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Conserved, Highly Specialized Olfactory Receptor Neurons for Food Compounds in 2 Congeneric Scarab Beetles, *Pachnoda interrupta* and *Pachnoda marginata*

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Abstract

Few studies have systematically addressed evolutionary changes in olfactory neuron assemblies, either by genetic drift or as an adaptation to specific odor environments. We have studied the sense of olfaction in 2 congeneric scarab beetles, *Pachnoda interrupta* Olivier and *Pachnoda marginata* Drury (Coleoptera: Scarabaeidae: Cetoniinae), which are both opportunistic polyphages, feeding mainly on fruit and flowers. The 2 species occur in dissimilar habitats: *P. interrupta* is found in dry savannah, and *P. marginata* in tropical parts of equatorial Africa. To study how these species may have adapted their sense of olfaction to their odor environments, we utilized single-unit electrophysiology on olfactory sensilla with a wide selection of food-related compounds. Despite the differences in habitat, we found that the species shared most of the physiological types of olfactory receptor neurons (ORNs) encountered, although their proportions frequently varied between the species. The high degree of conservation in olfaction between the species implies that a similar sensory strategy is efficient for food search in both habitats. However, shifts in proportions of receptor neuron classes, and slight shifts in response profiles and/or presence of some ORN classes unique to either species, may reflect adaptation to a different set of hosts.

Key words: comparative study, electrophysiology, olfaction, polyphagous herbivore, single sensillum recordings

Introduction

Olfaction is of profound importance for food search in herbivorous insects (Dethier 1982). The olfactory system of a polyphagous herbivorous insect is faced with a task of detecting several hosts, distinguishing hosts from nonhosts, and possibly assisting in choosing between hosts. The odor profiles of hosts and nonhosts may be overlapping but could also contain elements unique to single plant species or to groups of hosts or nonhosts (Bernays and Chapman 1994).

At the peripheral level, insects employ olfactory receptor neurons (ORNs) to detect volatile compounds. The response spectra of ORNs are usually narrow and nonoverlapping at physiologically relevant concentrations. This is typical for pheromone-detecting ORNs, which display very high specificity and sensitivity in many different insect orders (Mustaparta et al. 1980), including scarab beetles (Wojtasek et al. 1998; Larsson et al. 1999). It also applies to a great extent to ORNs detecting host-related cues; most insect species have ORNs with a high degree of specialization toward compounds in the odor blends of their hosts (Wibe and Mustaparta 1996; Hansson et al. 1999; Røstelien et al. 2000; Bichão et al. 2005; Andersson et al. 2009).

However, to date few studies have compared olfactory receptor neurons in closely related species, and further investigations are needed to determine how the odor world of an insect affects its olfactory sense. Stensmyr et al. (2003) found a high degree of conservation in a subset of ORNs in species of the *Drosophila melanogaster* subgroup, despite a wide variety of habitats used by these species. *Drosophila sechellia* was a notable exception, having lost one type of sensillum and acquired a change in the response spectrum of another, most likely due to adaptation to a widely disparate host, morinda fruit (Dekker et al. 2006). A similar pattern was observed in a study of the *Rhagoletis* complex; ORNs in the different subspecies responded to the same palette of compounds and were equally common (Olsson et al. 2006a), although differences in ORN response threshold to ligands may be related to recent shifts to different hosts (Olsson et al. 2006a). Several common receptor neurons were found in 3 species of heliothine moths, and a highly conserved germacrene D receptor neuron constituted a large proportion of ORNs encountered (Stranden et al. 2003a, 2003b). A broader comparison of 2 geographically separated heliothine moths (both polyphagous herbivores) also indicated a high degree of conservation in the sense of olfaction with regard to detection of host volatiles (Røstelien et al. 2005).

The sorghum chafer, *Pachnoda interrupta* Olivier, and the fruit chafer, *P. marginata* Drury (Coleoptera: Scarabaeidae: Cetoniinae), are opportunistic, highly polyphagous, and will readily adopt new hosts and have been recorded to feed on numerous plants, especially fruits (e.g., mango, banana, and papaya) and flowers (e.g., acacia, orange, and guava) (Schmutterer 1969; Clark and Crowe 1978; Grunshaw 1992). The full extent of their diet is likely as yet not covered, and the species show considerable overlap in food preferences. However, they have different geographic ranges, with *P. interrupta* occurring mainly in dry savannah and semiarid areas in the Sahel and *P. marginata* in tropical areas of equatorial Africa (Rigout 1989; Grunshaw 1992). Having evolved in environments with different potential food plants, as well as nonhosts, they could serve as excellent models for how olfactory systems of generalist species may adapt to different environments. In our study, we have compared the response of olfactory receptor neurons in *P. interrupta* and *P. marginata* to a large selection of food-related volatiles. Specifically, we addressed whether the species differed in the response spectra of their ORNs, in the frequency of their ORN classes, or in ORN grouping within sensilla.

Materials and methods

Model species

Whereas *P. marginata* reproduces throughout the year, *P. interrupta* is univoltine (Rigout 1989; Wolde-Hawariat et al. 2007). *Pachnoda interrupta* feeds on cultivated sorghum and became a pest in Ethiopia during the early 1990s (Hiwot 2000). Male and female *P. interrupta* were collected at Rasa (09°55'N, 40°05'E), located 255 km northeast of Addis Ababa, Ethiopia. *Pachnoda marginata* were reared in captivity starting from adults obtained from several different commercial and hobby breeders in Sweden. Both species were kept at SLU Alnarp, Sweden, in clear plastic boxes (30 × 12 × 22 cm; Cofa Plastics AB) with a 1:1:1 mixture of planting soil (Yrkesplantjord, Weibull Trädgård AB), peat (Växa trädgårdstorv, Econova Garden AB) and composted cow dung (Simontorps Bas, Weibull Trädgård AB). Boxes were kept at 25 °C, 70% relative humidity, and a 20:4 h light:dark cycle. The beetles were fed with bananas ad libitum. Adult beetles of both species were sexed based on the presence of a ventral, abdominal groove in males (Rigout 1989).

Scanning electron microscopy

For scanning electron microscopy, antennae from both species were excised by fine scissors and immersed in 70% ethanol overnight at 4 °C. The samples were dehydrated in 80%, 90%, and 100% ethanol, mounted on microscope holders, and coated with gold/palladium (3:2) using a JEOL JFC-1100 ion sputter (JEOL Skandinaviska AB). Specimens were studied with a LEO 435VP scanning electron microscope (Carl Zeiss) operated at 10 kV. Reanalysis was made of *P. marginata* antenna to make results fully comparable (Stensmyr et al. 2001).

Synthetic compounds and odor stimuli

A total of 85 compounds (Table 1, heading G1), followed by a subset of 37 compounds (Table 1, heading G2), were used in 2 phases of single sensillum recordings (see below). Synthetic standards for all experiments were purchased from Sigma-Aldrich (for purity, as listed by the manufacturer, and CAS number, see Table 1). The compounds include volatiles commonly found in flowers (Knudsen et al. 2006), volatiles from tropical fruit (Macku and Jennings 1987; Ibáñes et al. 1998; Boudhrioua et al. 2003; Carasek and Pawliszyn 2006; Clara et al. 2007; Pandit et al. 2009), and volatiles related to microbial degradation and fermentation (Chatonnet et al. 1992; Fischer et al. 2000; Xiao and Ping 2007). The large number of compounds used precluded the use of gas chromatography (GC)–based stimulation; although there is a risk of ORNs responding to impurities in the commercial compounds used, previous GC-single sensillum recordings (SSR) and behavioral work (Stensmyr et al. 2001; Larsson et al. 2003) have established that most compounds are relevant ligands at least for *P. marginata*, and we judged that the risk of misclassifying ORNs due to the same impurity being present in several commercial compounds was small. Stimuli were prepared by applying compounds to 1.5 × 1 cm pieces of filter paper that were placed in disposable glass Pasteur pipettes (VWR International). Truncated 1 ml pipette tips were put on the wide end of the Pasteur pipettes to reduce evaporation of the test compounds. In the first phase of single

Table 1 Synthetic compounds used for single-cell screening

G1	G2	Compound	S1	S2	CAS	%
1		4-Ethylphenol	A		123-07-9	99
1	4	4-Methylphenol	A	P	106-44-5	99
1	4	1,4-Benzoquinone	A	P	106-51-4	99
1	4	Toluquinone	A	P	553-97-9	98
1		Phenol	A		108-95-2	99
2	3	(*E*)-2-Hexenal	H	P	6728-26-3	98
2	3	(*E*)-2-Hexen-1-ol	H	P	928-95-0	96
2		(*E*)-2-Hexenyl acetate	H		2497-18-9	98
2	3	(*E*)-3-Hexen-1-ol	H	P	928-97-2	98
2	3	(*Z*)-3-Hexen-1-ol	H	P	928-96-1	98
2	3	(*Z*)-3-Hexenyl acetate	H	P	3681-71-8	98
3		Hexanal	H		66-25-1	98
3		1-Hexanol	H		111-27-3	98
3	2	Hexyl acetate	H	P	142-92-7	98
3	3	Nonanal	H	P	124-19-6	95
3		1-Nonanol	H		143-08-8	99.5
3		1-Octanol	H		111-87-5	99.5
3	3	(±)-3-Octanol	H	P	589-98-0	99
3		(±)-1-Octen-3-ol	H		3391-86-4	98
4	1	Anethole	H	P	4180-23-8	99
4	1	Benzaldehyde	H	P	100-52-7	99.5
4	1	Benzylalcohol	H	P	100-51-6	99
4	1	Eugenol	H	P	97-53-0	98
4	1	Methyl benzoate	H	P	93-58-3	99
4	1	Methyl anthranilate	H	P	134-20-3	99
4		2-Phenylethanol	H		60-12-8	98
4	1	2-Phenylethyl propionate	H	P	122-70-3	98
5	X	(±)-Acetoin	A	W	513-86-0	97
5	X	Racemic 2,3-Butanediol	A	P	513-85-9	99
5		Carvacrol	A		499-75-2	98
5		Cinnamic aldehyde	A		104-55-2	98
5		Methyl cinnamate	A		103-26-4	99
5	1	Methyl salicylate	A	P	119-36-8	99
5	1	Phenylacetaldehyde	A	P	122-78-1	90
5	1	Phenylacetonitrile	A	P	140-29-4	99
5		Thymol	A		89-83-8	99.5
6		Butyric acid	H		107-92-6	99
6		N-caproic acid	H		142-62-1	99.5
6	4	Isovaleric acid	H	P	503-74-2	98
6		Valeric acid	H		109-52-4	99.8
7		Isoamylalcohol	H		123-51-3	98
7	4	6-Methyl-5-hepten-2-one	H	P	78-70-6	99
7		Tetradecane	H		629-59-4	99.5
7		Tridecane	H		629-50-5	99.5
8	5	(±)-*beta*-Caryophyllene	H	P	87-44-5	98.5
8		(-)-*trans*-Citronellol	H		106-22-9	95
8	5	Geraniol	H	P	106-24-1	98
8	5	Geranyl acetate	H	P	105-87-3	98
8	5	(±)-Linalool	H	P	78-70-6	97
8	5	Linalool oxides	H	P	n/a	97
8		Methyl jasmonate	H		1211-29-6	95
8		Nerolidol	H		7212-44-4	98
9		(±)-*delta*-Decalactone	H		705-86-2	98
9	4	(±)-*gamma*-Decalactone	H	P	706-14-9	97
9		(±)-*gamma*-Hexalactone	H		695-06-7	98
9	4	(±)-*gamma*-Nonanlactone	H	P	104-61-0	97
9		(±)-*gamma*-Octalactone	H		104-50-7	97
9		(±)-*gamma*-Undecalactone	H		104-67-6	99
10		(±)-Ethyl 3-hydroxybutyrate	H		5405-41-4	97
10	2	(*Z*)-3-Hexenyl butyrate	H	P	16491-36-4	98
10		(*Z*)-3-Hexenyl isobutyrate	H		41519-23-7	98
10		(*Z*)-3-Hexenyl tiglate	H		67883-79-8	97
11	2	Butyl butyrate	H	P	109-21-7	98
11		Ethyl butyrate	H		105-54-4	99
11		Ethyl hexanoate	H		123-66-0	99
11		Ethyl propionate	H		105-37-3	99
11		Hexyl butyrate	H		2639-63-6	98
11		Methyl butyrate	H		623-42-7	99
11	2	Methyl hexanoate	H	P	106-70-7	99
11	2	Methyl octanoate	H	P	111-11-5	99
11		Methyl propionate	H		554-12-1	99
11		Propyl butyrate	H		105-66-8	99
12	2	Butyl isobutyrate	H	P	97-87-0	97
12		Hexyl hexanoate	H		6378-65-0	97
12		Isoamyl acetate	H		123-92-2	98
12		Isoamyl butyrate	H		106-27-4	98
12		Isobutyl acetate	H		110-19-0	99.8
12		Isobutyl isobutyrate	H		97-85-8	99

Table 1 Continued

G1	G2	Compound	S1	S2	CAS	%
12		Isopentyl isobutyrate	H		2050-01-3	98
12		Isopropyl acetate	H		108-21-4	99.8
13		Acetic acid	P		64-19-7	99
13		Acetone	P		67-64-1	99.9
13		Ethanol	P		64-17-5	99
13		Ethyl acetate	P		141-78-6	99.5
13		Propionic acid	P		79-09-4	99.5

G1 and G2, screening blend used in first and second screening phase, respectively (X denotes compounds tested singly); S1 and S2, solvent used in first and second screening, respectively; A, acetone; H, hexane; P, paraffin oil; W, water; CAS, Chemical Abstracts Service number; %, minimum purity in percent, as listed by manufacturer.

sensillum recordings, compounds were diluted in hexane (redistilled from 95%; Lab-scan), acetone (99.98% purity; Fisher Scientific AB), or paraffin oil (reagent grade; Merck KGaA) for some highly volatile compounds (Table 1, heading S1). A volume of 10 µl of a 1 µg/µl solution was applied to filter papers for a total amount of 10 µg. The same dilution procedure was used in dose–response experiments, except compounds were diluted to concentrations ranging from 1 pg/µl to 1 µg/µl in decadic steps, to achieve different concentrations when 10 µl of the diluted compound was applied to the filter paper in the stimulus pipette. In the second phase of single sensillum recordings, all compounds were diluted in paraffin oil (reagent grade; Merck KGaA), except for acetoin, which was diluted in water, because it was insoluble in paraffin oil at room temperature (Table 1, heading S2). A volume of 10 µl of a 100 ng/µl solution was applied to filter papers for a total amount of 1 µg. Control stimuli with only solvent were also prepared. Fresh stimuli were prepared before each recording session and kept at –18 °C until the start of the recording session, to avoid evaporation.

Single sensillum recordings

The method for single sensillum recordings follows that of Bengtsson et al. (2009). Insects were restrained with Parafilm (PM-992, Pecheney plastic packaging; Menasha) and fixed on microscope slides (ca. 76 × 26 mm; Menzel-Gläser) using dental wax (Surgident periphery wax; Heraeus Kulzer GmbH), with the lamellae held open on a wax surface using 2–3 mm long pieces of thin tungsten wire. A silver grounding electrode was inserted in the abdomen. Sensilla were classified into morphological types in a light microscope at ×750 magnification (Olympus BX51WI; LRI Instrument AB) and contacted with a tungsten electrode (diameter 0.12 mm; Harvard Apparatus Ltd) electrolytically sharpened in a saturated KNO_2 solution, using a DC-3K Rechts PM-10 piezo micromanipulator (Märzhäuser Wetzler GmbH). The signal from the ORNs was registered and amplified 10 times with a probe (INR-02; Syntech), amplified 200 times with a Syntech UN-06 AC/DC amplifier, and transferred to a computer through an IDAC-4-USB (Syntech), where it was visualized and analyzed with the software Autospike v. 2.2 (Syntech). A constant flow of 0.5 m/s of charcoal-filtered and humidified air was delivered through a glass tube with its outlet approximately 15 mm from the antenna. Stimuli were presented to the insect by inserting the stimulus pipette through a hole in the glass tube and blowing an air puff of 2.5 ml during 0.5 s through the pipette into the air stream, using a stimulus controller (Syntech SFC-1/b).

The net response to a stimulus was quantified as the number of action potentials (spikes) elicited during 0.5 s after the onset of the response, deducting the number of action potentials during 0.5 s immediately prior to the response. Each neuron was also subjected to blank stimuli (i.e., only solvent), and the net response to the blank was deducted from the response to the test compounds. The resulting value was doubled to obtain a value corresponding to spikes/s (Hz).

Large-scale SSR screens were mainly performed by J.B. and H.K. To minimize any impact from personal idiosyncrasies of method (despite careful standardization of the protocol), these 2 authors performed an equal share of the recordings from both species. Initially, a broad screening (phase 1) for SSR-active compounds in both *P. interrupta* and *P. marginata* was performed using a stimulus set of 85 compounds (Table 1). Contacted cells were first subjected to control stimuli and then tested for response to set blends of 4–10 of the 85 compounds (Table 1, heading G1). For all screening stimuli that elicited a positive response of approximately ≥40 Hz, the pipettes loaded with the compounds in the blend(s) were brought from the freezer and tested individually after thawing at room temperature for 5 min. Recordings from *P. interrupta* have been used to make a rudimentary ORN-type classification for this species, in order to identify putative field attractants (Bengtsson et al. 2009). Due to limited and seasonal availability of *P. interrupta*, dose–response experiments were performed exclusively on *P. marginata*.

Based on the results from the first screening, 37 compounds (Table 1, heading G2) were selected for an in-depth SSR screen (phase 2). Contacted cells were subjected to control stimuli first and then to blend stimuli in random order (Table 1, heading G2; set blends of 5–10 of the 37 compounds, with 2,3-butanediol and acetoin tested as single compounds). If the contacted cell responded to any blend or single compound at approximately 40 Hz or above, it was subjected to testing with all 37 compounds.

Statistical analysis

Responding ORNs from the second phase were subjected to cluster analysis, with average linkage and Euclidean distance (SPSS 13.0 for Windows; SPSS Inc.). The characters used in

this analysis were the responses of each ORN (defined by spike-counting as described above) to the 37 compounds used for stimulation. Based on the cluster analysis, a dendrogram was constructed. Fisher's exact test for proportions was used to test for significant differences in occurrence of ORN classes between the species (Minitab 16; Minitab Inc.).

Results

Scanning electron microscopy

Pachnoda interrupta and *P. marginata* exhibited highly similar antennal morphology, with the 3 apical antennal segments flattened into lamellae and sensilla located on the inner surfaces of the lamellae (Figure 1). The majority of sensilla in both species were of the placodea morphological type. Two morphological types of sensilla placodea were identified: grooved placodea, which were set apart from the surrounding area by a narrow groove along their edge, and smooth placodea, which were adjoined to the substrate (Figure 1E,F). Approximately 55% of the sensilla were smooth placodea, whereas around 40% were grooved placodea. The remaining 5% were smooth peg or coeloconic sensilla (Figure 1C,D). All lamellar surfaces had similar distributions of the morphological types of sensilla, and the surface of a lamella could roughly be divided into a smooth area and a heterogeneous area defined by smooth and grooved sensilla placodea, respectively (circled in Figure 1A,B). The smooth area contained almost exclusively smooth placodea, whereas the heterogeneous area contained a mixture of the other sensillum types (Figure 1C,D). The border between the smooth and heterogeneous areas was not sharp, but had a gradual transition in the proportion of grooved to smooth placodea (Figure 1E,F).

Single sensillum recordings

In both *P. interrupta* and *P. marginata*, we obtained stable contacts with grooved and smooth sensilla placodea, but not from smooth peg or coeloconic sensilla, and our results thus stem from sensilla placodea. Each placoid sensillum typically contained 2 ORNs, but in most cases (88%), only one ORN responded to stimulation. In most cases when 2 responding ORNs were present in a single sensillum, they could be distinguished by the amplitudes of their action potentials (spikes) (Figure 2). However, in some cases, neurons were difficult to distinguish based on spike amplitude. Simultaneous stimulation with 2 compounds was then used to test whether single or multiple neurons were activated (Figure 3,4). If multiple neurons were simultaneously activated, a distinct pattern of asynchronous firing could be observed, which did not occur if only one neuron was activated. Differences between neurons in amplitude decrease during response (''pinching'') also helped distinguish multiple active from single active neurons.

In the first phase, we recorded from 102 responding neurons in *P. interrupta* and 98 in *P. marginata*. We found no apparent sexual dimorphism in the ORN arrays of either species and therefore pooled data from males and females. For selected compounds, we performed dose–response trials in *P. marginata*. Out of the 85 compounds used in the first phase, 37 compounds that appeared to be best ligands for, or to segregate between, different putative ORNs, were chosen for use in the second phase of the screening. In the second phase we recorded from a total of 109 responding neurons in *P. interrupta* and 116 in *P. marginata*. These recordings were obtained from roughly the same number of male and female individuals in each species. For both species, the highest proportion of responding cells was found in grooved placodea.

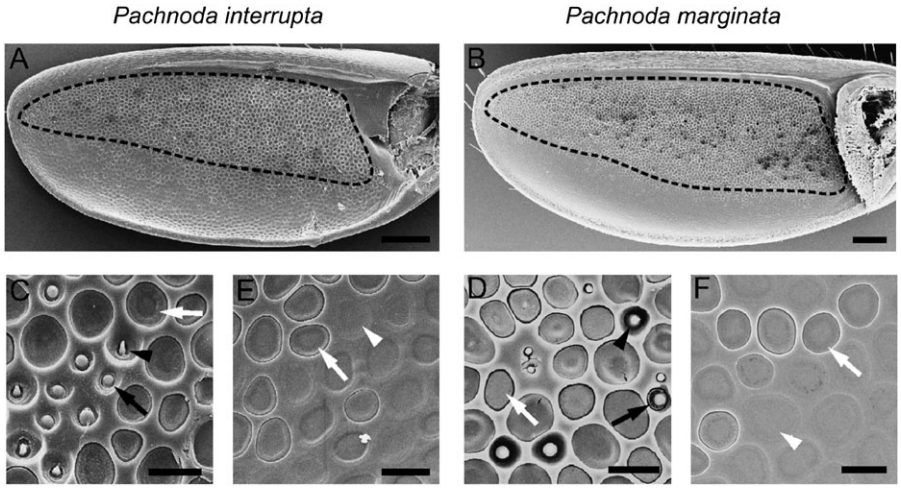

Figure 1 Scanning electron micrographs of *Pachnoda interrupta* and *P. marginata* antenna. **(A, B)** Antennal lamella showing 2 zones: a heterogeneous area (enclosed by a dashed line) and a smooth area, in *P. interrupta* and *P. marginata*, respectively. Scale bars are 100 μm. **(C, D)** A view of the heterogeneous area where several morphological types of sensilla are present: smooth peg (black arrow), grooved peg (black arrowhead), and grooved placodea (white arrow), in *P.* interrupta and *P. marginata*, respectively. Scale bars are 10 μm. **(E, F)** A view of the border between the smooth and heterogeneous area, showing smooth placodea (white arrowhead) and grooved placodea (white arrow), in *P. interrupta* and *P. marginata*, respectively. Scale bars are 10 μm.

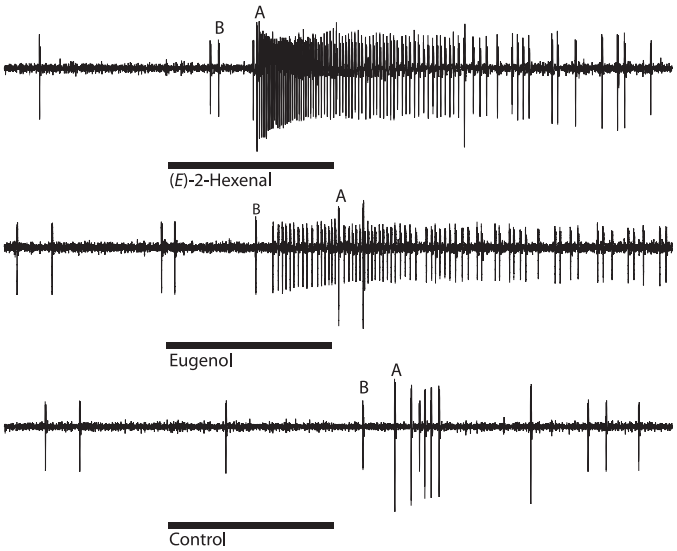

Figure 2 Single sensillum recordings from a grooved sensillum placodeum in *Pachnoda marginata*. The 2 ORNs present were differentiated by their spike amplitudes. The ORN responding to (*E*)-2-hexenal (denoted "A" above) had a higher amplitude than the ORN responding to eugenol (B). The horizontal bar denotes the stimulation period (0.5 s).

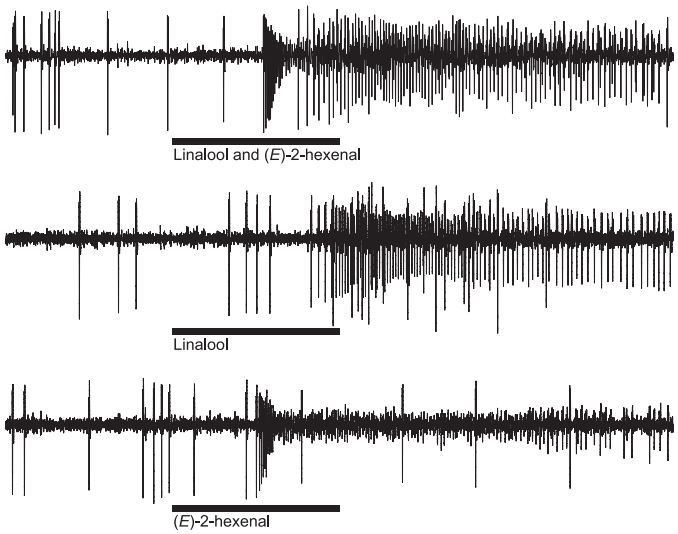

Figure 3 Single sensillum recordings from a grooved sensillum placodeum in *Pachnoda interrupta*. ORN responses to linalool and (*E*)-2-hexenal were present and could not be distinguished based on amplitude. Simultaneous stimulation with both compounds was used to determine whether responses originated from one or 2 ORNs. Two different neurons were found to be responding, with an asynchronous firing pattern, and differences in amplitude decrease during response. The horizontal bar denotes the stimulation period (0.5 s).

In *P. interrupta*, 38% (91 out of 239) of the contacted grooved placodea contained at least one responding neuron, and 43% (80 out of 184) in *P. marginata*. Responding neurons were more commonly found in the contacted smooth placodea in *P. marginata* (27%; 23 out of 84) than in *P. interrupta* (4%; 4 out of 96) ($P = 0.000013$, Fisher's exact test).

The dendrogram created based on cluster analysis shows that ORNs formed groups defined by response, rather than species (Supplementary Figure A, Figure 5). These response-based groups were used to define the ORN classes used in our study, and average response spectra were calculated for them (Figure 6). For all neuron classes that were found in both species, there were no differences in response pattern to ligands based on species, and means were based on pooled responses from both species. In most cases, little overlap was found between ORN classes: only 5% of compounds eliciting a response do so for more than one ORN class. Care must thus be taken in the analysis of the branching pattern connecting the ORN classes. Classes that do show overlap group together (e.g., the methyl salicylate, methyl benzoate, and methyl anthranilate classes, named for their best ligand). However, between classes with no overlap in strong, unequivocal responses, chance patterns in weak responses could influence the branching pattern.

Several ORN classes found in only one species were rare and only occurred once or twice in the second phase, for example, 2-phenethyl propionate, geraniol, and sulcatone (Figure 5). To increase sample size and make comparisons more robust, we included ORNs from the first phase, which could be retrospectively identified based on results from the second screen. With both the first and second phase, we had recordings from a total of 875 sensilla, out of which 374 contained one or more responding neurons. Most ORNs from the first phase could be classified according to response into the classes defined by the cluster analysis, with a few remaining as unclassified. In the total sample, including both the first and second phase, 18 of the 27 ORN classes were found in both species (Table 2). A majority of these shared ORN classes were common enough in the second phase of the screening that cluster analysis (Supplementary Figure A) and comparisons of means (data not shown) showed their response spectra to be indistinguishable between the 2 species, demonstrating a high degree of functional conservation in response specificity. Almost all common ORN classes (>10 neurons in total) occurred in both species. Only a few ORN classes, most notably 2-phenethyl propionate, methyl benzoate, *gamma*-nonalactone, and acetoin were found only in *P. marginata* (Table 2), whereas ORNs for 2,3-butanediol and methyl anthranilate appeared only in *P. interrupta*. Out of these, Fisher's exact test for proportions indicated that the methyl benzoate and *gamma*-nonalactone ORN classes were more frequent in *P. marginata* than in *P. interrupta* (Table 2). If sensilla in both grooved and smooth areas were pooled, nonanal ORNs were significantly more common in *P. marginata* (data not shown). A further 3 classes were significantly different in frequency between the species if Bonferroni correction was not applied (Table 2, *P* values in bold).

Most of the 27 ORN classes were functional specialists, detecting a low number of compounds with a high degree of

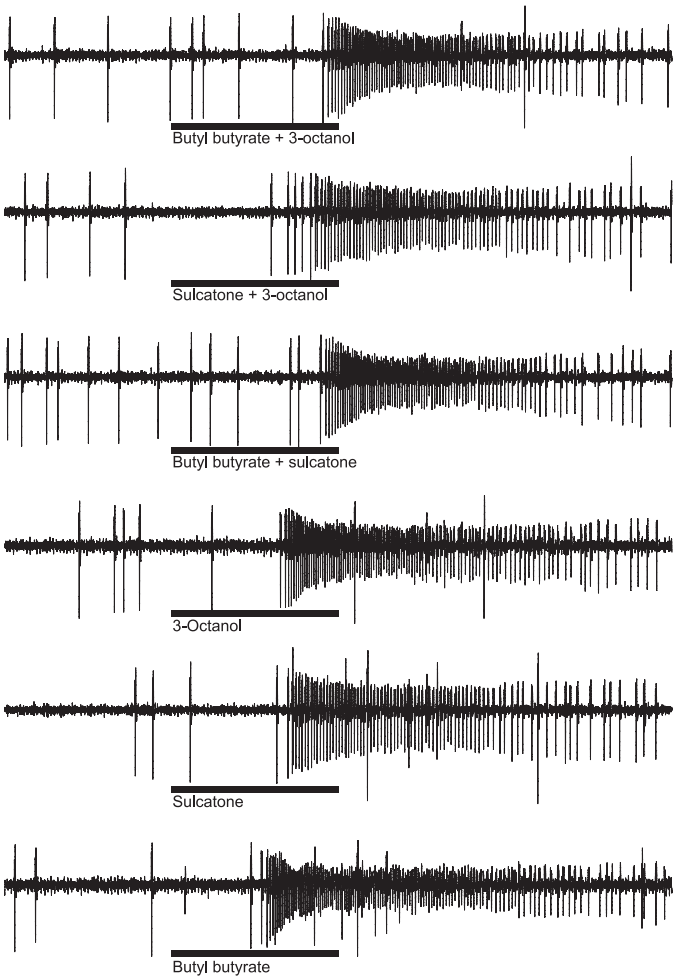

Figure 4 Single sensillum recordings from a grooved sensillum placodeum in *Pachnoda interrupta*. ORN responses to butyl butyrate, 3-octanol, and sulcatone were present and could not be distinguished based on amplitude. Simultaneous stimulation with all dual combinations of the compounds was used to determine whether responses originated from one or 2 ORNs. A single neuron was found to be responding to all compounds, as simultaneous stimulation with 2 compounds in no case led to asynchronous firing patterns or differences in amplitude decrease during response. The horizontal bar denotes the stimulation period (0.5 s).

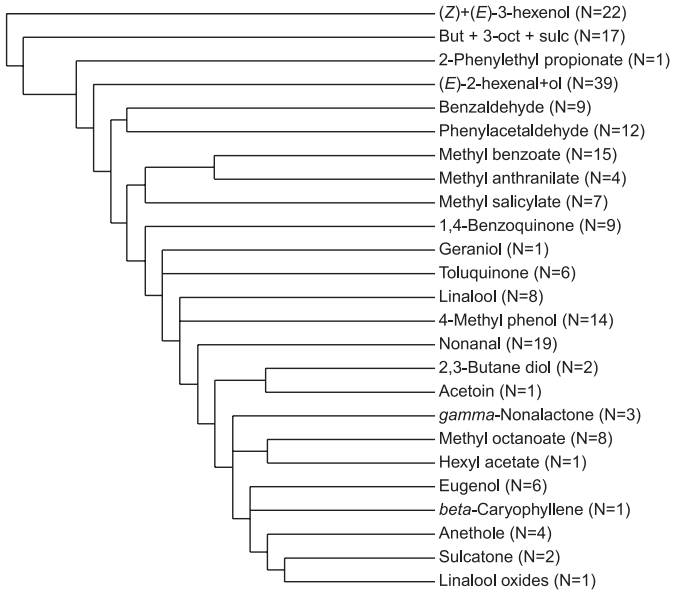

Figure 5 Dendrogram showing olfactory receptor neuron classes in *Pachnoda interrupta* and *P. marginata*. The dendrogram is based on cluster analysis using average linkage and Euclidean distance on data from the second phase of the single sensillum screening. It has been condensed to show ORN classes instead of individual ORNs (see Supplemental Figure A for the full dendrogram). "N" denotes number of ORNs belonging to a class, counting in both species.

specificity (Figure 6). Dose–response experiments with *P. marginata* on selected ORN classes corroborated this specificity: at lower doses, response spectra of ORNs narrowed further (Figure 7). This indicates that the doses used in both phase 1 and 2 were in the upper part of the range, reducing the likelihood of missing secondary responses. The 3 most common ORN classes responded to green leaf volatiles (GLVs) and occurred in both species. The (*E*)-2-hexenal class was the most common, and also responded to (*E*)-2-hexenol and, to a lesser extent, (*E*)-3-hexenol (Figure 6). It thus showed some overlap in secondary ligands with the third most common ORN class, which mainly responded to (*Z*)-3-hexenol, but also (*E*)-3-hexenol and (*E*)-2-hexenol. However, these classes constitute the exception rather than the rule, and a majority of the ORN classes were even more narrow, responding strongly only to a single compound, like the second-most common ORN class, whose sole strong response was to nonanal (Figure 6). An exception was the fourth most common ORN class, which had the widest response spectrum encountered in the study, and responded to butyl butyrate, butyl isobutyrate, methyl hexanoate, 3-octanol, and sulcatone. Being unique in responding to such widely different compounds, it was subject to extensive testing prior to our conclusion that it indeed constituted a single ORN type (see, e.g., Figure 4).

Two pairs of neuron classes showed extensive overlap, with only one member of each pair being found in each species, indicating that they may represent examples of divergent evolution of a single ancestral class. The ORN class 2,3-butanediol in *P. interrupta* and the acetoin class in *P. marginata* (Figure 6) appeared to be mutually exclusive ORN classes responding with an overlapping response pattern to 2 highly similar stimuli (Table 2). The other example was represented by the methyl anthranilate class and the methyl benzoate class, respectively. These 2 classes had overlapping response patterns to each of these 2 compounds (Figure 6). Both classes were also unique in that they were found exclusively in the smooth sensilla placodea. Three other ORN classes were found both in smooth and grooved placodea but in a majority of cases occurred in the latter (Table 2).

Few responding neurons occurred together in a single sensillum, and almost none in repeatable combinations, which precluded extensive comparisons of ORN pairing between

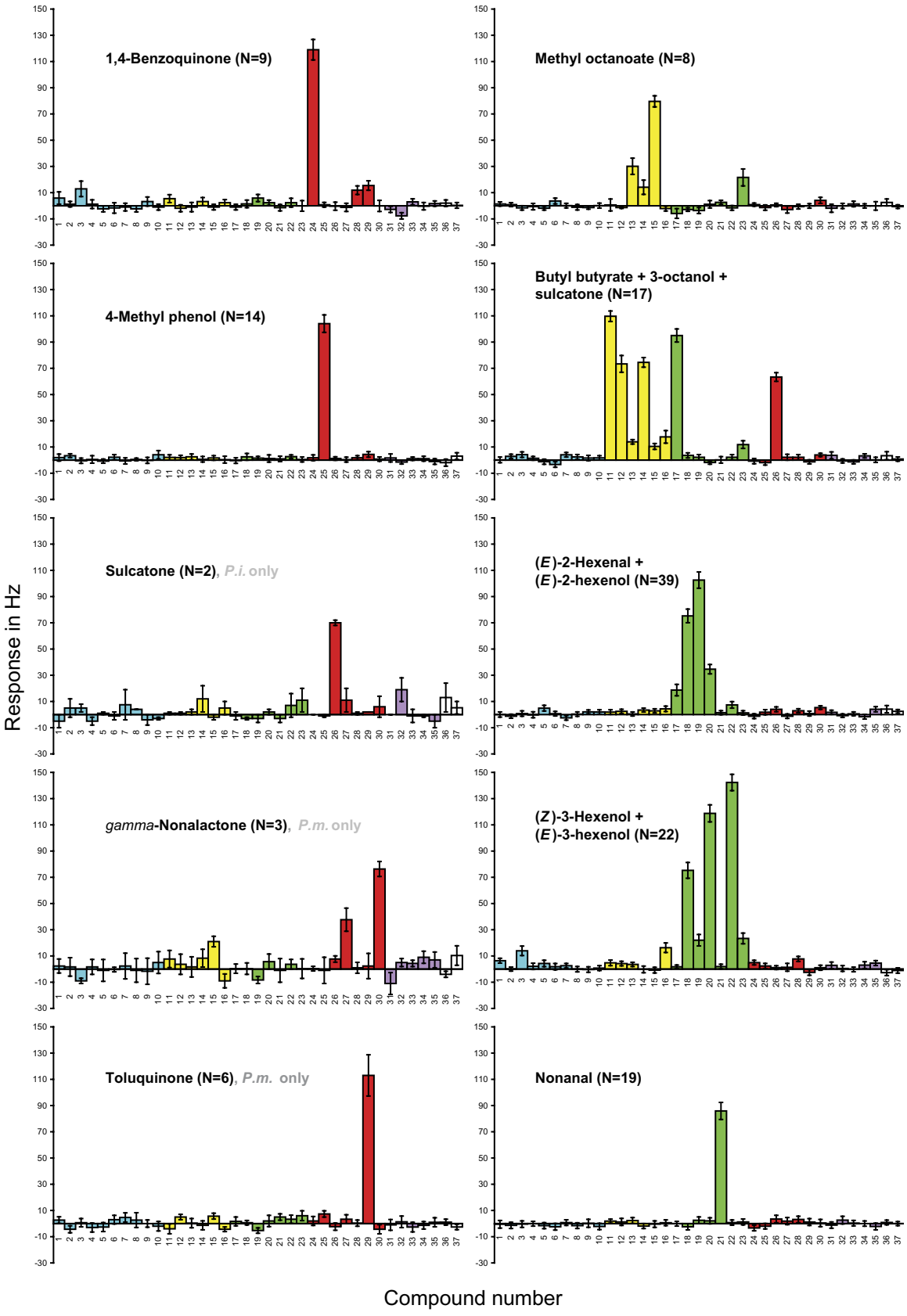

Figure 6 Continued

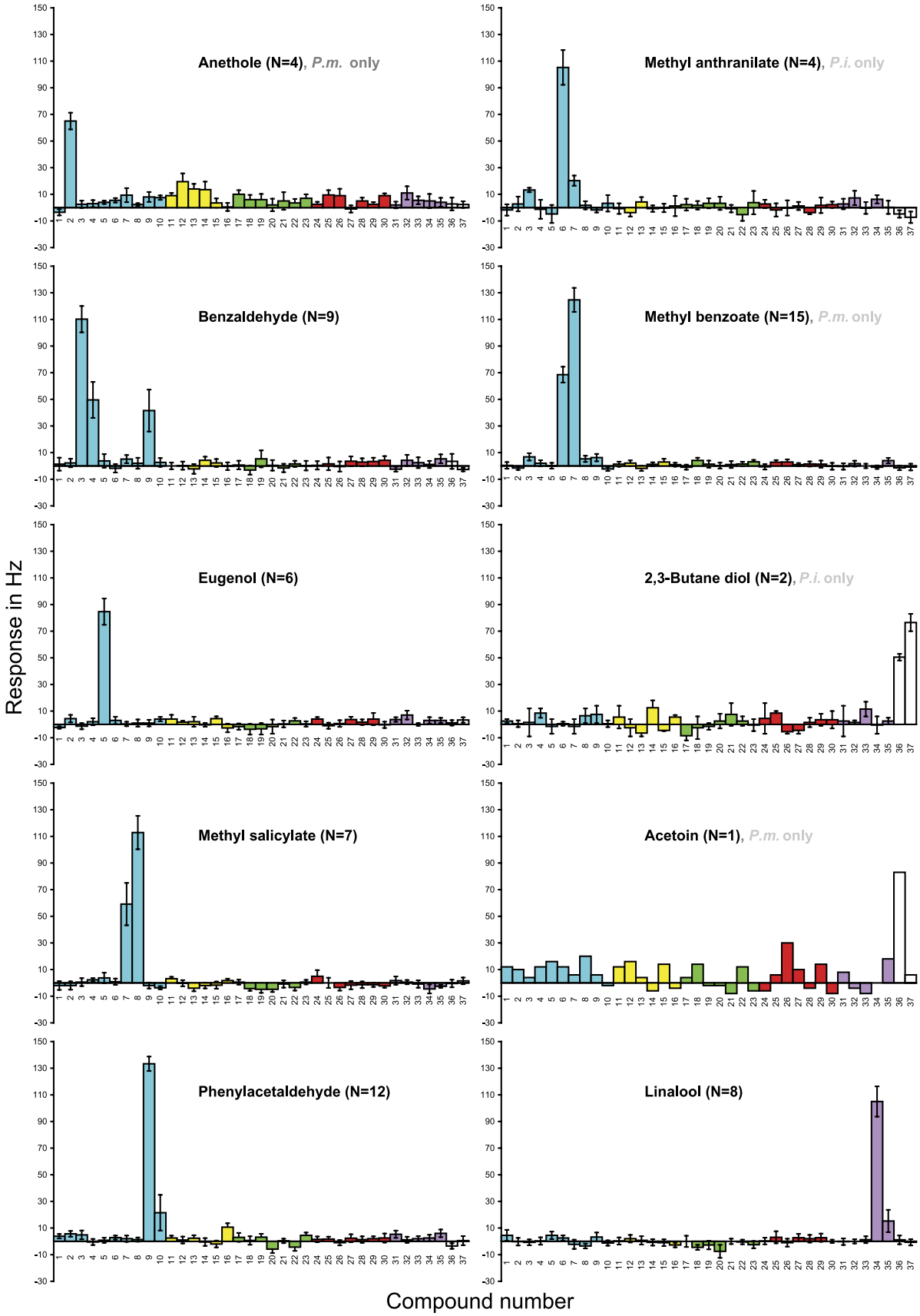

Figure 6 Continued

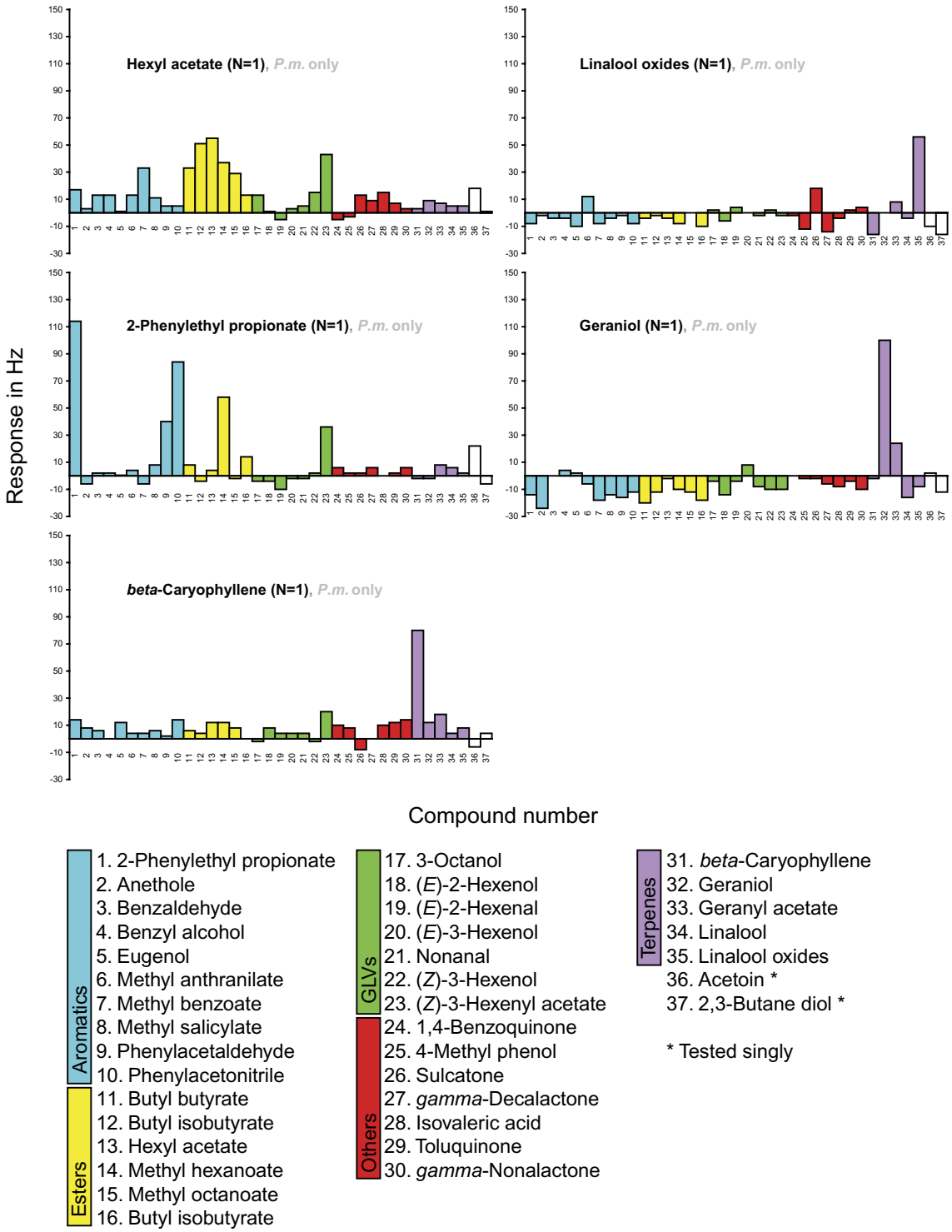

Figure 6 Average response of ORN classes determined in Figure 5 (means ± standard error) to ligands in the second phase of screening (using a stimulus loading of 1 μg). "*P.i.* only" and "*P.m.* only" denotes classes only found in *Pachnoda interrupta* or *P. marginata* during the second screening phase. Other classes were found in both species. In legend, numbers and colors denote screening compounds and screening blends, respectively.

the 2 species. In the most common pairing, occurring in both species, 48% of nonanal neurons encountered in the first phase were found together with isovaleric acid neurons. This combination was the only one that was encountered more than 3 times. Other combinations included GLV neurons with ORNs for different terpenes, esters, or aromatics, for example, (*E*)-2-hexenal+ol with linalool or eugenol and (*Z*) + (*E*)-3-hexenol together with the butyl butyrate+3-

Table 2 Prevalence of ORN classes in *Pachnoda interrupta* and *P. marginata*

Class	*P.i.*	*P.m.*	*P*, Fisher's
Grooved placodea			
(*E*)-2-Hexenal + ol	23	22	0.642, NS
Nonanal	12	23	**0.014,** NS
(*Z*) + (*E*)-3-hexenol	21	13	0.482, NS
But+3-Oct+Sulc	16	10	0.552, NS
4-Methyl phenol	12	12	0.678, NS
Methyl salicylate	15	8	0.396, NS
Isovaleric acid	6	13	0.058, NS
Linalool	13	5	0.157, NS
Benzaldehyde	9	9	0.811, NS
Phenylacetaldehyde	9	5	0.592, NS
Methyl octanoate	12	1	**0.008,** NS
Eugenol	5	8	0.266, NS
Anethole	4	6	0.356, NS
Gamma-nonalactone	0	9	0.001*
Toluquinone	1	8	**0.013,** NS
1,4-Benzoquinone	3	6	0.312, NS
Beta-caryophyllene	6	1	0.137, NS
2-Phenethyl propionate	0	5	**0.018,** NS
2,3-Butanediol	5	0	0.068, NS
Acetoin	0	2	0.201, NS
Sulcatone	3	2	≈1, NS
Geraniol	1	2	0.590, NS
Methyl benzoate	0	2	0.201, NS
Linalool oxides	1	1	≈1, NS
Hexyl acetate	0	1	0.448, NS
Unclassified	25	17	
Total responding neurons	202	191	
Total sensilla with 1+ responding neuron	182	163	
Nonresponding sensilla	181	132	
Total sensilla	363	295	
Percent responding sensilla	50.1%	55.3%	
Smooth placodea			
Methyl benzoate	0	15	0.000001*
Methyl anthranilate	4	0	0.140, NS
Nonanal	1	6	**0.022,** NS
1,4-Benzoquinone	0	2	0.173, NS
Beta-caryophyllene	1	0	≈1, NS
Smooth placodea			
Unclassified	0	1	
Total responding neurons	6	24	
Total sensilla with 1+ resp. neuron	6	24	
Nonresponding sensilla	121	67	
Total sensilla	127	91	
Percent responding sensilla	4.7%	26.4%	

"*" Denotes significant differences and NS denotes nonsignificant differences in ORN frequency between the species, respectively (Fisher's exact test for proportions, with Bonferroni correction). If Bonferroni correction is not applied, some further ORN classes are significantly different in frequency (*P* values in bold).

octanol+sulcatone class. Although no systematic testing with dual stimuli was performed, response in one ORN seemed independent from the other (for an example, see Figure 3). As part of the protocol, all ORNs were, however, stimulated with set blends of compounds in order to determine whether they responded at all. Dual and multiple compound mixtures generally seemed to have little effect on the number of spikes elicited, which were generally equal to that elicited by the ligand giving the strongest response alone. Care must be taken in the interpretation of these results, however, as the mixture pipettes were used for all ORNs (to determine whether they responded or not), whereas single-compound pipettes were only used when one or more of the mixture pipettes gave a response of >40Hz.

We found no ORNs responding to typical fermentation and degradation compounds such as acetic acid, acetone, ethanol, propionic acid, and just a single response to ethyl acetate (data not shown). ORN responses to other fermentation odorants were encountered, for example, to 2,3-butanediol, acetoin, and isovaleric acid. The latter was not encountered in the second phase, however, despite numerous nonanal neurons found, with which it was commonly co-compartmentalized in the first phase. Despite the considerably lower stimulus intensities in the second phase, this was the only neuron class found in the first phase that was not encountered in the second phase.

Discussion

Despite the dissimilarity between their habitats, *P. interrupta* and *P. marginata* showed a remarkable degree of conservation in their array of olfactory receptor neurons detecting food volatiles. Our results indicate that divergence between the species was in most cases manifested as differences in proportions of ORN classes, rather than changes in the response specificity of individual ORN classes (Table 2). The high degree of conservation indicates that a similar sensory setup can be efficient for food search in widely disparate

habitats. In part, this may be due to the presence of stereotyped volatile pollination and fruit dispersal signals emitted by plants, which might be employed in food search. The ORN classes identified in the 2 species were functional specialists, with a low level of overlap (Figure 6). Less than 5% of ligands that were detected by ORNs activated more than one class of olfactory neuron, and all identified ORN classes were most strongly excited by a ligand not detected by any other ORN class. Our findings thus suggest that olfactory sensory channels in chafers mostly detect either single ligands or very narrow spectra of nonoverlapping subsets of ligands.

The 2 species exhibited a conserved antennal morphology (Figure 1), and although most smooth placodea did not contain any responding ORNs, particular ORN classes were generally found in either grooved or smooth placodea and seldom in both. Similar to chafers from the subfamily Rutelinae, ORNs responding to presumed food-related odors were almost exclusively found in the heterogeneous area of lamella (Hansson et al. 1999; Larsson et al. 1999, 2001), and 22 out of 27 ORN classes were found exclusively in grooved placodea (Table 2). Whereas ORNs responding to methyl anthranilate have also been found in smooth placodea in *Anomala cuprea* (Larsson et al. 2001), in Ruteline scarabs almost all smooth sensilla placodea have responded to pheromone compounds (Hansson et al. 1999; Larsson et al. 1999). Although a study on *P. interrupta* (Bengtsson et al. 2010) implied that phenylacetaldehyde is a pheromone compound, we found that an ORN type in grooved placodea detected this compound. This contrasts with the pattern in Ruteline scarabs, but in *Osmoderma eremita*, a scarab closely related to the Cetoniinae, the relatively rare pheromone-detecting ORNs are found in both the smooth and heterogeneous areas (Svensson and Larsson 2008).

Several possible strategies for using olfaction in host search have been proposed, of which the "token stimulus" and "compound ratio" are among the more commonly suggested. In the former, herbivores mainly use volatile compounds unique to their particular hosts (Bruce et al. 2005; Fraenkel 1959), whereas in the latter, herbivores identify hosts based on specific ratios of compounds common to multiple hosts and nonhosts (Visser 1986). The former case seems to be true for some specialist herbivorous insects, where, for example, the presence of isothiocyanate defensive compounds in Brassicaceae is used as a host-specific stimulus by the cabbage seed weevil, *Ceutorhynchus assimilis* (Blight et al. 1995) and the cabbage aphid *Brevicoryne brassicae* (Nottingham et al. 1991). A lack of host-specific compounds could in part explain why most herbivores seem to utilize specific ratios of common compounds, but for oligo- or polyphagous species, it could also be a more efficient strategy for detecting multiple hosts (Masson and Mustaparta 1990), and could facilitate learning behavior (Cunningham et al. 2004). The use of common compounds also implies that host shift could occur with relatively small changes to the sense of olfaction, as has been found in single sensillum studies of species within the *Rhagoletis* complex (Olsson et al. 2006a, 2006a). The high level of overlap in ORN types and frequencies between *P. interrupta* and *P. marginata* does thus not preclude that they could utilize different hosts.

In view of their diet, which emphasizes fruits and flowers, it is perhaps surprising that such a large proportion of the ORNs encountered in *P. interrupta* and *P. marginata* responded to GLVs, which are generally associated with the vegetative parts of plants. The cupreous chafer, *A. cuprea*, likewise has a large proportion of ORNs responding to GLVs but, unlike the 2 *Pachnoda* species, has leaves as a major part of its diet (Larsson et al. 2001). In *Melolontha* chafers, the GLVs released by female feeding even forms a sexual kairomone essential for male attraction to females (Ruther et al. 2001, 2002). Several of the most attractive compounds for both *P. interrupta* and *P. marginata* (Larsson et al. 2003; Wolde-Hawariat et al. 2007; Bengtsson et al. 2009) are aromatics or esters, detected by relatively rare ORN classes. It is thus possible that several of these aromatic or ester compounds may act as token stimuli, being highly attractive in their own right. Single compounds have been observed to be strong attractants for several scarab species (Donaldson et al. 1990). Compared to dispensers with aromatics and esters, dispensers with GLVs generally attracted fewer *Pachnoda*, but in some cases GLVs seemed to have synergistic effects in blends (Larsson et al. 2003; Bengtsson et al. 2009). The detection of GLVs could be involved in blend-based host recognition in *P. interrupta* and *P. marginata*. Combining several esters or aromatics in a blend can also increase attraction, with varying degrees of redundancy observed for blends with more than 2 components (Larsson et al. 2003; Bengtsson et al. 2009). In bark beetles, GLVs have been implied as indicators of nonhosts (Zhang and Schlyter 2004), as GLVs present in deciduous trees repel the conifer-feeding beetles. However, a similar scenario may perhaps be less likely for the 2 *Pachnoda* species, as they feed on a wide range of plants, making such clear patterns of nonhost-specific GLVs unlikely.

D. melanogaster is perhaps the insect model that most closely resembles cetoniid beetles in its attraction to decaying fruit and other organic matter. It has several receptor neurons that are close functional analogues to those of *Pachnoda* beetles (Hallem and Carlson 2006). Interestingly, these compounds do not seem to constitute token stimuli for *D. melanogaster* to the same degree; in contrast, mixtures often appear to be necessary for attraction (Zhu et al. 2003; Ruebenbauer et al. 2008; Becher et al. 2010). This could potentially be related to the differing role of the signals in the 2 insect groups, which for *Pachnoda* exclusively signals food, whereas in *D. melanogaster* it also constitutes an oviposition cue.

The differences in ORN class frequency observed between *P. marginata* and *P. interrupta* could reflect the availability of different hosts between the habitats. Results from behavioral experiments also seem to mirror the divergence in response

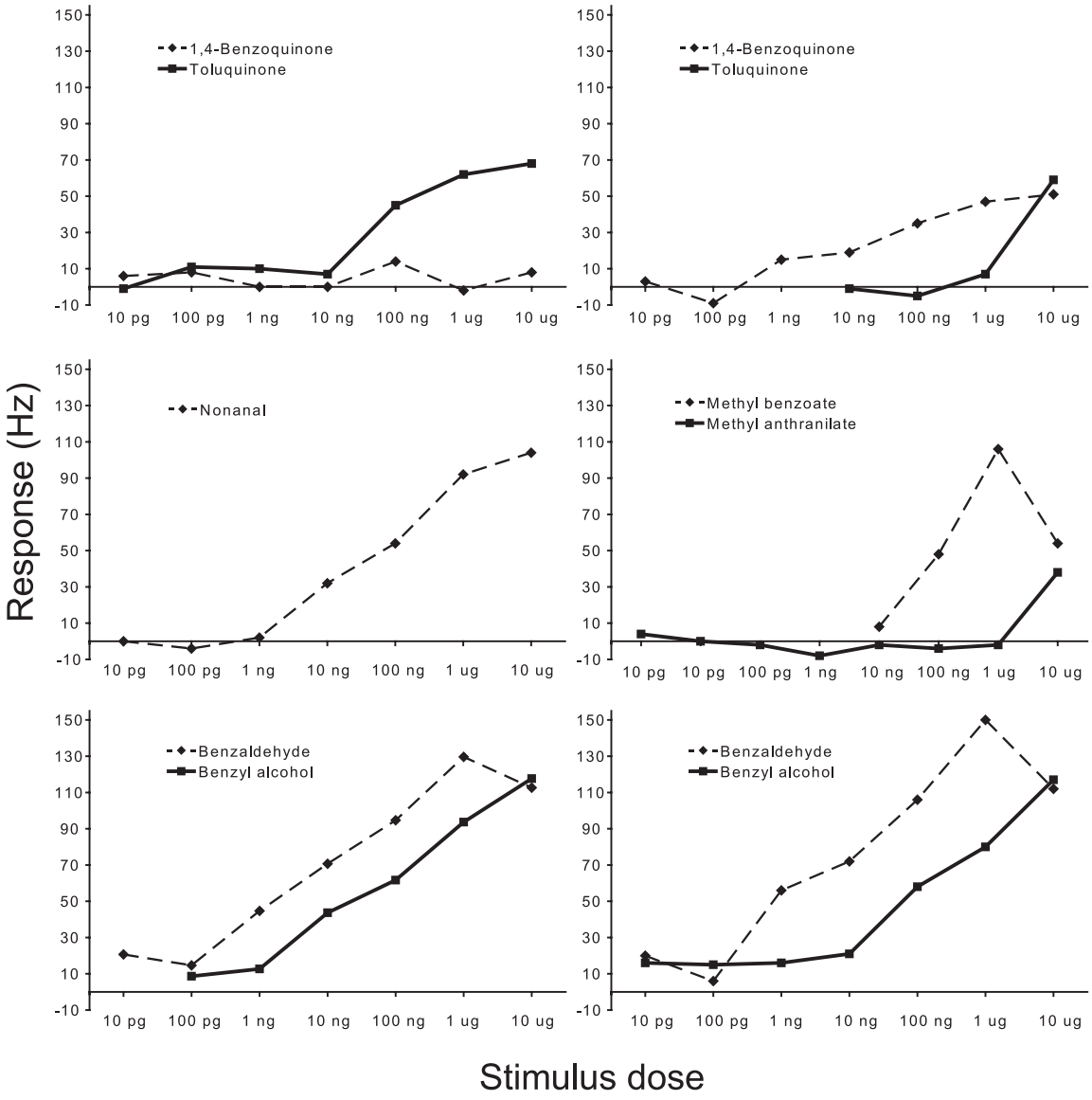

Figure 7 Dose–response curves for *Pachnoda marginata* ORNs, based on single sensillum recordings. Selected ligands were used for stimulation in a decadic range of loadings. Each diagram is based on recordings from a single ORN.

spectra between *P. marginata* and *P. interrupta* indicated for the 2 ORN classes acetoin and 2,3-butanediol. *Pachnoda marginata* was strongly attracted to acetoin, but not 2,3-butanediol (Larsson et al. 2003), which, however, is a strong attractant for *P. interrupta* (Bengtsson et al. 2009). This correlation between response spectra and behavior also seems to hold for the seemingly related ORN types methyl benzoate and methyl anthranilate, respectively. *Pachnoda marginata* is strongly attracted to both methyl anthranilate and methyl benzoate, both of which were detected by the ORN type in this species, whereas *P. interrupta* is strongly attracted only to methyl anthranilate, and not to methyl benzoate, which was a weak ligand in this species.

Despite the inclusion of decaying fruit in their diet, we did not encounter neuron classes tuned to acetic acid, acetone, ethanol, or propionic acid, which are all compounds commonly associated with fermentation and microbial degradation, in either *P. interrupta* or *P. marginata*. Acetic acid appears to be an essential component of *Drosophila* attraction to fermenting fruit (Zhu et al. 2003; Becher et al. 2010). It cannot be ruled out that ORNs present in either coeloconic or smooth peg sensilla detect these compounds as we only managed to get intermittent contacts to these morphological sensillum types. In *D. melanogaster*, coeloconic sensilla have been shown to contain ORNs that detect fermentation and degradation compounds such as isovaleric acid, propionic acid, and butyric acid (Yao et al. 2005). However, ORNs responding to fermentation and degradation compounds such as acetoin, 2,3-butanediol, and 4-methyl phenol (Chatonnet et al. 1992; Fischer et al. 2000; Xiao and Ping 2007) were

found. Some of these compounds, for example, 4-methyl phenol, are more often related to decaying animal matter than fermenting plant material. The ratio between them might be used to distinguish suitable hosts (e.g., rotting fruit) from carrion or other nonhosts.

The presence of a large overlap in ORN classes between *P. interrupta* and *P. marginata* implies that for olfaction, a similar sensory apparatus is efficient for host detection in both habitats. The 2 species may potentially differ in their higher level processing of this sensory information, but they may also utilize a similar strategy for food search. Such a shared strategy could be based around compounds shared by multiple hosts, either from vegetative parts, for example, GLVs, or compounds used to attract symbionts for pollination and seed dispersal, for example, esters and aromatic compounds. Shared compounds may also be found in different systems of fermenting or microbially degrading fruits, which are part of the diet of the 2 beetles.

Supplementary material

Supplementary Figure A can be found at http://www.chemse.oxfordjournals.org/

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