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# Neural coding merges sex and habitat chemosensory signals in an insect herbivore

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Understanding the processing of odour mixtures is a focus in olfaction research. Through a neuroethological approach, we demonstrate that different odour types, sex and habitat cues are coded together in an insect herbivore. Stronger flight attraction of codling moth males, *Cydia pomonella*, to blends of female sex pheromone and plant odour, compared with single compounds, was corroborated by functional imaging of the olfactory centres in the insect brain, the antennal lobes (ALs). The macroglomerular complex (MGC) in the AL, which is dedicated to pheromone perception, showed an enhanced response to blends of pheromone and plant signals, whereas the response in glomeruli surrounding the MGC was suppressed. Intracellular recordings from AL projection neurons that transmit odour information to higher brain centres, confirmed this synergistic interaction in the MGC. These findings underscore that, in nature, sex pheromone and plant odours are perceived as an ensemble. That mating and habitat cues are coded as blends in the MGC of the AL highlights the dual role of plant signals in habitat selection and in premating sexual communication. It suggests that the MGC is a common target for sexual and natural selection in moths, facilitating ecological speciation.

## 1. Introduction

Odours typically are blends of several chemicals, in specific proportions, and the olfactory system decodes and discriminates these multi-dimensional signals rapidly and precisely. A current question is how odour blends are represented in olfactory circuits and to what extent the neural odour space reflects their ecological and evolutionary significance [1–4].

For reproduction, animals largely rely on two types of olfactory signals: sex pheromones distinguish conspecific mates, and habitat odours signal food sources for adults and offspring. Both sex and habitat odours are important mediators of premating reproductive isolation and speciation [5–7], and the neural circuitry underlying the integration of these two types of chemosensory cues is, therefore, an important target for sexual and natural selection. The interaction of sexual and natural selection is thought to be a powerful driver of speciation [8–10].

Insect herbivores are particularly suitable for studying the interaction between mating and habitat cues, especially host plant odours, owing to the importance of these signals for their ecology and evolution. Host plant shifts have probably contributed to the remarkable diversification of plant-feeding insects [11,12], and most of these rely on sex pheromones for mate finding [13,14].

Plant volatiles are recognized as sex pheromone modulators in many insect species [15,16]. Although the behavioural interaction between pheromones and host plant volatiles is well established, little is known about the neurophysiological correlates. Research on the processing of odour blends in the primary olfactory

centre in the brain, the antennal lobe (AL), has focused mainly on sex pheromones or on plant volatiles, whereas the combination of these two classes of compounds has been investigated only recently [17–19].

Separate investigation of pheromones and plant volatile stimuli has led to the idea of a functional specialization of sensory processing in the AL, and that these two odour classes are represented in morphologically different regions of the AL of male moths. The macroglomerular complex (MGC) is considered to be dedicated to pheromone coding and the sexually isomorphic, ordinary glomeruli to the coding of plant volatile information [20]. Recent studies in the silk moth *Bombyx mori* and the noctuid moth *Agrotis segetum*, however, do not corroborate a strict segregation of the two subsystems, and indicate that the MGC receives lateral input from the AL [17–19].

In the codling moth *Cydia pomonella* (Lepidoptera, Tortricidae), a reconstruction of the glomerular structure of the AL, combined with electrophysiological recordings, suggested significant crosstalk between the pheromone and general odour subsystems [21]. Codling moth is a key pest of apple, and its sex pheromone and the behavioural role of host plant volatiles have been carefully studied [22].

We investigated the neurophysiological mechanisms regulating the interaction between female sex pheromone and behaviourally active host plant odorants, using functional imaging of the AL and intracellular recordings (IRs) of projection neurons (PNs) that transmit olfactory signals to higher brain centres. The finding that the MGC is dedicated to blends of social and environmental odours adds to our understanding of the role of chemosensory cues in premating reproductive isolation and plant–insect ecology. It also provides a new incentive for the refinement of sustainable insect control methods based on behaviour-modifying chemicals.

## 2. Material and methods

### (a) Insects

Experiments were carried out with 2- to 3-day-old unmated codling moth *C. pomonella* (Lepidoptera, Tortricidae) males, which were reared for several generations on an artificial diet (Adermatt Biocontrol, Grossdietwil, Switzerland). The males were kept at  $70 \pm 5$  per cent relative humidity,  $23^\circ\text{C}$ , under a 16 L:8 D photoperiod, and they were fed with sugar water.

### (b) Odour stimuli

Test odours included the main component of codling moth female sex pheromone, codlemone, (*E,E*)-8,10-dodecadienol (more than 99.6% chemical and isomeric purity, Shin-Etsu Chemical Co., Tokyo) and three plant volatiles, (*E*)- $\beta$ -farnesene (93.4% pure), butyl hexanoate (97.8%, both from Bedoukian Research Inc., Danbury, CT, USA) and pear ester, (*E,Z*)-2,4-decadienoate (87.4%, Sigma Aldrich).

For functional imaging and IRs, solutions of test compounds in  $10 \mu\text{l}$  re-distilled hexane were applied on filter paper ( $0.5 \times 1 \text{ cm}$ ), approximately 1 h before tests. After the solvent evaporated during 1 min, one or two filter papers (compound blends) were inserted into a Pasteur pipette. Codlemone was tested at amounts of from 1 ng to 1  $\mu\text{g}$ , plant compounds from 10 ng to 10  $\mu\text{g}$ , in decadic steps. A continuous charcoal-filtered and moistened airstream ( $500 \text{ ml min}^{-1}$ ) passed through a glass tube (10 mm i.d.) over the antenna. A stimulus controller (SFC-2/b, Syntech, Kirchlachen, Germany) injected a 0.5 s puff ( $500 \text{ ml min}^{-1}$ )

through the pipettes into this glass tube. Odours were presented in randomized order. Pipettes with filter paper loaded with  $10 \mu\text{l}$  of solvent were used as control.

For behavioural tests, synthetic compounds were released from a piezo sprayer [23]. Compound dilutions were delivered at  $10 \mu\text{l min}^{-1}$  to a  $20 \mu\text{l}$  glass capillary tube with a drawn-out tip. A piezo-ceramic disc vibrated the capillary at approximately 100 kHz, producing an aerosol, which evaporated a few centimetres downwind from the capillary tip at a constant rate and known chemical purity. Codlemone was tested at  $0.1 \text{ pg min}^{-1}$ , and plant compounds at 1 and  $100 \text{ pg min}^{-1}$ .

### (c) Behavioural assay

Wind tunnel experiments were conducted according to Knight *et al.* [24]. A fan pulled air through a charcoal filter, through a series of screens, at  $0.25 \text{ m s}^{-1}$  into the tunnel ( $1.6 \times 0.6 \times 0.6 \text{ m}$ ). Exhaust was expelled outside the building. Room lighting was computer-controlled to gradually decrease during a 60 min dusk period, between full light level (1330 lux) and the dark period (25 lux). Ten batches of five moths were flown consecutively to each lure, during the first 3 h of the scotophase. Male moth behaviour was recorded for up to 6 min. The following types of behaviour were recorded: wing fanning, take-off, upwind flight and contact with the screen. Proportional data were adjusted with Bartlett's correction for small sample size. An angular transformation was used to normalize proportional data prior to analysis of variance (ANOVA; STATISTIX v. 9, Analytical Software, Tallahassee, FL, USA). An  $\alpha$ -level of 0.05 was used to establish significance, Tukey's method was used to compare means.

### (d) Functional imaging

Individual moths were secured in a 1 ml plastic pipette, with the head protruding from the narrow end, and fixed by dental wax (Surgident, Heraeus Kulzer Inc.). The head capsule was opened between the antenna and the eyes; muscle, glands, trachea, neural sheath and the oesophagus were removed to expose the AL [25]. A calcium-sensitive dye (calcium green-2-AM dye) was dissolved in 20 per cent Pluronic F-127 in dimethyl sulfoxide (Molecular Probes, Eugene, OR, USA), and diluted in moth Ringer solution to  $30 \mu\text{M}$ , and then applied to the brain, leaving the preparation in a dark and cold ( $5^\circ\text{C}$ ) environment for 3 h.

Recordings were made *in vivo* after incubation and washing, using an Olympus microscope ( $20\times$  air objective NA 0.50; filter settings: dichroic 500 nm, emission LP 515 nm). The preparation was illuminated at 475 nm. Stimulation started at frame 12 and lasted 1 s. Images were binned twice ( $320 \times 240$  pixel) to increase signal-to-noise ratio. TILL PHOTONICS imaging software (Gräfelfing, Germany) was used to record sequences of 40 frames (4 Hz, 200 ms exposure time), and noise was removed by a Gaussian filter. The response magnitude was calculated as the average  $\Delta F/F$  for each frame, where  $F$  was estimated using a linear function fitted to the parts of the calcium fluorescence decay curve outside the potential response. The onset of the signal was set to the time of the first frame with a positive average  $\Delta F/F$ . For statistical analysis, a Kruskal–Wallis test was followed by a Mann–Whitney *U*-test with Holm–Bonferroni correction. A three-dimensional map of the codling moth AL [21] was used to link the active area to AL glomeruli.

### (e) Intracellular recordings

Insect preparation and recordings were carried out as described by Trona *et al.* [21]. During recordings, the brain was superfused with pH 6.9 Ringer solution delivered from a flow system. A silver ground electrode was in contact with the ringer solution. Using a micromanipulator, the AL was randomly penetrated with an electrode that was drawn from a heated glass capillary (0.5 mm i.d., Sutter Instrument Co., Novato, CA, USA) with

the tip filled with 1 per cent neurobiotin (Vector Laboratories, Burlingame, CA, USA) dissolved in 0.25 mM KCl, and the remaining part was filled with 1 mM KCl.

After recordings, the AL interneuron was stained with a depolarizing current (0.5–0.7 nA, 15 min). The brain was dissected from the head capsule and stained following the protocol of Trona *et al.* [21]. Stained neurons were viewed in a laser scanning confocal microscope (Zeiss LSM 510, Carl Zeiss, Jena, Germany) with a  $40 \times 1.4$  oil-immersion differential interference contrast objective. Alexa Fluor 488, fluorescein Avidin and Alexa Fluor 546-labelled structures were excited with an argon laser 488 nm (with a 505 nm long-pass filter) and a He-Ne laser (with a 560 nm long-pass filter). Stacks of X–Y confocal images ( $1024 \times 1024$  pixel) were scanned at  $0.7 \mu\text{m}$  step size.

Only complete recording sessions of the entire set of test stimuli were evaluated. Responses were calculated from the number of net-spikes during 500 ms (number of spikes 500 ms before stimulus onset subtracted from the number of spikes 500 ms after stimulus onset). Net-spikes in response to control were subtracted from the net-spikes in response to odour stimuli; blend responses were considered to be synergistic/suppressive when the number of net-spikes in response to blends was significantly higher/lower than the sum of net-spikes in response to the single compounds (*G*-test).

### 3. Results

#### (a) Behavioural assay

Blends of the main sex pheromone component, codlemone, and host plant volatiles attracted significantly more codling moth males than single compounds (figure 1). All three plant volatiles tested: (*E*)- $\beta$ -farnesene, butyl hexanoate and pear ester, elicited upwind orientation flights. Blending codlemone at  $0.1 \text{ pg min}^{-1}$  and plant volatiles at  $100 \text{ pg min}^{-1}$  significantly increased landings at the source, compared with codlemone alone (figure 1).

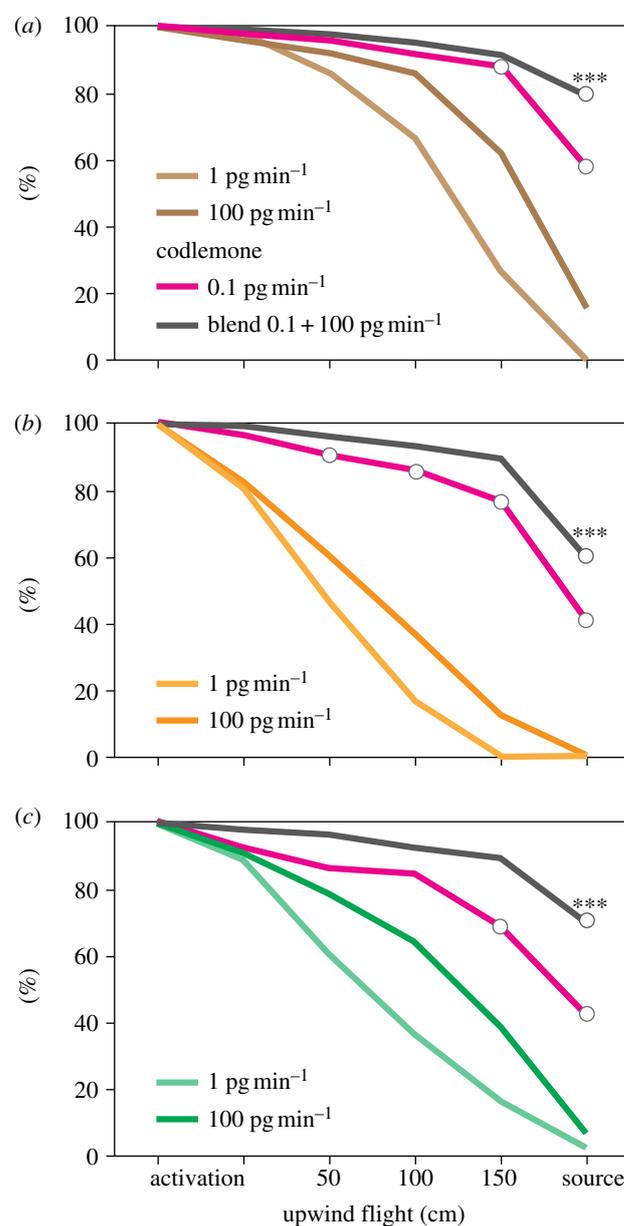
#### (b) Functional imaging

Calcium signals revealed distinct glomerular activity patterns for each odorant tested (figure 2). A threshold dose of codlemone (10 ng) elicited a significant response in the MGC, including the cumulus (Cu) and nearby satellite glomeruli (20 and 37; figure 2*b*). Plant volatiles alone did not elicit any response in the Cu, they instead activated satellite glomeruli and glomeruli outside the MGC (figure 2*c–e*). A threshold dose of pear ester (100 ng) was active in the satellite glomeruli 20 and 37, which also responded to codlemone (figure 2*c*) plus glomerulus 11 outside the MGC.

Blends of 10 ng codlemone plus 100 ng of each plant volatile compound produced a strong synergistic interaction in the Cu (figure 3*a,e*). This synergistic effect was not seen at a 10-fold higher dose (figure 3*a*). Although several of the glomeruli surrounding the Cu responded to plant volatiles and codlemone (figures 2*b–e* and 3*e*), there was no synergistic interaction in these glomeruli: outside the Cu, the activity elicited by blends was significantly lower than the sum of the activity elicited by the single compounds (figure 3*b–d*).

#### (c) Intracellular recordings

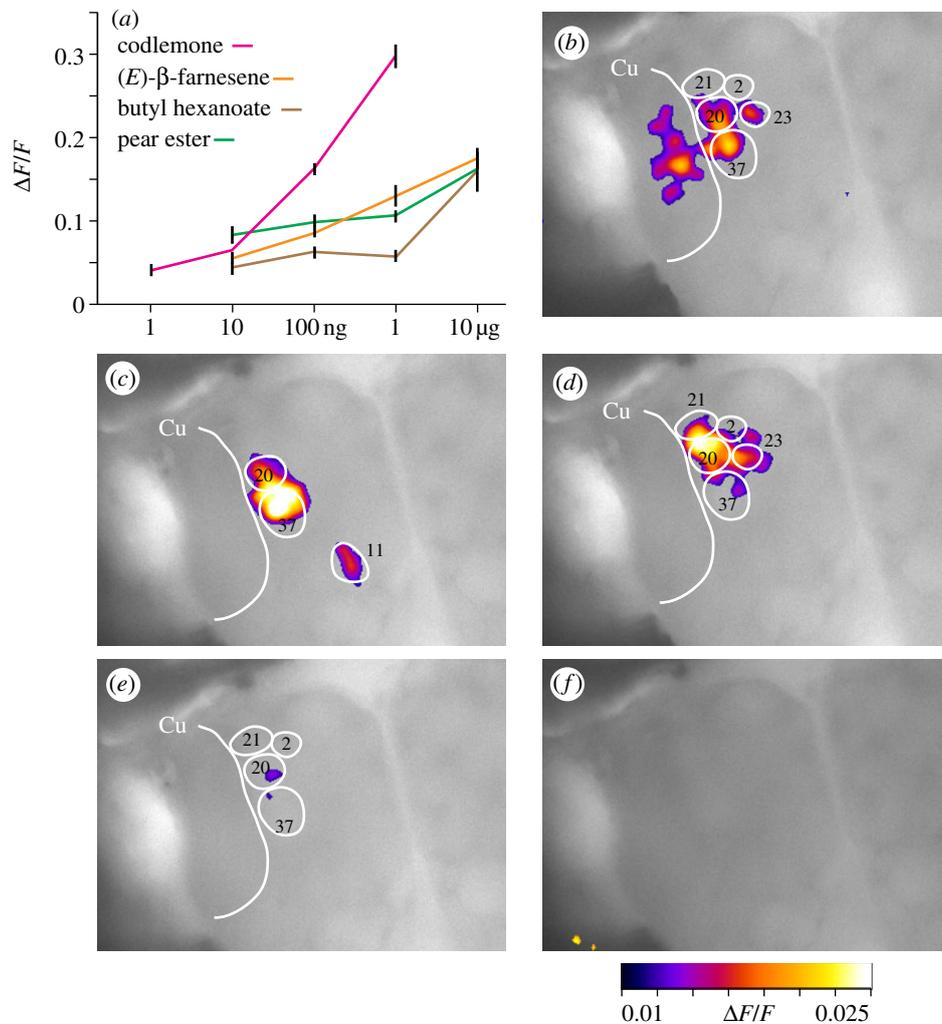
Figures 4 and 5 show the blend response of AL output neurons. Based on a dose–response test with single compounds (figure 4*a*), codlemone and individual plant volatiles were



**Figure 1.** Wind tunnel attraction of codling moth *C. pomonella* males ( $n = 50$ ) to the main pheromone compound codlemone (released at  $0.1 \text{ pg min}^{-1}$ ) and to plant volatiles (*a*) butyl hexanoate, (*b*) (*E*)- $\beta$ -farnesene, (*c*) pear ester, at 1 and  $100 \text{ pg min}^{-1}$ . Grey lines show attraction to 1:1000 blends of codlemone with these plant volatiles. Landings at the source are significantly increased in response to each of these two-component blends, compared with pheromone alone ( $***p < 0.001$ , two-way ANOVA; butyl hexanoate  $F_{4,45} = 45.0$ ,  $\beta$ -farnesene  $F_{4,45} = 23.75$ , pear ester  $F_{4,45} = 24.08$ ). Empty circles in the codlemone response curve show significant differences between codlemone and single plant volatiles alone ( $p < 0.0001$ , two-way ANOVA; butyl hexanoate  $F_{4,45} = 23.35$ ,  $\beta$ -farnesene  $F_{4,45} = 53.96$ , pear ester  $F_{4,45} = 20.68$ ).

combined in a ratio of 1:10 and 1:1000. The number of synergistic, suppressive and additive responses of AL neurons to blends of codlemone and plant volatiles, in the Cu and surrounding glomeruli is shown in figure 4*b,c*.

Analysis of 69 successful recordings demonstrates that odour blend interaction was not merely additive ( $p < 0.05$ , *G*-test). Of the neurons showing a synergistic blend response, 52 per cent responded only to blends, and not to single compounds. Suppressive responses comprised both a decreased excitatory phase (53%) and complete response suppression (47%; figure 4*b*).



**Figure 2.** Calcium imaging of the codling moth male AL upon stimulation with single odourants, sex pheromone (codlemone) and three plant volatiles. (a) Dose–response relationships of odour-evoked calcium signals, using an increasing dose of codlemone ( $n = 19$ ), pear ester ( $n = 23$ ),  $\beta$ -farnesene ( $n = 14$ ) and butyl hexanoate ( $n = 19$ ). Glomerular activation patterns in response to (b) 10 ng codlemone, (c) to 100 ng of pear ester, (d) (E)- $\beta$ -farnesene and (e) butyl hexanoate, respectively, and (f) in response to the solvent (hexane). Data points show means and standard errors (s.e.), glomeruli numbers correspond to the three-dimensional atlas of the codling moth AL [21].

Twenty-nine neurons were successfully stained: 11 PNs arborizing in the Cu, five PNs in satellite glomeruli surrounding the Cu, 10 PNs in glomeruli outside the MGC and, in addition, three local interneurons (LNs). The Cu was innervated by uni-glomerular PNs (figure 5a), and by one multi-glomerular PN that also arborized in the satellite glomerulus 20 (figure 4d). Spike frequency histograms for selected PNs in response to compound blends are shown in figure 5. A statistical comparison of the blend effects in stained PNs revealed a significant difference: synergism occurred almost exclusively in the Cu, whereas blend stimulation of glomeruli outside the MGC mostly had an additive or suppressive effect (figures 4c and 5c).

## 4. Discussion

### (a) Neural ensemble coding of sex pheromone and host plant odour in the macroglomerular complex of the male moth antennal lobe

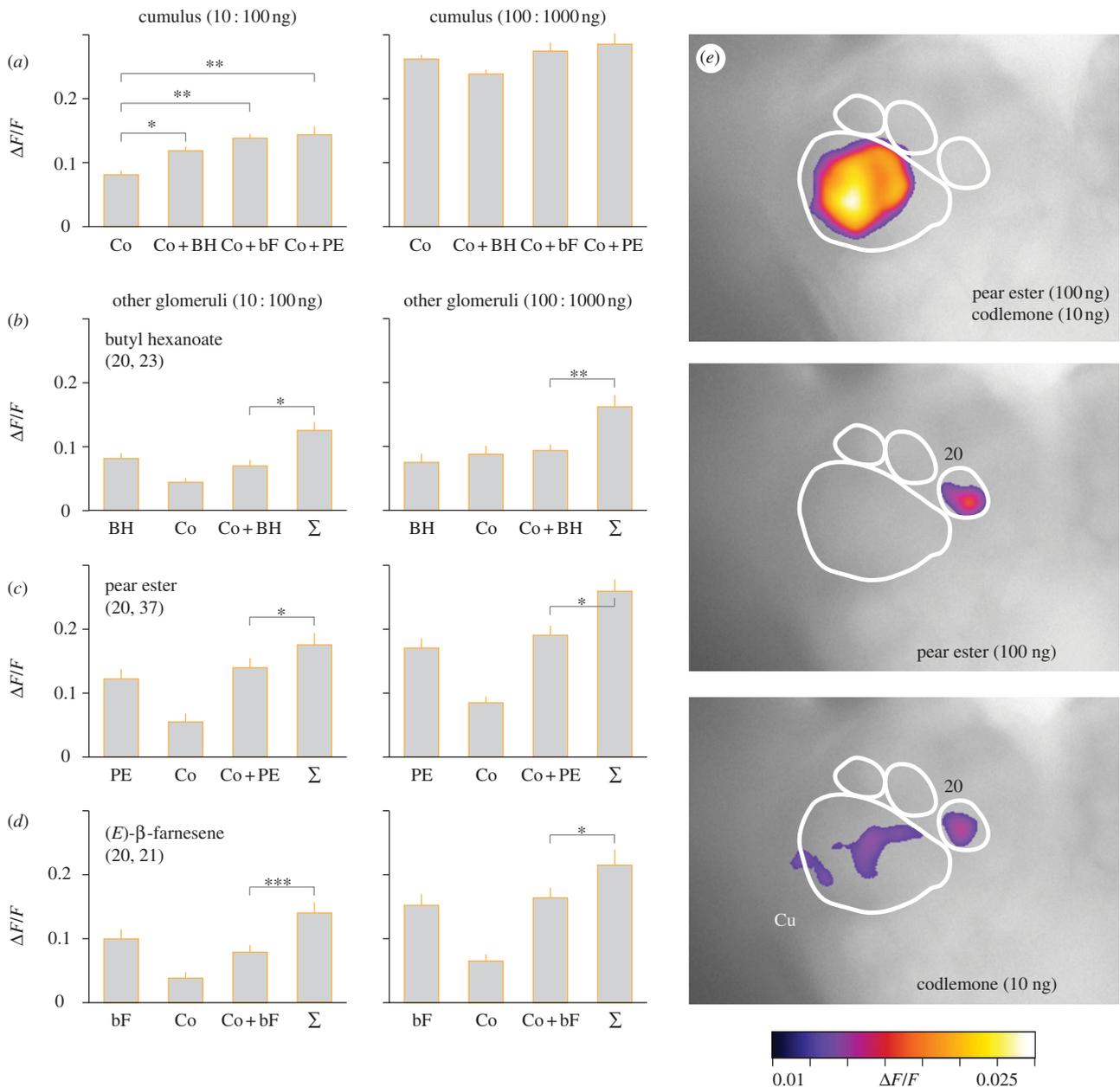
Understanding how stimulation with a blend of odourants generates a unique perception in the brain is a current research question. What adds to the complexity of olfactory coding is the integration of separate, independent signals—

sex and habitat odours—which are together required to generate appropriate behavioural responses during mate finding.

We combined functional imaging and IRs to study odour blend processing in the codling moth *C. pomonella*, and show that the behavioural synergism between sex pheromone and host plant odourants is mirrored neurophysiologically. The MGC in the AL integrates signals from conspecific insects with habitat odours, and synergistic interactions between these two classes of odours occur both at the input and output level. This demonstrates that processing of sex pheromone and plant volatiles, which insects encounter as an ensemble in nature, does not use functionally separate pathways [17,18].

Blend enhancement and suppression in the AL may stem from odour interference in antennal sensory neurons [19,26] and ultimately at the olfactory receptor level [27]. However, in codling moth, pheromone–plant volatile blends enhance the Cu response while they simultaneously suppress surrounding glomeruli in a ‘centre-surround’ manner. Such complex coding may instead rely on lateral excitatory or inhibitory interconnections between glomeruli through LNs [2,28]. Functional studies of LNs will be essential to understand olfactory processing in the AL.

IRs of PNs, which connect the AL to higher brain centres, further corroborate that the MGC processes blends of plant



**Figure 3.** Calcium imaging of the codling moth male AL following stimulation with two-component blends of sex pheromone (codlemone) and plant volatiles, butyl hexanoate, pear ester and  $\beta$ -farnesene. Odour-evoked activity was measured in the Cu and other responding glomeruli. Response in the Cu (a), showing a synergistic blend interaction for 10 : 100 ng blends ( $*p < 0.05$ ,  $**p < 0.01$ , Kruskal–Wallis test followed by Mann–Whitney  $U$ -test with Holm–Bonferroni correction,  $n = 30$  males). At a higher dose, blends (100 : 1000 ng) were not significantly different from codlemone ( $p = 0.36$ , Kruskal–Wallis test,  $n = 30$  males). Response of glomeruli outside the Cu (b–d) to plant compounds, codlemone, their blends and the summed responses to single compounds ( $\Sigma$ ): butyl hexanoate, satellite glomerulus 20 and glomerulus 23 ( $*p < 0.05$  and  $**p < 0.01$ ,  $n = 26$ ; b); pear ester, satellite glomeruli 20, 37 ( $*p < 0.05$ ,  $n = 30$ ; c);  $\beta$ -farnesene, satellite glomeruli 20, 21 ( $***p < 0.001$  and  $*p < 0.05$ , one-sided  $t$ -test,  $n = 31$ ; d). Bars show the standard error of the mean (s.e.m.). Representative recording of codlemone, pear ester and their blend (e). Glomeruli numbers correspond to the atlas of codling moth AL [21].

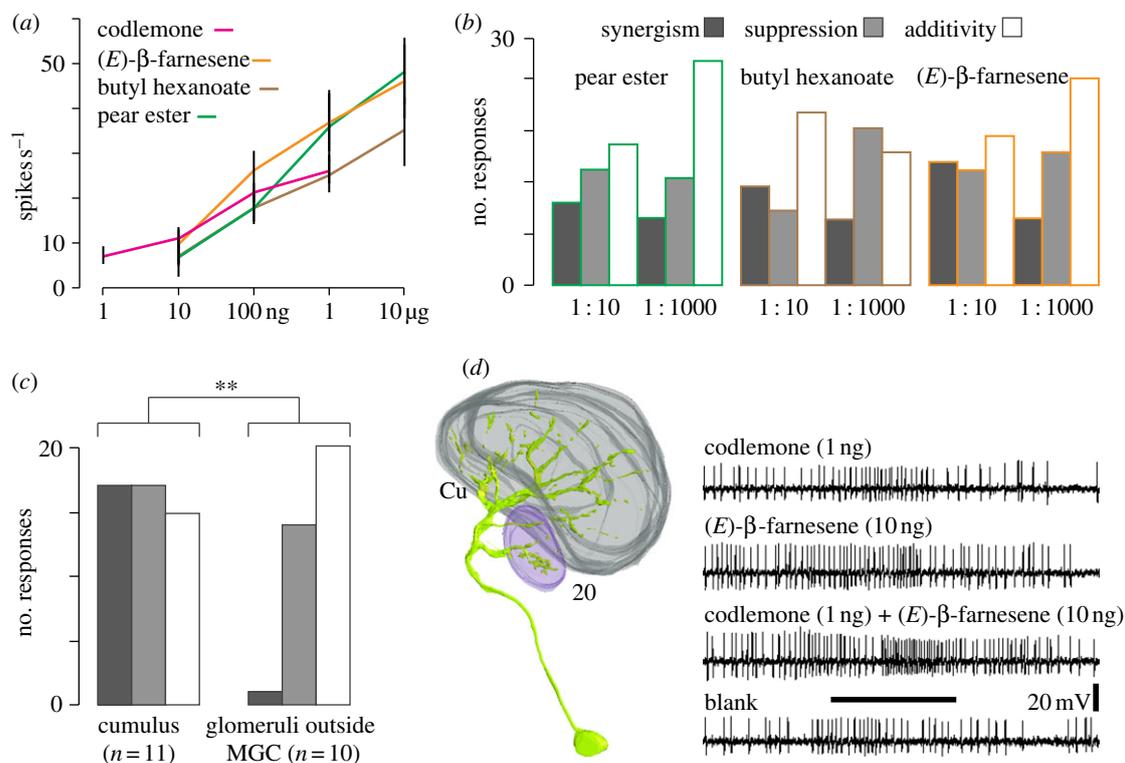
volatiles and sex pheromone. Synergistic, blend-specific responses have been shown in the silk moth *B. mori* [17] and in codling moth, where PNs innervate the Cu and satellite glomeruli of the MGC [21].

An antagonistic interaction modality was shown in the black cutworm *Agrotis ipsilon*. A floral volatile, which inhibits male attraction to pheromone, suppresses the pheromone response in the AL [18] and in PNs innervating the MGC [19]. This suggests that odours with different ecological roles may differently affect pheromone coding. A wiring diagram of input and output signals in the codling moth AL, based on a more complete panel of ecologically

relevant odorants, from host and non-host plants or associated mutualistic micro-organisms [29,30], will reveal whether glomerulus morphology and position in the AL correlates with the behavioural role of the respective key stimuli [31].

### (b) Behavioural and ecological physiology of pheromone–plant odour blend perception

Mate recognition in insects, and especially in habitat-specific plant-feeding species, involves two main elements: sexual communication and recognition of larval and adult



**Figure 4.** Responses of AL neurons to single compounds and binary blends. Intracellular recordings of AL neurons with increasing doses of codlemone ( $n = 12$ ), butyl hexanoate ( $n = 10$ ), pear ester ( $n = 11$ ) and  $\beta$ -farnesene ( $n = 12$ ) (a). Histograms of synergistic, suppressive and additive responses of 69 physiologically characterized interneurons to blends of codlemone and plant volatiles (b). Number of synergistic, suppressive and additive responses of neurons innervating Cu and glomeruli outside the MGC (\*\* $p < 0.005$ ,  $\chi^2$ -test) (c). Three-dimensional reconstruction of a multi-glomerular PN innervating the Cu and the satellite glomerulus 20, showing a synergistic response to a blend of codlemone and ( $E$ )- $\beta$ -farnesene. The horizontal bar shows the stimulus period (500 ms) (d).

food plants, which frequently serve as rendezvous sites. Both mate and host finding largely rely on olfactory signals [14,32] that play a fundamental role in speciation [6,33].

In the codling moth, host plant odour is part of the mate-finding signal. The plant volatiles chosen for this study are distinctive for the main hosts, pear and apple, respectively. They mediate female attraction for oviposition [29,34–37], and they synergize male attraction to female sex pheromone. The MGC, in the olfactory centre of the moth brain, is the focal point for processing blends of pheromone and these plant signals.

Speciation is thought to be facilitated by multiple-effect or ‘magic’ traits, which are subject to divergent selection and which contribute to non-random mating [9,10]. The MGC interconnects mate and host choice, and would accordingly be considered as a multiple-effect trait. Host choice seemingly is under divergent selection in codling moth, which forms distinct host races on apple, pear, walnut, plum and apricot. These differ in spring emergence and diapause initiation, in close association with host flowering and fruit maturation [38,39], and the genetically distinct walnut strain is adapted to toxic walnut metabolites [40–42]. Females of several strains preferentially oviposit on their respective host fruit [29,38].

A comparison of the female sex pheromones of closely related *Cydia* species further corroborates the role of plant volatiles in reproductive isolation. Only few species share the same pheromone, but these all feed on host plants belonging to different families. For example, pea moth *C. nigricana*

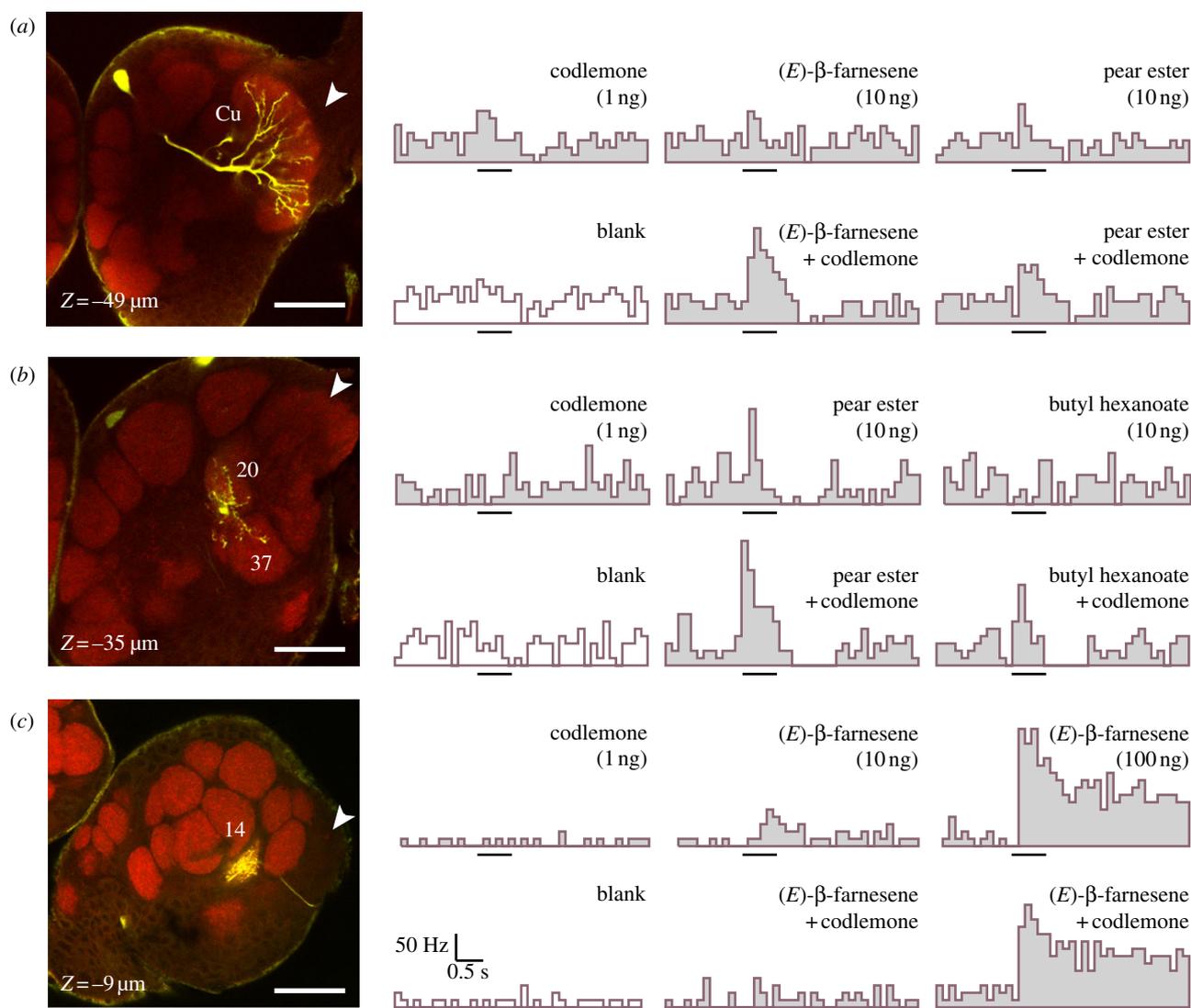
(Leguminosae) and pear moth *C. pyrivora* (*Pyrus*), the sibling species of codling moth, use codlemone acetate ( $E,E$ )-8,10-dodecadienyl acetate, which is a strong pheromone antagonist in codling moth males [43].

Pheromone and host odour communication is also highly integrated in other insects, for example in *Drosophila* [44] and in bark beetles, where non-host volatiles, as opposed to host volatiles, have an antagonistic effect on host and mate finding [45]. In the two pheromone races of the European corn borer *Ostrinia nubilalis*, male preference for females of the same race leads to premating isolation [46,47], which is reinforced by preferential attraction to volatiles of their respective host plants, mugwort and maize [48,49].

Ecological speciation, following host plant shifts, has probably contributed to the remarkable diversity of phytophagous insects [11,33]. Our study provides physiological data which suggest that mate recognition systems evolve in concert with chemosensory adaptation to new hosts and ecological niches, and that sexual selection cannot be separated from natural selection in male insect herbivores.

### (c) Practical implication

Our knowledge of codling moth chemical ecology has led to the successful development of species-specific and safe population control by pheromone-mediated mating disruption. In spite of orchard applications on 200 000 ha [50], the behavioural mechanisms underlying the disruption of mating are still under debate [51,52], and a better



**Figure 5.** Single confocal sections and spike frequency histograms (spikes  $s^{-1}$ ) of physiologically and morphologically characterized PNs in the codling moth male AL. (a) Synergistic responses of a PN innervating the Cu to blends of codlemone with pear ester and  $\beta$ -farnesene. (b) Synergistic responses of a multi-glomerular PN, innervating the satellite glomeruli 20 and 37, to blends of codlemone with pear ester and butyl hexanoate. (c) Suppressive responses of a PN innervating the glomerulus 14, to a blend of codlemone and (*E*)- $\beta$ -farnesene at different blend ratios. Confocal sections: entrance of the antennal nerve (arrowheads), depth from anterior side of the AL (*Z*), scale bars (50  $\mu$ m), glomeruli numbers correspond to the three-dimensional AL atlas [21]. Histograms: stimulus period (bars, 500 ms).

understanding of them will give leads for improvement. Our study demonstrates that it will be useful to consider the physiological and behavioural effect of plant volatiles on mating disruption, because, in nature, pheromone and plant volatiles are perceived together.

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