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In the text of this paper, the proportions of two compounds in the plant-derived volatile mixture tested, i.e. (*E*)-2-hexenal and benzaldehyde, were inadvertently exchanged.

In the second paragraph of the 'Chemicals and mixtures' section on p. 3389, it is stated that the mixture mimicking bioactive peach shoot volatiles was composed of (*Z*)-3-hexenyl acetate, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, benzaldehyde and benzonitrile, at ratios of 69.74:14.62:13.24:2.25:0.15 vol./vol., respectively. The proportions of the compounds in the mixture should read: 69.74:14.62:2.25:13.24:0.15 vol./vol.

The authors apologize for this error but confirm that the correct proportions of compounds were used throughout the study and therefore results and conclusions remain unaffected.

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

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SUMMARY

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.

Key words: insect, olfaction, behavior, odor ratios, odor mixtures, calcium imaging, antennal lobe, olfactory glomeruli.

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 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 (Bichão 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., 1999; 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 et al., 1992; Riffel 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.

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 (Convion Ltd, Winnipeg, MB, Canada), with L:D 16h:8h, 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 naïve.

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 69.74:14.62:13.24:2.25:0.15 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 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 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

Table 1. Odors used in the trials with mated female oriental fruit moths

Odor(s)	Abbreviation	Ratios of the first constituent of the mixture to benzonitrile (m:B)	Benzonitrile concentration (ng μl^{-1})		
			10^{-4}	10^{-3}	10^{-2}
Mixture with no benzonitrile	mB0	100:0	0	0	0
Mixture with benzonitrile decreased 100×	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
Mixture with benzonitrile increased 100×	mB100	86.69:13.31	22	220	2200
Mixture with benzonitrile increased 1000×	mB1000	39.42:60.58	220	2200	22,000
Mixture with benzonitrile increased 5000×	mB5000	12.53:87.47	1100	11,000	110,000
Benzonitrile alone	B	0:100	100.4	1004	10,040

Abbreviations and ratios of the first constituent in the mixture (m) to benzonitrile (B) shown for each odor. Absolute concentrations of benzonitrile (ng μl^{-1}) 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.

trials were carried out as described in Piñero and Dorn (Piñero and Dorn, 2007) and Piñero et al. (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 $740 \pm 10 \text{ ml min}^{-1}$ 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 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 $\times 20$ 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: $1.58 \pm 0.27 \text{ m s}^{-1}$, $19.3 \pm 0.56^\circ\text{C}$) to avoid contamination from external odors in the room. The brain was kept in buffer (in mmol l^{-1} 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^{-1} 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 8×8 binning on chip was applied to a spatial resolution of $1.57 \mu\text{m} \times 1.57 \mu\text{m pixel}^{-1}$. 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 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 Foundation for Statistical Computing, Vienna (Austria), www.R-project.org]. 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 3–5) were plotted using R [R Foundation for Statistical Computing, Vienna (Austria), www.R-project.org].

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. 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 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 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. 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. 2B). This glomerulus was thus considered as the reference glomerulus and was used to normalize responses across females. The different mixtures tested elicited strong activation of two additional glomeruli, the benzonitrile

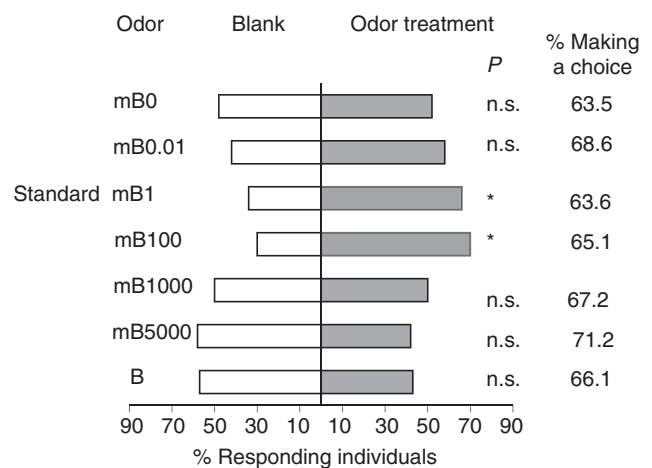


Fig. 1. Behavioral responses of mated female oriental fruit moths in a Y-tube olfactometer to synthetic odors derived from peach volatiles: five mixtures differing only in the proportion of benzonitrile (mB0.01 to mB5000), a mixture with no benzonitrile (mB0) and benzonitrile alone (B). Preference for 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: * $P<0.05$; n.s.= $P>0.05$.

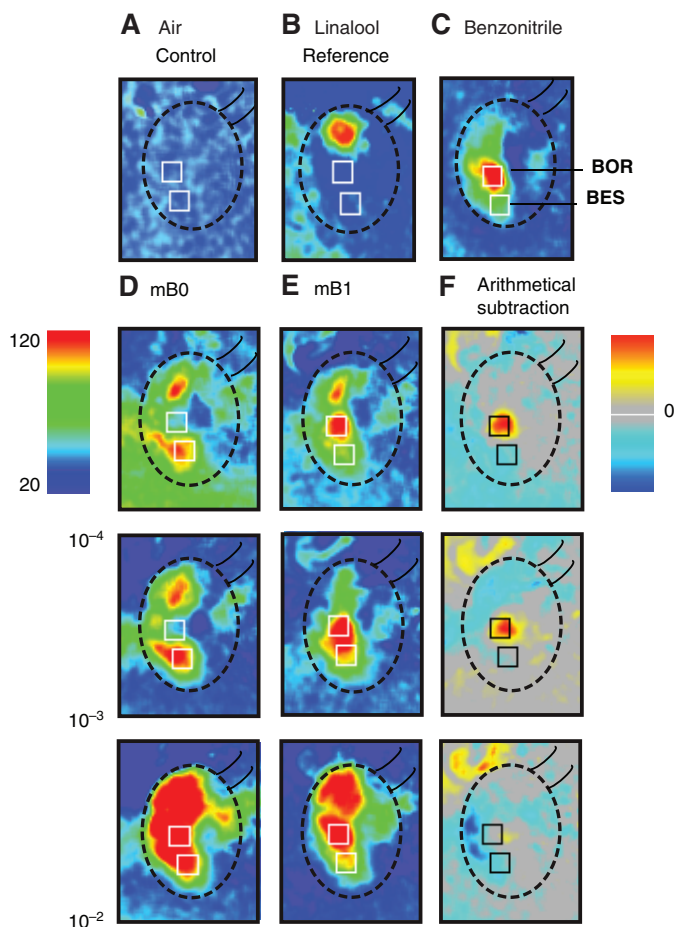


Fig. 2. Spatial odor response patterns in the right antennal lobe (AL) of a representative mated female oriental fruit moth using calcium imaging. Differential odor response profiles measured as relative changes in fluorescence ($\Delta F/F$) and in false-color coding shown for two selected glomeruli (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.

optimum response glomerulus ‘BOR’, and the benzonitrile sensitive glomerulus ‘BES’ (for details see below) (Fig. 2C,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. 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. 2E) and mB0 (Fig. 2D), and compared the calculated result (Fig. 2F) with the response to benzonitrile alone (Fig. 2C). 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. 3B). In the BES glomerulus, responses to the mixture without benzonitrile (mB0) (Fig. 2D) were stronger than those evoked by the mixtures containing benzonitrile (Fig. 2E), even at low concentrations of benzonitrile (compare with Fig. 3E). 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. 3A,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. 3B,E) and the second (Fig. 3C,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. 3A–F) and the air control (Fig. 2A).

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.

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. 3B). 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. 3B).

Responses to the second odor pulse were lower and less dose dependent in comparison with the first pulse (Fig. 3C). 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. 4A,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. 4A), as is also reflected in the synopsis of responses shown in Fig. 4B. Significant inhibitions

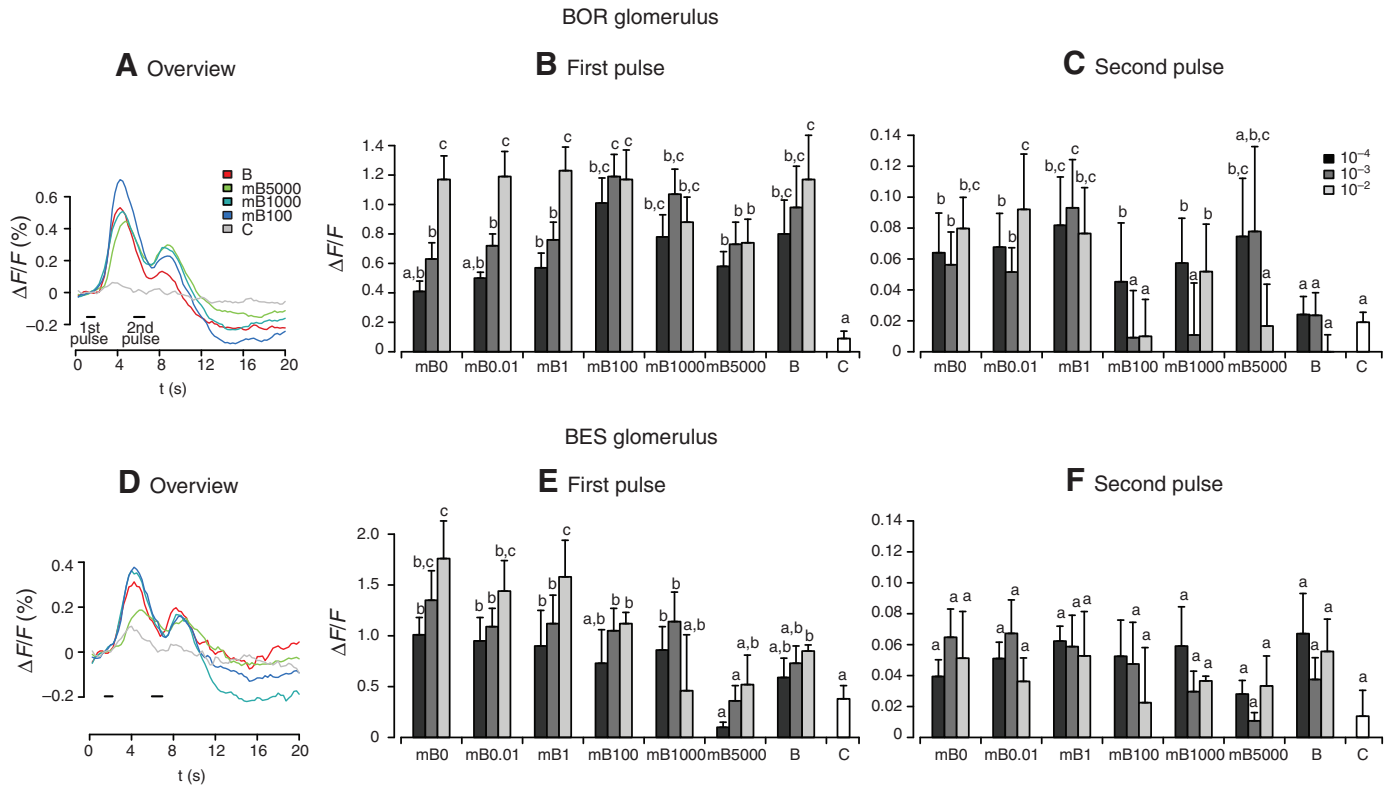


Fig. 3. Time traces and dose–response relationships elicited by two consecutive pulses of the same odor in two selected glomeruli inside the antennal lobes (ALs) of mated female oriental fruit moths. Tested were five different peach-derived synthetic mixtures (mB0.01, mB1 standard mixture, mB100, mB1000 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 (\pm s.e.m.). Bars followed by the same letters are not significantly different from one another (Fisher's LSD *post-hoc* test).

were only noted for the mixture mB1000 at 10^{-3} and 10^{-2} and for mB5000 at 10^{-2} (Fig. 4A,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. 3B), inhibitions could only be the result of network activity (Fig. 4B).

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. 3E). 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. 3E). 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. 3F).

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. 4C,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. 4C,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. 5A), 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. 5B) 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. 5B) 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).

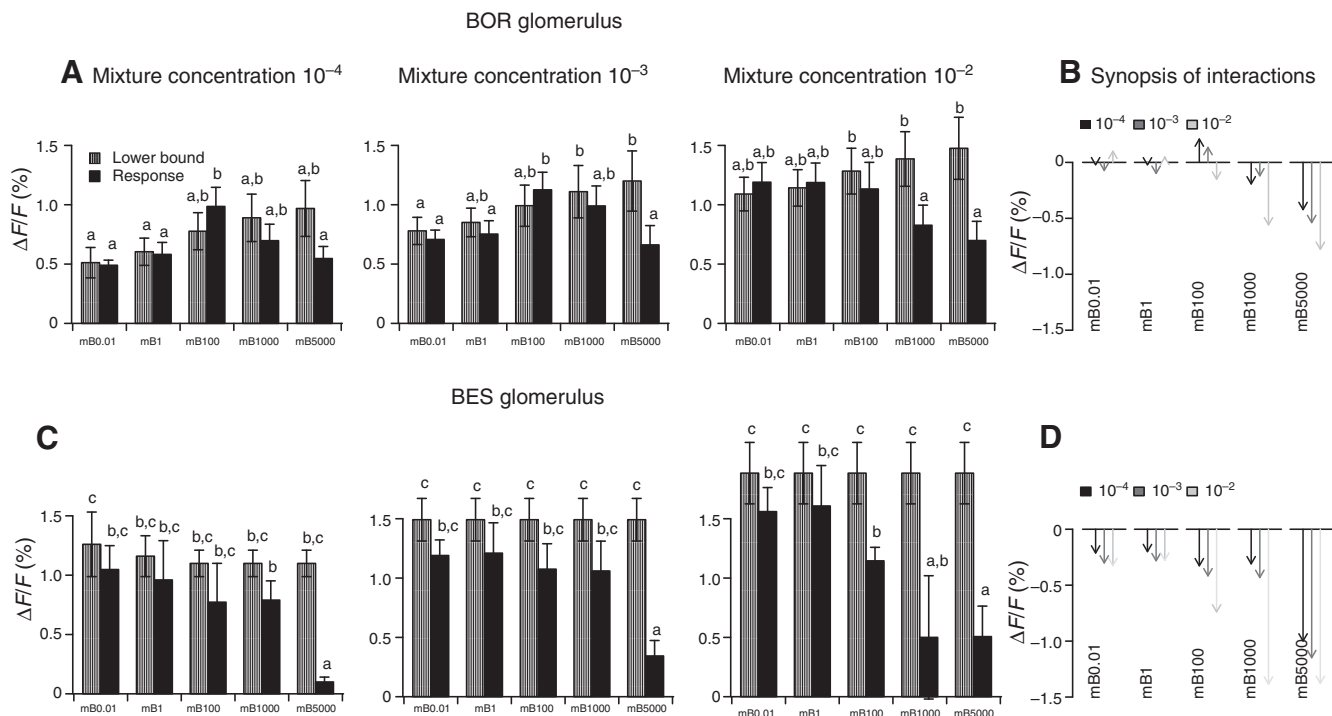


Fig. 4. Mixture interactions calculated for two selected glomeruli (BOR and BES, same individuals as in Fig. 3) identified inside the antennal lobes (ALs) of mated female oriental fruit moths (absolute concentrations of 10^{-2} , 10^{-3} and 10^{-4}). Responses to a given odor (black bars) and its calculated lower bound (striped 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.

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* (Riffel et al., 2009b). 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

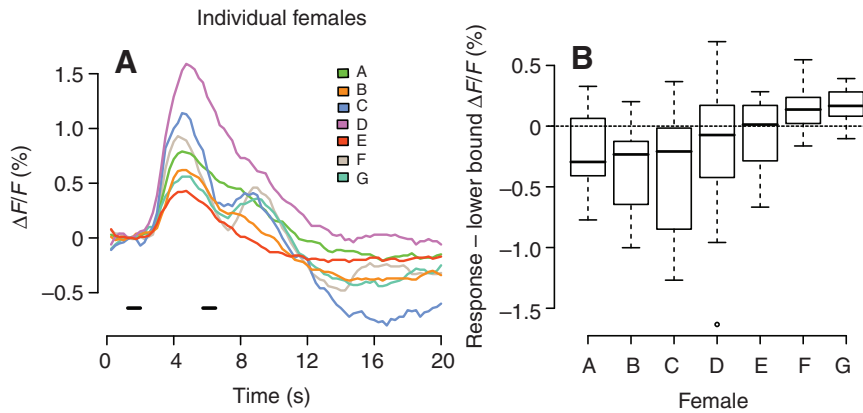


Fig. 5. (A) Individual time traces recorded inside the BOR glomerulus in response to a representative mixture (mB100, 10^{-3}). Each color represents a different female ($N=7$, same individuals as in Fig. 3). The stimulations (1 s each) are indicated as black lines. Note the variability across individuals 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 $1.5\times$ inter-quartile range). Negative values indicate inhibitory mixture interactions; positive values indicate the lack of inhibitory effects.

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 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., 2009a), 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 (e.g. Christensen et al., 1989; Christensen et al., 1993; Vickers et al., 1998) and locusts (e.g. 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 and Galizia, 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. benzoinitrile 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).

In conclusion, the behavioral and physiological evidence presented here suggests that recognition of volatile blends might not be as ratio-specific as previously assumed. Our results also show that the processing of mixture-related information inside the AL is not uniform across glomeruli and that the final processing of this information likely takes place in higher-order brain centers along the olfactory pathway. Future research should address further effects of changing ratios of plant volatile constituents on insect behavior, such as female flight in wind tunnels, and on the processing of odor information in higher-order brain centers. These studies could provide us with more evidence as to disentangle the neural mechanisms underlying odor perception in insects and to understand how processing of olfactory information ultimately leads to behavior.

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