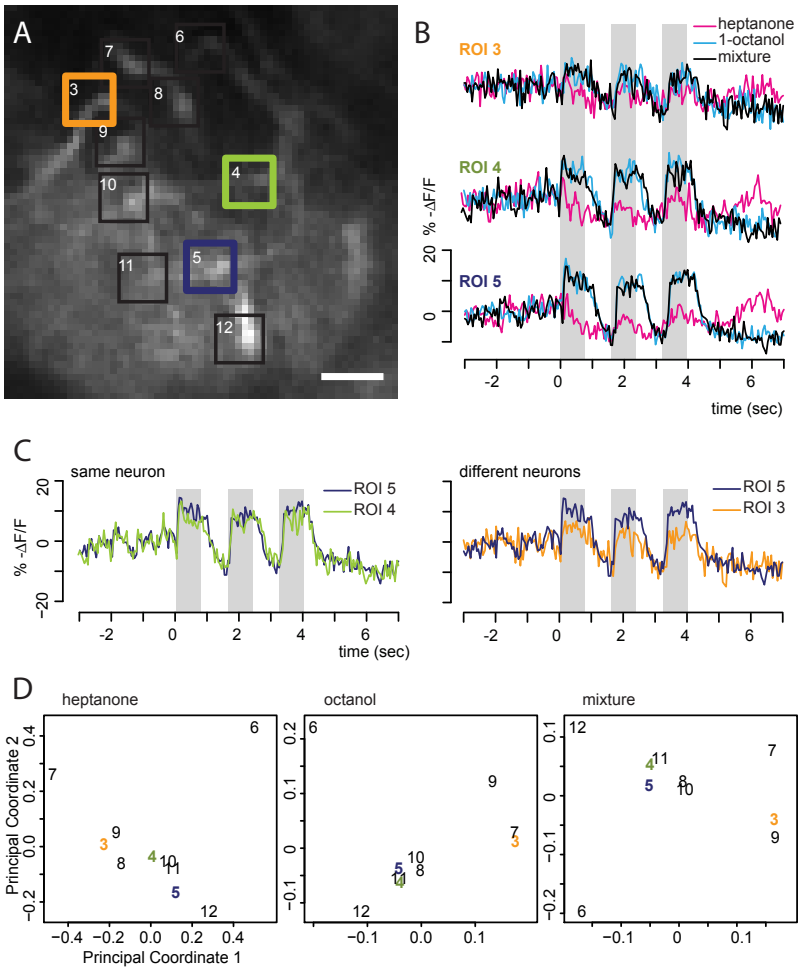

Figure 6.3: Responses of 3 neurite sections within a glomerulus. A) Morphological view of PN neurites in a glomerulus (scale bar = 10 µm), with indicated ROIs for response time trace extraction. B) Mean time traces of 4 odor responses of the ROIs to two odors and their binary mixture. C) responses of different neurites to the odor mixture (same data as in B, but sorted for neuron identity: responses in neurites of the same neuron (4 and 5) are more similar then from ROIs of presumably different neurons (3 and 5). D) Responses of ROIs of the same neuron (4, 5, 11) are similar and cluster in the CMD plot. Acquisition rate was 20 Hz.

stained and others are not, or that the neurons took up different amount of calcium dye, resulting in different ratios of calcium and dye in different neurons of the same glomerulus. Different dye to calcium ratios might result in slightly different response strength and kinetics. Whatever the origin of the variability between neurites is, the fact that we were able to record and detect minute differences between neurite section responses suggests that our method can acquire data of a quality from single neurites that suffices quantitative and qualitative analysis and is therefore potentially useful to study the role of single neurons within a network response.

6.6 Acknowledgments

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CHAPTER 7

General Discussion

This doctoral thesis investigates the role of insects antennal lobe in the processing of odorants. The main subject was to investigate the role of the antennal lobe in processing and discrimination of similar odor mixtures. We changed two parameters of an odor mixture, concentration ratio of the components and timing of the components. From the chapters 2 to 5, we gained the view that the concentration ratio can be different in odor mixtures emanating from the same odor source, but the timing of components cannot: Mixtures from one odor source are always "synchronous mixtures", whereas odor mixtures rendered by intermingling odors from different sources form "asynchronous mixtures" - due to turbulent and disruptive propagation of airborne odorants. In different manuscripts, we investigated mixture interactions in both kind of mixtures. But what are the consequences for an animal when odors are mixed? Are odor mixtures recognized as such by a sensory system?

7.1 How do odor mixtures differ from single-substance odorants?

Odors evoke stereotypic spatiotemporal response patterns in the brain. Both the components and the mixture evoke a subset of glomeruli, and high concentrations generally excite more glomeruli than low concentrations (Carlsson and Hansson, 2003; Sachse and Galizia, 2003). So, information about whether an odor is a mixture or a pure substance, or even how many components are in the mixture, cannot be deduced merely by the information which glomeruli are active. Hence, the information about odors can only be extracted from a mixture if one of the following preconditions are given:

1. The animal has an innate "pattern template" of the odor, e.g. for pheromones or kairomones.

2. the system has experienced and memorized the components before, for example by learning to associate the odor with a reward.

3. the mixture pattern can be deconstructed into the components pattern.

The first two preconditions imply that an odor is recognized by generalization. The pattern elicited by the odor must not be too dissimilar from the template or learned odor. That means, if the composition of the target odor changes to much, it will not be recognized any more. In chapter 2 of this study, we investigated the processing of odor mixtures that have an innate meaning for the animal, and how the behavioral and physiological responses change, when the composition of the odor mixture changes. We showed that the appetitive behavioral response correlated with the physiological response of a special glomerulus, and that the relation between the responsiveness (of both behavior and physiology) and the concentration of the key component was not linear. We conclude that by inhibitory mixture interactions, the response of a glomerulus is controlled, and the behavior of the animal is mediated by that glomerulus. I hold the view that for an unexperienced system, synchronous mixtures and monomolecular odorant conceptually make no difference, i.e. synchronous mixtures are not recognized as such. Both synchronous mixtures and pure odorants elicit a distinct spatiotemporal response patterns and a distinct percept and contain no information about the number of the constituents. After all, we name odors by and associate odors with single objects, and seldom by mixtures of them. For deconstructing the pattern to segregate the sub-patterns (precondition 3), the contours of the sub-patterns need to be clear. This would be possible if they differ on an additional dimension of information, e.g. time. Thus, this mechanism can only be applied on asynchronous odor stimuli. Interestingly, we all know this phenomenon from vision, where two similarly patterned objects, or an object in front of a similarly patterned background (for example, a camouflaged animal), can easily be separated as soon as they move independently (=asynchronous) from each other. The timing of "figure" and "ground" onset is a crucial parameter underlying this separation (Usher and Donnelly, 1998; Hancock et al., 2008). That means, the information about the two objects differ in the time dimension. Similar mechanisms are known from audition: Concurrent sounds can be better segregated when there is a temporal offset between them (Bregman and Pinker, 1978; Zera and Green, 1993; Carlyon, 2004; Lipp et al., 2010). In chapter 3, we show that this also holds true for the segregation of olfactory stimuli.

7.2 The role of the antennal lobe in odor object segregation

By acknowledging animals to respond differently to synchronous and asynchronous mixtures, the question arise, where and how this is accomplished by the neuronal systems. Since the antennal lobe has an important role in processing and reshaping odor responses, including the involvement of mixture interactions that are involved in synthetic or analytical mixture processing (Lei and Vickers, 2008), we sought in this region for neural correlates of odor object segregation. In chapter 4, we found inhibitory mixture interactions that were stronger in asynchronous mixtures with 5 and 50 ms delay. The odor stimuli in this study lasted between 0.8 and 1 second. Interestingly, the inhibitory phases were not during the odor response, but around 3 seconds after the odor onset, i.e. at least 2 seconds after the stimulus offset. By that, we were not able to conclude whether this late inhibitory phase is established only *after* an odor response, thus associated with a post-odor activity pattern that , or whether the inhibition was associated with the stimulus onset, and might be also active *during* an odor response. In chapter 5, we used longer odor stimuli that consisted of a train of 3 odor pulses. We analyzed the inhibitory responses separately for every pulse. In the response to the first odor pulse, we did not find asynchronous mixture suppression. In the response third pulse, they were strongest. Thus we concluded that the inhibitory phase is not associated with a post-odor response, but rather dependent on the time after stimulus onset, suggesting the involvement of short-term plasticity. Since the inhibition is effective at the earliest one second after stimulus onset, it might be $GABA_B$ driven. $GABA_B$ receptors were found in olfactory receptor neurons in the antennal lobe (Root et al., 2008), and have been reported to play a role in enhancing dissimilarity between odors in projection neuron responses (Wilson and Laurent, 2005). Thus they might indeed be important for the separability of similar odors or odor mixtures. Future studies on asynchronous mixture processing should involve behavioral and physiological experiments with $GABA_B$ antagonists, for example CGP54626, to test whether $GABA_B$ is mediating asynchronous mixture suppression in the late odor response and the ability to better segregate odorants from an asynchronous mixture. Also, plasticity on longer timescales might play a role for the ability to segregate odors from a mixture. We are currently testing the hypothesis that repeated stimulation with asynchronous mixtures shift the projection neuron ensemble responses of asynchronous mixture away from the synchronous mixture towards the components.

7.3 Odor object segregation in vertebrates

Despite very similar circuitry and mechanisms in odor processing (Strausfeld and Hildebrand, 1999; Kaupp, 2010), insects and mammals differ in a fundamental principle: Whereas insect's antennae are continuously exposed to the odor world, vertebrates are sniffing, thereby experiencing the odor world in batches. It is therefore questionable whether sniffing animals can perform odor object segregation with the same strategy (exploiting onset asynchrony), and if so, whether this can happen on similar timescales. Although olfactory neurons of mammals are precise enough to process stimuli with 10 ms acuity (Smear et al., 2011), it is open whether onset asynchrony on these timescales is used for odor-object segregation. The asynchrony between two odorant filaments needs to survive the turbulences of sniffing all the way through vertebrates nostrils up to the olfactory epithelium, what suggests that greater time differences might be necessary. At least, mammals use temporal differences on longer timescales, which involve adaptation, for odor-background segmentation (Kadohisa and Wilson, 2006; Linster et al., 2007).

7.4 Conclusions

We conclude that inhibitory mixture interactions in incoherent mixtures (delay suppression) that alter the response patterns in the antennal lobe output neurons (projection neurons), also alter the perception. Evidence arises from behavioral responses and physiological responses: Both show more similarity between asynchronous mixtures and component responses than between synchronous mixtures and component responses. Note that in chapter 3 and 4, we used the same odor stimuli with the same olfactometer, and that there is a correlation between perceptual and physiological odor similarity (Guerrieri et al., 2005). Taken together, the results of the 3 chapters on asynchronous mixture processing provide evidence to suggest an extension of the term "object recognition" from the boundaries of vision into the world of olfaction, as suggested already by Stevenson and Wilson (2007). For what it's worth, the word "object" was used by a couple of studies in the context of olfaction, although using definitions with different precision, ranging from a smelly object that can be "categorized" (Gottfried, 2009) to a position on a unidimensional, hedonic scale (Yeshurun and Sobel, 2010). The knowledge of how odors and odor mixtures are processed by sensory systems give insight in how neuronal networks grasp the outside world. Certainly, object recognition is not an ability that can attributed to one sense, but works best if all senses of an organism cooperate.

CHAPTER 8

Miscellaneous

8.1 Summary

This doctoral thesis investigates the role of the insect antennal lobe in the processing of odors. The main subject was to investigate the role of the antennal lobe in processing and discrimination of similar odor mixtures. We distinguish two odor mixture qualities: "Synchronous mixtures" are homogeneous blend that comprise fixed concentration ratios over time. These are odors that emanate from one source, or object. On the other hand, "asynchronous mixtures" form when odors of different sources intermingle. Due to filamentous propagation of odor plumes, they feature temporal incoherence between their components and changing concentration ratios. Whereas synchronous mixture often have their own meaning which is given by the ratio of the components, for example host plant odors, the meaningful information needs to be extracted from asynchronous mixtures. This requires a different processing of synchronous and asynchronous mixtures.

Host plant odors belong to synchronous mixtures. The ratios of the odor constituents differ between phenological stages of the plant. In chapter 2, we investigated the effect of ratios of host plant volatile constituents on herbivore insect attraction and olfactory information processing. We tested a synthetic mixture of bioactive peach shoot volatiles with different concentrations of one of the mixture constituents, benzonitrile, on oriental fruit moth *Cydia (=Grapholita) molesta* females. Y-tube olfactometer bioassays showed that female attraction to the mixture was maintained while increasing the benzonitrile level up to 100 times. Further increases led to behaviorally ineffective mixtures. Then, we recorded odor-evoked neural activity patterns in the antennal lobes, the main olfactory center of the brain, using calcium imaging. Benzonitrile-containing mixtures elicited strong activation in two glomeruli, which were found to process mixture-related information in specific ways. Activation in one glomerulus directly paralleled behavioral effects of the different ratios tested whereas a deviating pattern was noted in the other glomerulus. Our results indicate that the ratio of constituents in a volatile mixture can be varied to a certain degree without reducing female attraction. Thus, volatile blends in nature might vary quantitatively within a certain range without affecting odor-guided host location. Neurophysiological results showed that the processing of mixture-related information inside the antennal lobes is not uniform across glomeruli. Thus, final processing of this information probably takes place in higher-order brain centers.

Airborne odors rarely occur as pure, isolated stimuli. Still, how odor objects are segregated from a background, and how it is determined which of many concurrent stimuli belong to the same object, is a challenging unsolved problem both in neuroscience and in technical application. While this phenomenon has been investigated in depth in vision and audition, it has hardly been investigated in olfaction. In chapter 3,

we show that temporal difference in stimulus onset is sufficient for odor-object segregation: Honeybees recognize a learned odor better from a mixture with a novel odor, when the onset of either component is delayed by only 6 ms. This suggests that the temporal resolution of the olfactory system is much faster than previously thought.

Using in vivo calcium imaging of projection neurons in the honeybee, we then studied the neuronal mechanisms of odor-object segregation in the antennal lobe. In chapter 4, we show that asynchronous mixtures elicit response patterns that are different from synchronous mixtures: The responses to asynchronous mixtures contain more information about the constituent components. With longer onset shifts, more features of the components were present in the mixture response patterns. Moreover, we found that the processing of asynchronous mixtures activated more inhibitory interactions than the processing of synchronous mixtures. This means that the average response strengths to asynchronous mixtures are weaker.

Mixing odors from different sources, that intermingle in the air, results in intermittent stimuli. In chapter 5, we studied the effect of short term sensory experience on the processing of asynchronous odor mixtures. We applied odorants and their synchronous and asynchronous mixtures as trains of 3 odor pulses, and recorded the projection neuron responses with high speed calcium imaging. We found inhibitory mixture interaction in the responses to asynchronous mixtures for the second and third, but not for the first odor pulse, suggesting that the processing of these stimuli involves the delayed activation of an inhibitory network. Moreover, we found that response latencies to odorants did not differ in the various mixture situations, but increased when the same odor was repeated. This confirms that projection neurons are sensitive to millisecond short stimulus onset asynchrony and shows that processing of asynchronous mixtures might involves short-term plasticity in the antennal lobe.

The antennal lobe output is organized in bunches of ca. 5 projection neurons per glomerulus. The role of the different neurons within one glomerulus could either be channel splitting (conveying different response parameters) or redundancy, but is unknown since simultaneous recording from PNs of the same glomerulus has not been performed. In chapter 6, we show that simultaneous recording of calcium signals from single neurites within the same glomerulus is possible using a two photon laser scanning microscope. We recorded responses to simple and monomolecular odors for the measurement of dose-response curves, and to temporally complex stimuli and odor mixtures allow for the recording of temporal kinetics and eventually ties in with the previous manuscripts (chapters 2 - 4). Our results were consistent with results from previous, conventional calcium imaging studies. Moreover, we show that 1) signals from different neurites can be clearly separated at temporal resolutions of 20 Hz, 2) the penetration depth allows for imaging of glomeruli 100 µm below the AL surface. An application of this new approach would for example be the investigation of the function of channel splitting for the processing of asynchronous mixtures, by parallel

recording of multiple PNs within the same glomerulus.

8.2 Zusammenfassung

Diese Doktorarbeit untersucht die Rolle des Antennallobus von Insekten in der Verarbeitung von Düften. Im Mittelpunkt stand hierbei die Funktion bei der Unterscheidung von ähnlichen Duftmischungen. Wir definieren zwei unterschiedliche Qualitäten von Duftmischungen: Zum einen gibt es "synchrone Mischungen", das sind homogene Stimuli in denen die Konzentrationsverhältnisse der Mischungskomponenten in kurzen Zeiträumen stabil sind. Dies ist bei Duftmischungen der Fall, die von einem duftenden Objekt stammen. Zum anderen gibt es "asynchrone Mischungen". Sie entstehen, wenn sich Düfte aus verschiedenen Quellen überlagern. Aufgrund der filamentösen und turbulenten Natur der Duftausbreitung sind die Konzentrationsverhältnisse in diesen Mischungen nicht stabil und die Komponenten sind zeitlich inkohärent. Während synchrone Mischungen durch die genauen Konzentrationsverhältnisse oft eine Bedeutung tragen, zum Beispiel der Duft einer Wirtspflanze, so müssen die Bedeutungen aus asynchronen Mischungen erst extrahiert werden. Dieser Umstand verlangt eine differenzielle Verarbeitung von synchronen und asynchronen Duftmischungen.

Die Verhältnisse zwischen den Konzentrationen von Duftstoffen einer Wirtspflanze unterscheiden sich in den phänologischen Stadien. In Chapter 2 untersuchen wir den Effekt dieser Konzentrationsunterschiede auf die Attraktivität der Pflanze für herbivore Insekten, sowie auf die Verarbeitung dieser Düfte im Gehirn. Wir prüften die Wirkung einer künstliche Mischung bioaktiver Duftstoffe von Pfirsichtrieben auf weibliche Individuen des Pfirsichwicklers (*Cydia (=Grapholita) molesta*). Dabei wurde die Konzentration einer der Duftstoffe, Benzonitril, variiert. Bis zu einer 100-fachen Erhöhung der Benzonitrilkonzentration war die Mischung für die Weibchen attraktiv, bei noch höheren Konzentrationen nicht mehr. Durch Kalziumbildgebung zeichneten wir die Neuronenaktivität im Antennallobus zu diesen Stimuli auf. Mischungen mit Benzonitril aktivierten zwei Glomeruli besonders stark. Diese verarbeiteten Informationen über die Mischung auf ihre eigene Weise: Die Aktivität des einen Glomerulus entsprach genau der Attraktivität der verschiedenen Mischungen. Das Aktivitätsmuster des anderen Glomerulus wich allerdings von dieser Regel ab. Die Ergebnisse zeigen, dass das Verhältnis der Komponenten in gewissem Rahmen variieren kann, ohne dass die Attraktivität auf die Weibchen reduziert wird. Die neurophysiologischen Ergebnisse zeigen dass die Verarbeitung der Duftmischung in unterschiedlichen Glomeruli auf unterschiedliche Weise geschieht.

Luftgetragene Düfte sind selten reine und isoliert auftretende Substanzen. Trotz dessen ist sowohl aus neurowissenschaftlicher wie aus der Sicht technischer Anwendungen ungelöst, wie Düfte aus einem Dufthintergrund segregiert werden können, und wie bestimmt werden kann, welche von vielen gleichzeitig auftretenden Duftstoffen zum gleichen Duftobjekt gehören. Während ähnliche Phänomene für die Sinne des Sehens

und Hörens gut erforscht sind, gibt es noch kaum Arbeiten dazu für den Geruchssinn. In Chapter 3 zeigen wir, dass zeitliche Unterschiede zwischen den Komponenten einer Duftmischung die Segregierung von Duftobjekten begünstigt: Honigbienen *(Apis mellifera)*, welche gelernt haben, dass ein Duft einer Belohnung durch Zucker vorausgeht, reagieren auf eine Mischung aus diesem und einem anderen Duft stärker, wenn zwischen den Komponenten ein Zeitversatz von 6 Millisekunden herrscht. Die zeitliche Auflösung des olfaktorischen Systems ist also schneller als bisher gedacht.

Mit Hilfe von Kalziumbildgebung an Projektionsneuronen im Antennallobus der Honigbiene studierten wir dann die neuronalen Mechanismen, die dieser Duftobjekt-Erkennung zugrundeliegen. In Chapter 4 zeigen wir, dass asynchrone Mischungen neuronale Aktivitätsmuster hervorrufen, die mehr Informationen über die Komponenten enthalten als Antworten auf synchrone Mischungen. Mit längeren Zeitunterschieden zwischen den Komponenten werden die Antworten denen der Komponenten immer ähnlicher. Außerdem treten mehr inhibitorische Mischungsinteraktionen auf, d.h. die Antworten der Neurone werden schwächer.

Wenn sich Düfte von verschiedenen Objekten turbulent in der Luft vermischen, entstehen unterbrochene, pulsartige Duftstimuli. In Chapter 5 untersuchen wir den Effekt kurzfristiger Erfahrung durch wiederholte, gepulste Stimulation auf die neuronale Verarbeitung der asynchronen Mischungen. Mit hoher Frequenz von bis zu 200 Hz zeichneten wir an einem konfokalen Mikroskop die neuronale Antwort ausgewählter Glomeruli auf eine Abfolge von 3 Pulsen auf, um räumliche und zeitliche Antwortparameter zu erfassen. Mischungsinteraktionen traten nur für asynchrone Mischungen auf, und das erst im 2. und 3. Puls, aber nicht im ersten Puls. Dies weist darauf hin, dass die Prozessierung von asynchronen Mischungen mit einer verzögerten Aktivierung eines inhibitorischen Netzwerken einhergeht. Darüberhinaus sind die Antwortlatenzen auf Duftstimuli für die verschiedenen Mischungssituationen gleich, nehmen jedoch von Puls zu Puls zu. Diese Studie bestätigt, dass Projektionsneurone zeitliche Unterschiede von wenigen Millisekunden verarbeiten. Außerdem scheint kurzfristige Plastizität durch ein inhibitorisches Netzwerk beteiligt zu sein.

Der Antennallobus gibt die Information nach Prozessierung anhand ca. 5 Projektionsneurone an höhere Hirnregionen weiter. Warum die Ausgabe in mehreren Projektionsneuronen organisiert ist, ist nicht hinreichend bekannt. Zum einen könnte die gleiche Information an verschiede Ziele gehen, zum anderen könnten in den verschiedenen Neuronen unterschiedliche Information transportiert werden. Um Fragen wie diese zu lösen, müssen Duftantworten von Projektionsneuronen mit entsprechend hoher räumlicher Auflösung in allen drei Raumdimensionen gleichzeitig aufgenommen werden. In Chapter 6 zeigen wir Beispiele dass diesen Anforderungen mit einem Lasers-Scanning-Mikroskop und Zwei-Photonen-Anregung von Kalziumsonden entprochen werden kann. Wir erstellten eine Dosis-Wirkung Beziehung für die Duftantworten einzelner Neuriten und zeichneten auch die Antworten auf zeitlich komplexe Stimuli und Mischungen

auf und knüpfen so an unsere vorhergehenden Studien an. Die Duftantworten der Zwei-Photonen Technik entsprechen qualitativ denen konventioneller Kalziumbildgebungsverfahren. Neuriten und deren Antworten waren klar voneinander abgrenzbar, während die zeitliche Auflösung mit 20 Hz gegenüber physiologischen Aufnahmen mit Epifluoreszenz ebenfall um ein vielfachen höher war. Die Eindringtiefe lag mit 100 mW Laserleistung bei ca. 100 µm. Mit Hilfe dieser Technik wäre eine Untersuchung der Rolle verschiedener Projektionsneurone des gleichen Glomerulus bei der Prozessierung von asynchronen Mischungen denkbar.

8.3 List of publications and declaration of self-contribution

1. Chapter 2: Published as
   Behavioral and Neurophysiological Responses of an Insect to Changing Ratios of Constituents in Host Plant-Derived Volatile Mixtures
   A. J. Najar-Rodriguez, C. G. Galizia, J. Stierle and S. Dorn
   2010. The Journal of Experimental Biology 213, 3388-3397.
   doi: 10.1242/jeb.046284

   Self contribution: analyzed the physiological data, created figures 3-5, wrote according parts of the paper.

2. Chapter 3: Published as
   The Speed of Smell: Odor-Object Segregation within Milliseconds
   P. Szyszka, J.S. Stierle, S. Biergans and C. G. Galizia
   2012. PLoS ONE 7(4): e36096.
   doi: 10.1371/journal.pone.0036096

   Self contribution: designed experiments together with PS, analyzed the data for figure 1, wrote parts of the paper.

3. Chapter 4: Published as
   Millisecond Stimulus Onset-Asynchrony Enhances Information about Components in an Odor Mixture
   J.S. Stierle, C. G. Galizia and P. Szyszka
   2013. The Journal of Neuroscience, 33(14): 6060 – 6069.
   doi: 10.1523/JNEUROSCI.5838-12.2013

   Self contribution: designed research together with PS and CGG, performed research together with PS, analyzed the data, wrote major parts of the paper.

4. Chapter 5: In preparation
   Short-term Plasticity in the Insect Olfactory System Enhances Sensitivity for Stimulus Onset Asynchrony
   J.S. Stierle, C. G. Galizia and P. Szyszka

   Self contribution: designed research together with PS and CGG, performed research, analyzed the data, wrote the manuscript.

5. Chapter 6: In preparation
   Calcium Imaging of Single Neurites of Projection Neurons in the Honeybee Antennal Lobe
   J.S. Stierle, C. G. Galizia and P. Szyszka

   Self contribution: designed research together with PS and CGG, performed research, analyzed the data, wrote the manuscript.

8.4 Abbreviations

AL	antennal lobe
ANOVA	Analysis of Variance
B	benzonitrile
BES	benzonitrile sensitive glomerulus
BOR	benzonitrile optimum response glomerulus
CMD	classical multidimensional scaling
EAG	electro-antennogram
GABA	gamma-aminobutyric acid
H	1-hexanol
LIS	linalool sensitive glomerulus
LN	local neuron
N	1-nonanol
ORN	olfactory receptor neuron
PC	principal component
PCA	principal component analysis
PN	projection neuron
RH	relative humidity
ROI	region of interest
s.e.m.	standard error of the mean
SC	stronger component
SD	standard deviation

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